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Modulation of the secondary metabolism of a *Cophinforma mamane* endophytic fungal strain by epigenetics and cultivation-based approaches

Romina Géraldine Pacheco Tapia

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THÈSE

**En vue de l'obtention du
DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE**

Délivré par l'Université Toulouse 3 - Paul Sabatier

**Présentée et soutenue par
Romina Geraldine PACHECO TAPIA**

Le 25 octobre 2021

**Modulation du métabolisme secondaire d'une souche endophyte
de Cophinforma mamane par des approches épigénétiques et
basées sur les conditions de culture**

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Développement**

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LIST OF ABBREVIATIONS

| | |
|------------------|---|
| TDKP | Thiodiketopiperazine |
| DKP | Diketopiperazine |
| CM | <i>Cophinforma mamane</i> |
| UHPLC | Ultra High-Performance Liquid Chromatography |
| HRMS | High Resolution Mass Spectrometry |
| LC-MS | Liquid Chromatography – Mass Spectrometry |
| PI | Positive Ionization |
| OSMAC | One Strain – Many Compounds |
| HDAC | Histone Deacetylase |
| DNMT | DNA methyltransferase |
| AZA | 5-azacytidine |
| SB | Sodium butyrate |
| NIC | Nicotinamide |
| HSL | Homoserin lactone |
| QS | <i>Quorum</i> sensing |
| RT | Retention time |
| PCA | Principal Component Analysis |
| PLS-DA | Partial Least-squares Discriminant Analysis |
| PDB | Potato Dextrose Broth |
| MEA | Malt Extract Agar |
| ISP2 | International Streptomyces Project-2 Medium |
| CZA | Czapek solution Agar |
| TSA | Trypticase soy agar |
| GNPS | Global Natural Products Social Molecular Networking |
| IC ₅₀ | Half-maximal Inhibitory Concentration |
| EC ₅₀ | Half-maximal Effective Concentration |

LIST OF SCIENTIFIC PRODUCTION

Publications

1. Book chapter (Elsevier), expected publication date April 2022

Book title: Medicinal plants as anti-infectives: current knowledge and new perspectives

Chapter title: Fungal endophytes: A source of anti-infectives

Authors: R. Pacheco, S. Ortiz, M. Haddad, M. Vansteelandt

2. Submitted manuscript

Journal: Fungal Biology

Title: Chemical modulation of the metabolism of an endophytic fungal strain of *Cophinforma mamane* using epigenetic modifiers and amino-acids

Authors: R. Pacheco, P. Vásquez-Ocmin, S. Duthen, S. Ortiz, P. Jargeat, C. Amasifuen, M. Haddad, M. Vansteelandt

3. Manuscript in preparation

Journal: Chemistry and Biodiversity

Title: Metabolome time-variations and induction of secondary metabolites through OSMAC approaches of an endophytic fungal strain of *Cophinforma mamane*

Authors: R. Pacheco, S. Ortiz, P. Jargeat, C. Amasifuen, M. Vansteelandt, M. Haddad

Poster presentation

1. **Analysis of metabolome time-variations of an endophytic fungal strain of *Botryosphaeria mamane* through untargeted LC-MS profiling.** R. Pacheco; M. Haddad; P. Jargeat; B. Cabanillas; C. Amasifuen; N. Fabre; M. Vansteelandt. European RFMF Metabomeeting 2020, 22 – 24 Janvier 2020, Toulouse – France

Oral presentations

1. **Epigenetic modifiers alter the secondary metabolite production of an endophytic fungal strain of *Botryosphaeria mamane* isolated from *Bixa orellana*.** R. Pacheco, M. Vansteelandt, P. Jargeat, B. Cabanillas, C. Amasifuen, N. Fabre, M. Haddad. IV Encuentro científico de Peruanos en Europa SINAPSIS 23 – 25 Octobre 2019, Ghent – Belgium
2. **Modulation non ciblée d'une souche fongique endophyte de *Cophinforma mamane* par le traitement de modificateurs épigénétiques et l'addition des acides aminés.** R. Pacheco. Treizièmes Journées de l'Ecole Doctorale EDSM. 21 – 22 Mai 2021, Toulouse – France

RÉSUMÉ

Modulation du métabolisme secondaire d'une souche endophyte de *Cophinforma mamane* par des approches épigénétiques et basées sur les conditions de culture

Dans le contexte actuel d'émergence croissante de souches microbiennes résistantes et de déclin du taux de découverte de nouveaux médicaments à partir de produits naturels, les champignons endophytes représentent une source potentielle de molécules actives aux structures chimiques nouvelles relativement peu étudiée. En effet, les champignons endophytes ont fait l'objet d'une attention accrue en raison de leur capacité importante à produire des métabolites chimiquement diversifiés ayant des propriétés antimicrobiennes, antiparasitaires, antivirales et de nombreuses autres bioactivités avec des applications potentielles dans les domaines de la santé et de l'agriculture. Dans cette étude, nous nous sommes concentrés sur une souche fongique endophyte de *Cophinforma mamane* (Botryosphaeriaceae) isolée de *Bixa orellana* (Bixaceae), à partir de laquelle 4 thiodikétopipérazines (TDKPs), les botryosulfuranols A-D, présentant une activité anti-leishmanienne ont précédemment été isolées. Afin de moduler le métabolisme secondaire de *C. mamane* pour mieux comprendre la production des TDKPs et espérer induire la production de métabolites anti-infectieux, nous avons utilisé différentes approches basées sur la modification des conditions de culture ainsi qu'une approche épigénétique. Des analyses UHPLC - spectrométrie de masse couplées à une approche par métabolomique non ciblée ont été utilisées pour traiter l'ensemble de nos résultats. L'étude cinétique réalisée sur 28 jours de culture a mis en évidence une production dans le temps très courte (jours 6 à 9) du botryosulfuranol C, un TDKP qui possède un pont disulfure dans sa structure. Il est également intéressant de noter que le traitement avec l'homosérine lactone (HSL), une molécule impliquée dans le *quorum*-sensing des bactéries gram-négatives, a induit la production du botryosulfuranol C ainsi que celle d'autres dicétopipérazines mises en évidence par réseau moléculaire. La production de métabolites, annotés comme étant des cyclopentapeptides et détectés à un stade précoce (jours 4 à 8), a été significativement induite en présence des co-cultures de *C. mamane* avec *Pseudomonas aeruginosa* et *Staphylococcus aureus*. En outre, le traitement de la culture de *C. mamane* avec différents modificateurs épigénétiques nous a permis d'identifier la 5-azacytidine (AZA) comme étant un puissant modulateur chimique du métabolome de *C. mamane* via l'induction de gènes silencieux ou faiblement exprimés. Ces résultats par approche métabolomique nous ont ainsi aidé à mieux comprendre l'effet des conditions de culture sur le métabolome de *C. mamane*, le but étant de trouver les conditions optimales de culture afin d'une part d'obtenir des rendements plus importants en TDKPs, nécessaires pour approfondir l'évaluation de leur activité antileishmanienne *in vivo* et d'autre part d'identifier puis isoler de nouveaux composés bioactifs produits par *C. mamane*.

Mots clés : champignons endophytes, *Cophinforma mamane*, modificateurs épigénétiques, co-culture, métabolomique, dicétopipérazines.

ABSTRACT

Modulation of the secondary metabolism of a *Cophinforma mamane* endophytic fungal strain by epigenetics and cultivation-based approaches

In the context of an increasing emergence of microbial resistant strains added to decline in drug discovery rate from natural products, endophytic fungi represent a potential source of new metabolites with novel and diverse chemical structures. Endophytic fungi have gained an increased attention due to their great capacity to produce chemically diverse metabolites with antimicrobial, antiparasitic, antiviral and many other bioactivities with potential applications in health and agriculture. In this study, we focused on an endophytic fungal strain of *Cophinforma mamane* (Botryosphaeriaceae) isolated from *Bixa orellana* (Bixaceae) and from which 4 antileishmanial thiodiketopiperazines (TDKPs), botryosulfuranols A-D, were previously isolated. We performed different culture-based approaches as well as an epigenetic approach in order to alter the secondary metabolism of *C. mamane* for a better understanding of TDKPs production and to induce the production of anti-infective metabolites. UHPLC – Mass Spectrometry analysis and untargeted metabolomics tools have been used to evaluate our results. The 28-days kinetic study highlighted a short time-frame production (day 6 – 9) of botryosulfuranol C, a TDKP that possesses a di-sulfur bridge on its structure. Interestingly, the treatment with homoserin lactone (HSL), a quorum-sensing molecule of gram-negative bacteria, induced the production of botryosulfuranol C and other diketopiperazines revealed by molecular networking. Also, the production of metabolites putatively annotated as cyclopentapeptides and their detection at an early production stage (day 4 – 8), was significantly induced during the co-culture with *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Moreover, treating *C. mamane* culture with different 4 epigenetic modifiers allowed us to identify 5-azacytidine (AZA) as a potent chemical modulator of the metabolome of *C. mamane* via the induction of silent or lowly expressed genes. These results will help us to improve the culture conditions of *C. mamane* to obtain better yields from TDKPs for further investigation of their *in vivo* antileishmanial activity and to isolate and identify other potential novel bioactive compounds revealed thanks to the metabolomics tools.

Keywords: endophytic fungi, *Cophinforma mamane*, epigenetic modifiers, co-culture, metabolomics, diketopiperazines

RESUME EN FRANÇAIS

Modulation du métabolisme secondaire d'une souche endophyte de *Cophinforma mamane* par des approches épigénétiques et basées sur les conditions de culture

I. Introduction générale

Les produits naturels ont été historiquement utilisés pour le développement de médicaments et de candidats médicaments. Actuellement, près de 50% du total des petites molécules approuvées comme médicaments, entre 1981 et 2019, sont dérivées ou inspirées de produits naturels (Newman & Cragg 2020). Un grand nombre de principes actifs proviennent de microorganismes, notamment de bactéries, d'actinomycètes et de champignons (Hutchings *et al.* 2019), avec 45% de la production de métabolites microbiens issue des micromycètes (Bérdy 2012), montrant ainsi l'importance des produits naturels microbiens.

Cependant, l'arsenal de médicaments à activité antimicrobienne devient très limité, parallèlement à l'émergence croissante de souches microbiennes résistantes qui nous conduit à des prévisions alarmantes de 10 millions de décès d'ici 2050, ainsi qu'à un énorme fardeau économique pour les systèmes de soins de santé du monde entier (Browne *et al.* 2020). En outre, l'Organisation mondiale de la santé (OMS) n'a signalé que six médicaments en cours de développement clinique dans la liste des anti-infectieux ciblant des agents pathogènes prioritaires, considérés comme innovants. Pour faire face à cette problématique, de nouvelles molécules avec de nouvelles structures et/ou de nouveaux mécanismes d'action sont nécessaires, et elles peuvent potentiellement être trouvées dans des sources inhabituelles comme dans des environnements peu explorés ou inexplorés tels que les endophytes (Challinor & Bode 2015).

Les endophytes sont des micro-organismes qui colonisent les tissus internes des plantes sans provoquer de symptômes apparents. Ceci est possible grâce à de multiples interactions entre la plante et son microbiome, avant, pendant et après l'invasion de la plante hôte, *via* des molécules de signalisation (Khare *et al.* 2018). Il est considéré que presque toutes les plantes sur Terre sont en interaction constante et proche avec des endophytes (Arnold 2007). Malgré cela, seulement 1 à 2% de toutes les plantes connues ont été étudiées pour leur composition endophytique (Strobel 2018).

La plupart des endophytes isolés et étudiés correspondent à des champignons endophytes qui sont capables de produire des composés chimiquement diversifiés (Gao *et al.* 2018; Zheng *et al.* 2021) avec des propriétés antibactériennes, antifongiques, antiparasitaires, antivirales, anticancéreuses, antioxydants, immunosuppressives et antidiabétiques (Manganyi & Ateba 2020; Adeleke & Babalola 2021). Un grand nombre de ces composés présentent des applications potentielles dans divers domaines

tels que la santé publique, l'agriculture et les industries pharmaceutiques (Gupta *et al.* 2020) avec un total de 245 demandes de brevets rapportées, entre 2001 et 2019, impliquant la production de métabolites secondaires et dans les processus de biotransformation (Torres-Mendoza *et al.* 2020). À cet égard, les champignons endophytes représentent une source renouvelable et prometteuse de produits naturels.

Dans cette étude, nous nous sommes intéressés à une souche fongique endophyte de *Cophinforma mamane* (D.E. Gardner) A. J. L. Phillips & A. Alves (Botryosphaeriaceae) isolée des feuilles de la plante médicinale *Bixa orellana* (Bixaceae). La collecte des échantillons de feuilles a été effectuée dans la forêt amazonienne du Pérou permettant ainsi l'isolement de 17 souches fongiques endophytes appartenant aux genres suivants: *Diaporthe*, *Nigrospora*, *Phyllosticta*, *Endomelanconiopsis*, *Sporothrix*, *Xylaria*, et *Cophinforma* (données non publiées). Un extrait brut de la souche E224 (*Cophinforma mamane*) a montré une activité antileishmanienne *in vitro* contre *Leishmania infantum* (valeur IC₅₀ de 1.96 µg/mL) et a conduit à l'isolement de quatre nouveaux alcaloïdes thiodikétopipérazines (TDKP), les botryosulfuranols A, B, C et D (Barakat 2018). Ces composés présentent des atomes de soufre sur les positions α- et β- des résidus de phénylalanine et deux centres spirocycliques en C-4 et C-2 jamais décrits dans la littérature (**Figure 1**).

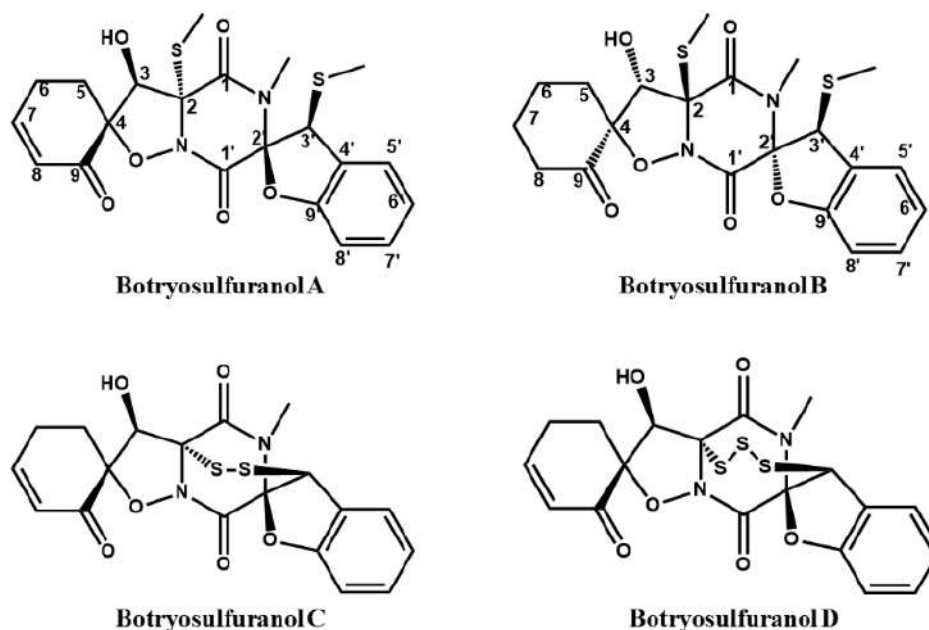


Figure 1. Structures chimiques des thiodikétopipérazines, botryosulfuranols A - D

L'activité antileishmanienne des quatre TDKPs a été évaluée *in vitro* contre les amastigotes axéniques de *Leishmania infantum*. Le botryosulfuranol D a présenté la plus forte activité avec une valeur de CI₅₀ de 0.03 µM et un indice de sélectivité (IS) de 190, suivi du botryosulfuranol C (CI₅₀ = 0.44 µM) ; du

botryosulfuranol A ($CI_{50} = 0.69 \mu\text{M}$) et du botryosulfuranol B ($CI_{50} = 3.87 \mu\text{M}$) (données non publiées). Les botryosulfuranols A, B et C ont également présenté une activité cytotoxique faible à modérée ($GI_{50} = 8.0 - 115.7 \mu\text{M}$) contre 4 lignées cellulaires cancéreuses (Barakat *et al.* 2019a). Bien que ces nouveaux composés aient présenté une activité antileishmanienne intéressante, les faibles quantités isolées n'ont pas permis d'approfondir leur activité biologique.

Un projet de pré-maturation, soutenu financièrement par la région Occitanie (porteur : N. Fabre), a été lancé avec l'objectif de poursuivre les recherches sur l'activité biologique par des tests *in vivo*. L'obtention de plus grandes quantités de molécules d'intérêt nécessaires aux tests *in vivo* a été possible grâce à une collaboration avec le Critt Bioindustries qui a pris en charge la fermentation du champignon à grande échelle. Parallèlement à ce projet auquel j'ai participé, notamment pour l'extraction et l'isolement des botryosulfuranols à partir des cultures en fermenteurs, la majeure partie de mon travail de recherche doctorale a porté sur l'étude des modifications du métabolisme de *C. mamane* soumis à différentes conditions de culture.

Il est connu que chez les champignons, le nombre de cluster de gènes est largement supérieur au nombre de groupes de gènes connus, impliqués dans la biosynthèse des métabolites secondaires isolés (Petit 2011) en raison de la présence de gènes silencieux ou faiblement exprimés (Brakhage & Schroeckh 2011) en conditions standards de laboratoire. Ainsi, différentes approches ont été appliquées afin d'induire la production de métabolites secondaires chez les champignons, telles que les méthodologies basées sur la culture, comme l'approche OSMAC, la co-culture avec d'autres micro-organismes et l'ajout d'éliciteurs chimiques aux milieux de culture, tels que les modificateurs épigénétiques. Lors d'un travail doctoral précédent, une étude portant sur *C. mamane* a évalué l'influence de deux modificateurs épigénétiques (acide suberoylanilide hydroxamique et valproate de sodium) (Triastuti *et al.* 2019) et d'une co-culture avec *Fusarium solani* (Triastuti *et al.* 2021), sur le métabolisme de la souche *C. mamane* E224. Ce travail avait mis en évidence la production de métabolites induits *de novo* dans ces conditions.

Afin d'éviter le ré-isolement de composés déjà connus, l'approche métabolomique est devenu un outil puissant pour l'analyse, l'identification et la quantification des petits métabolites (Zhao *et al.* 2018). La spectrométrie de masse (SM) est la technique la plus utilisée en raison de sa grande sensibilité, du faible volume d'échantillon nécessaire, de l'acquisition de données de fragmentation SM/SM pour l'élucidation structurale suivie d'une comparaison avec des bases de données spécifiques. En outre, l'association avec des outils informatiques et statistiques peut offrir une bonne vue d'ensemble qui simplifie les vastes analyses de données (Demarque *et al.* 2020). Plus précisément, la métabolomique non ciblée se concentre sur la détection d'autant de métabolites que possible pour élaborer un ensemble de données complet afin de mieux comprendre le métabolome de l'objet étudié.

Après une revue de la littérature, nous avons appliqué différentes approches culturales et épigénétiques pour modifier le métabolisme secondaire d'une souche fongique endophyte de *Cophiniforma mamane* (Botryosphaeriaceae) isolée de la plante médicinale *Bixa orellana* (Bixaceae). Une importance particulière a été accordée aux 4 thiodicétopipérazines bioactives précédemment isolées dans notre équipe par F. Barakat (Barakat *et al.* 2019a). Les deux objectifs principaux de cette étude sont :

- 1. Une meilleure compréhension de la production des botryosulfuranols précédemment isolés ainsi qu'une induction de leur production, et plus généralement l'induction des dicétopipérazines et autres alcaloïdes par une approche cinétique et OSMAC incluant l'utilisation de modificateurs épigénétiques et de précurseurs biosynthétiques.**
- 2. La production de nouveaux composés, notamment des métabolites anti-infectieux, par la co-culture de *C. mamane* avec des souches bactériennes pathogènes ainsi que des cultures en présence de parasites inactivés.**

Des analyses de CLUHP-SM couplées à des outils de métabolomique ont été utilisées afin d'atteindre nos objectifs.

Afin de répondre à notre premier objectif, une étude des variations temporelles de la production de métabolites secondaires a été réalisée. Cette analyse a été suivie par l'application de l'approche OSMAC consistant à utiliser différents milieux de culture et différentes conditions de lumière comme facteurs pouvant induire le métabolisme secondaire de ce champignon endophyte.

Le métabolisme de cette souche fongique soumise à la présence de modificateurs épigénétiques dans le milieu de culture a également été analysé, afin de mettre en évidence, via la production de nouveaux composés, une éventuelle stimulation de transcription de gènes silencieux ou faiblement exprimés en conditions standards de laboratoire. Nous avons également évalué l'impact de l'ajout d'acides aminés dans le milieu de culture sur le métabolisme, et notamment sur la production de dicétopipérazines et autres alcaloïdes via une stimulation des voies de biosynthèse spécifiques.

Enfin, pour répondre à notre deuxième objectif, des parasites de *Leishmania infantum* thermiquement inactivés ont été ajoutés à la culture du champignon endophyte afin de simuler la présence d'autres micro-organismes qui pourraient induire la production de métabolites secondaires antileishmaniens, tels que les botryosulfuranols. Des co-cultures de *C. mamane* avec des souches bactériennes pathogènes vivantes ont également été réalisées afin d'induire la production de métabolites antibactériens.

II. Matériels et méthodes

a) Matériels biologiques

Matériel fongique

La souche E224 de *Cophinforma mamane* (D.E. Gardner) A. J. L. Phillips & A. Alves a été isolée à partir des feuilles fraîches de *Bixa orellana* L. collectées en novembre 2013 par Carlos Amasifuen et Mohamed Haddad dans la réserve nationale d'Allpahuayo Mishana dans la forêt amazonienne d'Iquitos, Pérou (coordonnées GPS : 3°58'2.3" S, 73°25'3.9" W). Les processus d'isolement, d'identification et de stockage réalisés pour cette souche ont été précédemment décrits dans la littérature (Triastuti *et al.* 2019; Barakat *et al.* 2019).

Brièvement, les tissus collectés ont été stérilisés avec de l'hypochlorite de sodium à 5%, de l'éthanol à 70% (2 fois) et rincés à l'eau distillée. Les feuilles stérilisées en surface ont été coupées en petits fragments (5x5 mm) et placées sur une boîte de Pétri contenant du milieu MEA et du chloramphénicol (100 mg/L). Les isolats purs ont ensuite été identifiés par biologie moléculaire, via le séquençage des ITS1 et ITS2. La séquence correspondant à celle de la souche *C. mamane* E224 est disponible dans la base de données GenBank sous le numéro d'accession MG457709.1. Les cryotubes sont conservés à -80 °C dans 30% de glycérol dans la mycothèque de l'UMR 152.

Matériel bactérien

Pour les expériences de co-culture, trois souches bactériennes provenant de l'American Type Culture Collection (ATCC) ont été utilisées: *Escherichia coli* ATCC® 25922™; *Pseudomonas aeruginosa* ATCC® 27853™ et *Staphylococcus aureus* ATCC® 25923™.

Parasites de Leishmania

Pour la culture de *C. mamane* et pour les tests biologiques, des parasites *Leishmania infantum* exprimant une activité luciférase ont été utilisés (MHOM/MA/67/ITMAP-263, CNR Leishmania, Montpellier, France).

b) Conditions de culture et procédures d'extraction

Les étapes générales de la méthodologie, de l'isolement des champignons à l'analyse métabolomique, réalisées au cours de ce travail de doctorat sont résumées dans le schéma suivant (**Figure 2**)

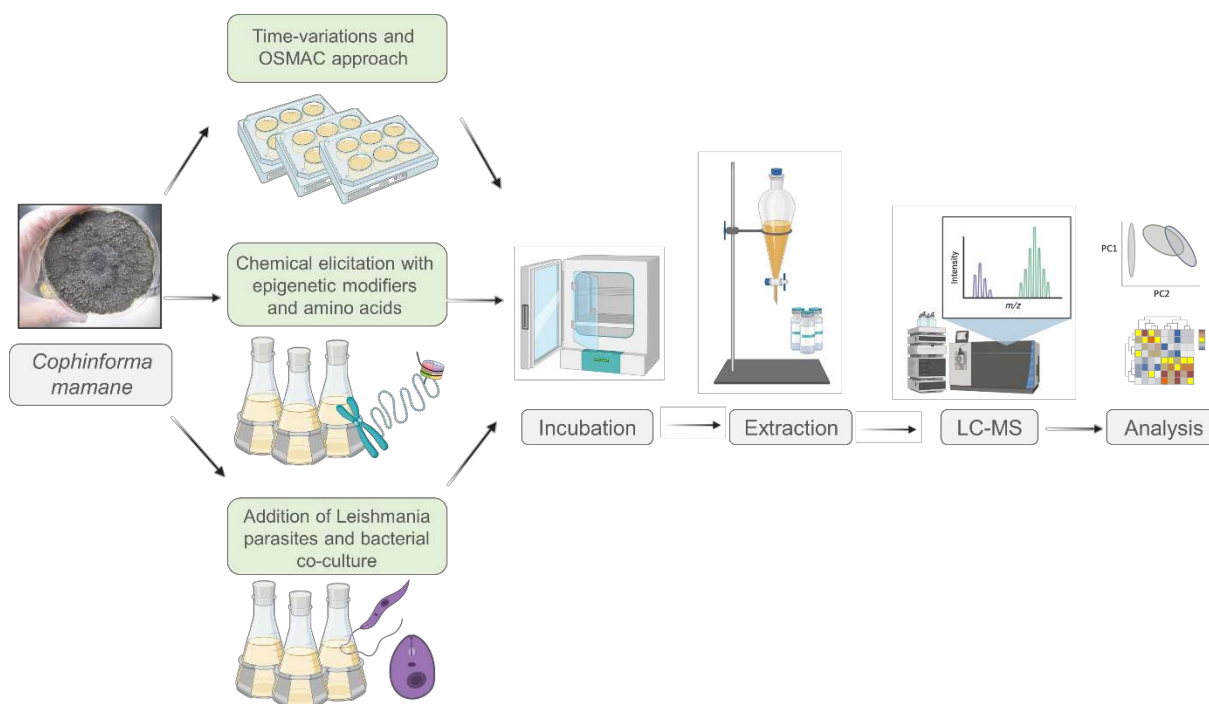


Figure 2. Étapes de la méthodologie générale: les différentes approches pour l'induction de métabolites secondaires de *C. mamane* (création propre avec biorender.com)

Étude cinétique et approche OSMAC

Pour l'étude cinétique, *C. mamane* a été incubé dans des plaques 6 puits contenant 4 mL/puits de MEA pendant 28 jours à 27°C sous lumière naturelle. A la fin de chaque jour de culture, une plaque de culture et une plaque témoin ont été placées à -80°C. Chaque culture (biomasse et milieu) a ensuite été lyophilisée. Le matériel sec a été transféré dans un tube de verre avec 10 mL de solvant. L'extraction a été réalisée à l'aide d'un mélange dichlorométhane-méthanol-eau 64:36:8 (v/v/v) préparé extemporanément (Bertrand *et al.* 2014).

En ce qui concerne l'approche OSMAC, *C. mamane* a été incubé dans des plaques 6 puits contenant du milieu CZA et du milieu ISP2 pendant 6 et 10 jours à 27 °C en présence ou en l'absence de lumière naturelle. A la fin de chaque temps d'incubation, les plaques de culture et les plaques témoins ont été placées à -80 °C. Chaque culture a ensuite été extraite avec 10 mL d'acétate d'éthyle. Le milieu et les mycéliums ont été broyés dans le solvant et soumis à un traitement par ultrasons pendant 60 minutes.

Dans les deux expériences (cinétique et OSMAC), les surnageants ont été filtrés à travers un coton de verre. Les filtrats ont ensuite été déshydratés sur du sulfate de magnésium puis filtrés à l'aide d'un papier filtre. Enfin, le solvant de toutes les phases organiques a été évaporé sous pression réduite (évaporateur rotatif KNF RC 600) à 40 °C afin d'obtenir les extraits bruts secs.

Élicitation chimique avec des modificateurs épigénétiques et ajout d'acides aminés

La souche *C. mamane* E224 a été cultivée sur milieu PDA pendant 7 jours. Puis le prélèvement d'un morceau de mycélium a permis l'inoculation de 50 mL de PDB. Après 3 jours de cette culture à 27°C, 2 ml ont été utilisés pour inoculer des flacons Erlenmeyer de 250 ml contenant 50 ml de PDB. Cette culture a été traitée avec 4 modulateurs épigénétiques différents, NIC, SB, AZA et HSL, dissous dans du DMSO et ajoutés aux 50 ml de PDB. NIC et SB ont été ajoutés à 1 µM, AZA a été ajouté à 25 µM et HSL a été ajouté à 100 µM comme concentrations finales. La fermentation a été réalisée pendant 2 semaines à 27 °C dans des conditions statiques. Toutes les conditions, y compris les contrôles négatifs, ont été réalisées en triplicata.

Pour l'ajout d'acides aminés, *C. mamane* a été cultivé dans du MEB et dans du MEB supplémenté avec 2 mg/L de L-Trp et L-Phe. La fermentation a été effectuée dans des flacons Erlenmeyer de 250 mL contenant 50 mL de MEB ou 50 mL de MEB supplémenté pendant 2 semaines à 27°C sous agitation constante. Toutes les conditions et les contrôles négatifs ont été réalisés en triplicata.

Pour les deux études et après deux semaines d'incubation, un partage liquide/liquide a été réalisé avec 50mL d'acétate d'éthyle sous 60 minutes de traitement aux ultrasons. Les mycéliums ont été retirés par filtration avec du coton de verre. Les phases organiques ont ensuite été déshydratées sur du sulfate de magnésium et filtrées avec du papier filtre. Enfin, elles ont été évaporées à sec sous pression réduite (évaporateur rotatif KNF RC 600) à 40 °C.

Ajout de parasites inactivés et co-culture avec des bactéries

Des cultures fraîches de promastigotes et d'amastigotes de *Leishmania infantum* ont d'abord été centrifugées à 2100 RPM pendant 10 minutes. Après élimination du surnageant, chaque culot a été remis en suspension dans de l'eau distillée et autoclavé à 121°C pendant 15 minutes afin d'inactiver les parasites.

La souche de *C. mamane* a été cultivée dans du MEB en présence de trois concentrations différentes d'amastigotes tués par la chaleur : C1=5.2 x 10⁵, C2=1.06 x 10⁶ et C3=2.12 x 10⁶ parasites/mL. La fermentation a été effectuée dans des flacons Erlenmeyer de 250 ml contenant 50 ml de MEB pendant 2 semaines à 27 °C et sous agitation constante. Toutes les conditions, y compris les contrôles négatifs, ont été réalisées en triplicata.

Après 2 semaines d'incubation, un partage liquide/liquide a été réalisé avec 50mL d'acétate d'éthyle sous 60 minutes de traitement aux ultrasons. Les mycéliums ont été retirés par filtration avec du coton de verre. Le surnageant a été filtré à l'aide de membranes en acétate de cellulose régénérée (0.45 µm) pour éliminer les débris cellulaires. Les phases organiques ont ensuite été déshydratées sur du sulfate de

magnésium et filtrées avec du papier filtre. Enfin, elles ont été évaporées à sec sous pression réduite (évaporateur rotatif KNF RC 600) à 40°C.

Pour les expériences de co-culture, les souches *Escherichia coli* ATCC® 25922™, *Pseudomonas aeruginosa* ATCC® 27853™ et *Staphylococcus aureus* ATCC® 29923™ ont d'abord été cultivées dans du TSA et incubés à 30°C pendant 24 h. Une colonie de chaque souche a ensuite été cultivée dans du bouillon LB à 30°C pendant 48 h à 150 RPM. En parallèle, *C. mamane* a été cultivé sur PDA pendant 7 jours avant d'inoculer 50 mL de PDB. Après 3 jours de culture à 27°C, 1 ml a été utilisé pour inoculer des flacons Erlenmeyer de 250 ml contenant 50 ml de PDB auquel sont ajoutés 200 µL de bouillon LB 48 h de chaque souche bactérienne. Ces co-cultures ont été mises à incuber à 27°C pendant 10 jours.

Un partage liquide/liquide a été réalisé avec 50mL d'acétate d'éthyle sous 60 minutes de traitement aux ultrasons. Les mycéliums ont été retirés par filtration avec du coton de verre. Les phases organiques ont ensuite été déshydratées sur sulfate de magnésium et filtrées avec du papier filtre. Enfin, elles ont été évaporées à sec sous pression réduite.

Les différentes conditions de culture utilisées dans ce travail sont résumées dans le **Tableau 1**.

Tableau 1. Résumé des conditions de culture pour la modification du métabolisme de *C. mamane*

| Etude | Milieu de culture | Temps d'incubation | Autres conditions |
|-----------------------------|-------------------|--------------------|--|
| Cinétique | MEA | 28 jours | 27°C |
| OSMAC | ISP2, CZA | 6 and 10 jours | 27°C, 24h de lumière et 24 h d'obscurité |
| Modificateurs épigénétiques | PDB | 14 jours | 27°C, sans agitation |
| Acides aminés | MEB | 14 jours | 27°C, 120 RPM |
| <i>Leishmania</i> inactivés | MEB | 14 jours | 27°C, 120 RPM |
| Co-culture | PDB | 10 jours | 27°C, sans agitation |

c) Evaluation de l'activité biologique

Un test d'activité antiparasitaire a été réalisé pour les extraits bruts obtenus à partir de la culture de *C. mamane* en présence des amastigotes thermiquement inactivés de *L. infantum*. Pour cela, les promastigotes de *L. infantum* ont été cultivés dans un milieu RPMI 1640 complété par 10% de sérum de veau foetal (FCS), 2 mM L-glutamine, 100 U/mL de pénicilline et 100 µg/mL de streptomycine, puis récoltés en phase logarithmique de croissance par centrifugation à 900 g pendant 10 min. Le surnageant a été soigneusement éliminé et a été remplacé par le même volume de milieu complet RPMI 1640 à pH 5,4 et incubé pendant 24 h à 24°C. Les promastigotes acidifiés ont ensuite été incubés pendant 24 h à

37°C dans un flacon ventilé pour transformer les promastigotes acidifiés en amastigotes axéniques (Sereno & Lemesre 1997). Les effets des extraits sur la croissance des amastigotes axéniques de *L. infantum* ont été évalués comme suit: des amastigotes de *L. infantum* ont été incubés (2×10^6 parasites/mL) dans des plaques stériles 96 puits avec chaque échantillon dissous dans du MeOH (concentration finale de 0.5 % v/v), en duplicata. Des témoins solvants (DMSO ou MeOH) et un contrôle positif (amphotéricine B), ont été ajoutés à chaque série d'expériences.

Après une période d'incubation de 48 heures à 37 °C, chaque puits de plaque a été examiné au microscope pour détecter toute formation de précipité. Pour estimer l'activité luciférase des amastigotes axéniques, 80 µL de chaque puits ont été transférés dans des plaques blanches à 96 puits, le réactif Steady Glow® (Promega) a été ajouté conformément aux instructions du fabricant, et les plaques ont été incubées pendant 2 min. La luminescence a été mesurée dans le compteur de luminescence Microbeta (PerkinElmer). La concentration efficace à 50 % (CE_{50}) a été définie comme la concentration du composé nécessaire pour inhiber de 50 % l'activité métabolique des amastigotes de *L. infantum* par rapport au contrôle. Les valeurs de la CE_{50} ont été calculées par analyse de régression non linéaire traitée sur des courbes dose-réponse, à l'aide du logiciel TableCurve 2D (version 5.0).

d) Analyse UHPLC-spectrométrie de masse

Des solutions méthanoliques à 2 mg/mL de tous les extraits ont été préparées pour l'analyse par chromatographie liquide à ultra-haute performance - MS. Des échantillons de contrôle de qualité (QC) ont été préparés en regroupant un aliquot de tous les extraits pour chaque expérience. Les analyses ont été réalisées sur un système UHPLC Ultimate 3000 (Dionex) couplé à un spectromètre de masse LTQ Orbitrap XL (Thermo Fisher Scientific, Hemel Hempstead, UK). Les échantillons ont été séparés sur une colonne C18 Acquity (100 × 2.1 mm i.d, 1.7 µm, Waters, MA, USA) équipée d'une colonne de garde. La phase mobile A était de l'eau ultrapure acidifiée avec 0.1% de FA et la phase mobile B était de l'acétonitrile acidifiée avec 0.1% de FA. Le gradient de solvant était le suivant : 0 min, 95 % A ; 10 min, 95 % B ; 12.5 min, 95 % B. Le débit était de 0.45 mL/min, la température de la colonne était réglée à 40 °C, la température de l'échantillonneur automatique était réglée à 15 °C et le volume d'injection fixé à 5 µL pour les extraits. La détection de masse a été réalisée à l'aide d'une source électrospray (ESI) en mode d'ionisation positive (PI) à un pouvoir de résolution de 15 000 [largeur totale à mi-hauteur (FWHM) à 400 m/z]. La plage de balayage de masse était de m/z 100-2000 pour tous les échantillons. La tension du spray d'ionisation était réglée à 3,5 kV et la température du capillaire était réglée à 300°C. Pour chaque scan MS complet, une fragmentation MS/MS des trois ions les plus intenses a été réalisée (mode « data dependant-scan ») en utilisant la dissociation induite par collision (CID) à 35 unités d'énergie arbitraires.

e) Analyse des données

Traitement des données

Les données LC-MS de chaque expérience ont été traitées séparément avec MS-DIAL version 4.7 (Tsugawa *et al.* 2015) pour l'extraction du signal de masse entre 100 et 1500 Da de 0.5 à 12.5 min. Les tolérances MS1 et MS2 ont été fixées à 0.01 et 0.025 Da, respectivement, en mode centroïde pour chaque ensemble de données. Les pics ont été alignés sur un fichier de référence QC avec une tolérance de temps de rétention (RT) de 0.05 min et une tolérance de masse de 0.025 Da. La hauteur minimale des pics a été fixée à 70 % en dessous de la ligne de base du chromatogramme d'ions totaux (TIC) observé pour les échantillons témoins. Les données MS-DIAL ont été nettoyées avec le flux de travail MS-CleanR en utilisant les paramètres par défaut (Fraisier-Vannier *et al.* 2020) : tous les filtres avec un rapport minimum de blanc fixé à 0.8, un écart-type relatif (RSD) maximum fixé à 30 et un défaut de masse relatif (RMD) allant de 50 à 3000. La différence de masse maximale pour la détection des relations entre les caractéristiques a été fixée à 0.005 Da et la différence de RT maximale a été fixée à 0.025 min. Les liens de corrélation de Pearson ont été considérés avec une corrélation $\geq 0,8$ et statistiquement significative $\alpha = 0.05$. Deux pics ont été conservés dans chaque cluster: le plus intense et le plus connecté aux autres ions.

Les caractéristiques conservées ont été annotées avec MS-FINDER version 3.5 (Tsugawa *et al.* 2016). Les tolérances MS1 et MS2 ont été fixées à 0.005 et 0.05 Da, respectivement. La recherche de formules chimiques a considéré les atomes C, H, O, N, P et S. L'annotation a été faite en utilisant les bases de données génériques internes de MS-FINDER: LipidMaps, YMDB, ChEBI, NPA, NANPDB, COCONUT, KNAPSack et UNPD. Seules les annotations ayant un score supérieur à 5 ont été retenues tout au long de l'analyse, tandis que les caractéristiques non annotées sont désignées comme "inconnues". Les données ont finalement été exportées sous forme de fichiers *.msp* pour les informations spectrales MSMS et de fichiers *.csv* pour les informations relatives aux métadonnées (surface des pics, temps de rétention, résultats des annotations, etc.).

Réseau moléculaire

Les fichiers MSP et de métadonnées générées après le flux de travail MS-CleanR ont été importés dans MetGem (version 1.3.4) (Olivon *et al.* 2018). Les données ont été filtrées en supprimant tous les pics MS/MS situés à ± 17 Da du *m/z* du précurseur. Les spectres MS/MS ont été filtrés par fenêtre en utilisant les 6 pics supérieurs dans la fenêtre de ± 50 Da dans tout le spectre. La tolérance des ions fragments MS/MS a été fixée à 0.2 Da. Un réseau de similarité de spectres de masse a été créé où les liens ont été filtrés pour avoir un score de cosinus supérieur à 0.7 et au moins six pics appariés. D'autres arêtes entre deux nœuds ont été conservées dans le réseau de similarité uniquement si chacun des nœuds apparaissait

dans le top 10 des nœuds les plus similaires de l'autre. Le réseau résultant a ensuite été importé dans Cytoscape (version 3.8.2, Institute for Systems Biology, Seattle, WA, USA) pour en améliorer la visualisation (Shannon 2003). Les nœuds ont été colorés en fonction de leurs conditions de culture et la taille des nœuds, quant à elle, indique leur surface de pic totale normalisée.

Analyses statistiques

Les analyses statistiques ont été réalisées en téléchargeant le fichier CSV sur la plateforme Metaboanalyst version 5.0 (<https://www.metaboanalyst.ca/>) (Chong *et al.* 2019). Les données ont été prétraitées, d'abord par normalisation (par la médiane) des échantillons, puis les variables ont été pondérées par auto échelle (centrée sur la moyenne et divisée par la racine carrée de l'écart-type de chaque variable). Une analyse en composantes principales (PCA) puis une analyse discriminante par moindres carrés partiels (PLS-DA) ont été réalisées. Enfin, le m/z et le temps de rétention des principales caractéristiques associées à leur aire de pic normalisée ont été reportés sur une carte thermique (regroupement hiérarchique) en utilisant l'ANOVA.

III. Résultats et discussions

Ce travail a porté sur l'étude des variations du métabolome sous différentes approches de culture d'une souche fongique endophyte de *Cophinforma mamane* (Botryosphaeriaceae), isolée de *Bixa orellana* (Bixaceae), une plante médicinale du Pérou. Cette thèse de doctorat est un travail qui poursuit les recherches menées par les docteurs Fatima Barakat et Asih Triastuti dans le cadre de leurs travaux de doctorat, et qui ont permis d'une part l'étude du métabolome de ce champignon (Triastuti *et al.* 2019, 2021) et d'autre part l'isolement de 4 composés antiparasitaires, les botryosulfuranols A, B, C (Barakat *et al.* 2019) et D (Barakat 2018) (données non publiées).

Nos principaux objectifs étaient les suivants :

- 1) Mieux comprendre la production du TDKP botryosulfuranols A-D et d'autres DKPs par des approches cinétiques, et d'induction de leur production notamment par l'ajout de modificateurs épigénétiques et d'acides aminés (bioprécurseurs) dans les milieux de culture

- 2) Induire la production de métabolites, notamment anti-infectieux.

Afin de mener à bien ce travail, différentes approches ont été suivies.

Une étude cinétique a été réalisée pour analyser les changements du métabolome au fil du temps, tandis que l'approche OSMAC a été appliquée afin d'observer l'influence des milieux de culture et de la

présence de lumière sur le profil chimique global de *C. mamane*. Pour tenter d'induire l'expression des gènes faiblement exprimés ou silencieux en conditions standards de laboratoire, différents éliciteurs chimiques ont été ajoutés à la culture fongique, dont trois modificateurs épigénétiques qui agissent au niveau des chromosomes (protéines histones) et une molécule impliquée dans le quorum-sensing bactérien (homosérine lactone). Afin de stimuler spécifiquement les voies de biosynthèse des alcaloïdes, y compris les TDKPs, botryosulfuranols A-D, les acides aminés L-tryptophane et L-phénylalanine ont été ajoutés comme précurseurs de biosynthèse dans la culture fongique.

Enfin, dans le cadre de la recherche de nouveaux anti-infectieux, *C. mamane* a été mis en co-culture avec 3 souches bactériennes pathogènes différentes. De plus, une méthodologie utilisant des parasites de *Leishmania* inactivés par la chaleur a été développée afin d'induire la production par *C. mamane* de métabolites antileishmaniens, y compris les TDKPs, botryosulfuranols A-D.

a) Modulation générale de la diversité chimique de *C. mamane*

Les méthodologies utilisées nous ont permis d'identifier différents groupes chimiques produits par *C. mamane* dans la plupart des conditions, comme les dicétopipérazines, les peptides cycliques, les acides gras à chaîne longue et moyenne, les eicosanoïdes, suivis des sphingolipides, des terpénoïdes, des quinones, des coumarines, des hydroxyridines et des benzénoïdes, qui sont les moins fréquents parmi les métabolites annotés. Parmi les méthodologies appliquées, l'ajout de modificateurs épigénétiques a été celle qui a le plus modifié le métabolisme général de *C. mamane*, et en particulier l'ajout de 5-azacytidine (un inhibiteur de l'ADN méthyltransférase) à la fois dans la régulation positive et négative des métabolites. De même, la culture de *C. mamane* dans le milieu de culture ISP2 pendant 10 jours dans des conditions lumineuses a semblé modifier le métabolisme général, à la fois dans la régulation positive et négative de nombreux métabolites, par rapport aux autres méthodologies utilisées.

L'un des principaux résultats, portant sur les métabolites annotés, nous a permis de constater que ce champignon endophyte produit un groupe de métabolites identifiés comme des cyclopentapeptides. Les pics chromatographiques correspondants ont été, dans la plupart des conditions, observés comme les pics principaux. L'étude cinétique nous a permis de mettre en évidence que ces métabolites apparaissent à un stade précoce (D4-D8) et diminuent rapidement après cette période. Cependant, dans une étude précédente portant sur la dynamique de la co-culture entre *C. mamane* et *Fusarium solani*, la production de ces cyclopentapeptides par les deux champignons avait été maintenue dans le temps après 10 jours de co-culture dans la MEA (Triastuti *et al.* 2021). Cette condition permettrait ainsi peut-être de favoriser la production de ces composés dans le temps, et ceci soulève l'hypothèse du rôle de ces composés dans les interactions fongiques.

De plus, la production de cyclopentapeptides a été régulée négativement en présence d'amastigotes de *Leishmania* inactivés par la chaleur, mais elle a été induite de manière significative en présence de bactéries vivantes, notamment *P. aeruginosa* (Gram-) et *S. aureus* (Gram +). Dans des études antérieures, les cyclopentapeptides fongiques ont présenté diverses activités biologiques contre d'autres champignons, bactéries, virus et parasites avec des applications potentielles (Wang *et al.* 2017). Cependant, leur rôle écologique a été moins discuté et à cet égard, certains auteurs ont suggéré leur rôle dans la communication inter-espèces, comme dans le cas des cyclopentapeptides produits par le champignon endophyte *Fusarium decemcellulare* (Li *et al.* 2016) ou les hexocyclopeptides produits par le champignon endophyte *Fusarium solani* (Wang *et al.* 2015). La régulation négative de la production de cyclopentapeptides par *C. mamane* en présence de parasites inactivés par la chaleur et la régulation positive en présence de bactéries vivantes, renforce leur rôle potentiel en tant que molécules de communication entre différentes espèces microbiennes, mais des recherches supplémentaires sont nécessaires afin de comprendre les mécanismes de déclenchement, comme cela est connu pour le processus de *quorum-sensing* bactérien.

b) Modulation de la production des TDKPs, botryosulfuranols A-D

Les méthodologies appliquées dans cette thèse de doctorat avaient également pour objectif d'induire la production de 4 TDKPs, précédemment isolées de *C. mamane*, les botryosulfuranols A-C (Barakat *et al.* 2019b) et D (données non publiées). Un résultat intéressant issue de l'étude cinétique est la production à court terme, par rapport aux autres TDKPs, du botryosulfuranol C (**Figure 3**), une TDKP qui possède un pont disulfure dans sa structure qui pourrait jouer un rôle important sur son activité biologique (Jiang & Guo 2011; Barakat *et al.* 2019b). Il est suggéré que l'organisme producteur de TDKPs avec pont disulfure pourrait activer des mécanismes de défense qui impliquent l'ouverture du pont disulfure pour éviter sa toxicité comme cela a été observé pour la biosynthèse de la gliotoxine produite par *Aspergillus fumigatus* (Scharf *et al.* 2014), ce qui pourrait expliquer la production à court terme de botryosulfuranol C observée chez *C. mamane*.

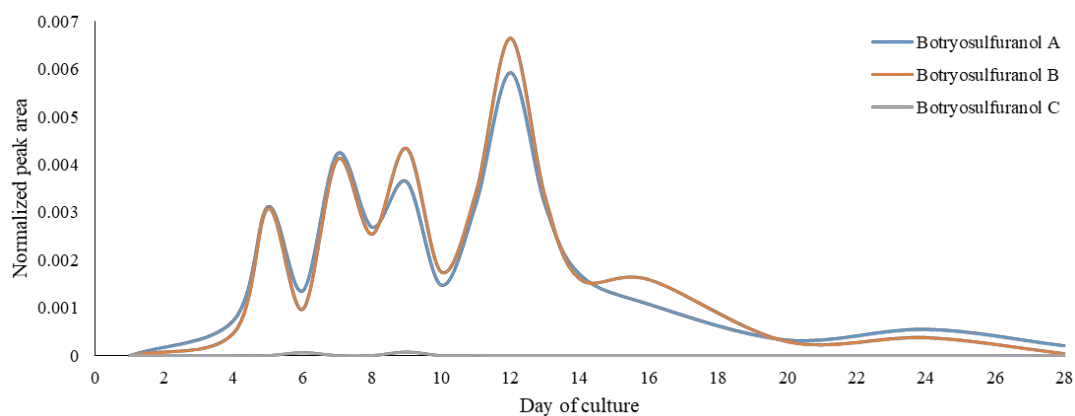


Figure 3. Tendance de production des TDKP en fonction de leur aire de pic normalisée

De plus, la culture de *C. mamane* dans ISP2 pendant 6 jours et dans des conditions d'obscurité a semblé exercer un effet retardateur sur la production de TDKPs pendant l'étude de l'approche OSMAC (**Figure 4**). Il est connu que les gènes codant pour ce type de composés répondent à différents signaux environnementaux, y compris la lumière (Brakhage 2013), tandis qu'une grande partie du génome fongique répond à différentes intensités et longueurs d'onde de la lumière (Yu & Fischer 2019). Ce résultat met en évidence que la lumière peut influencer la production de ces métabolites par *C. mamane* et que c'est un facteur important à prendre en compte lors de la culture dans des conditions de laboratoire et lors de fermentations à grande échelle dans des bioréacteurs au niveau industriel.

D'autres expériences impliquant différentes longueurs d'onde lumineuses lors de la culture de *C. mamane* seront envisagées.

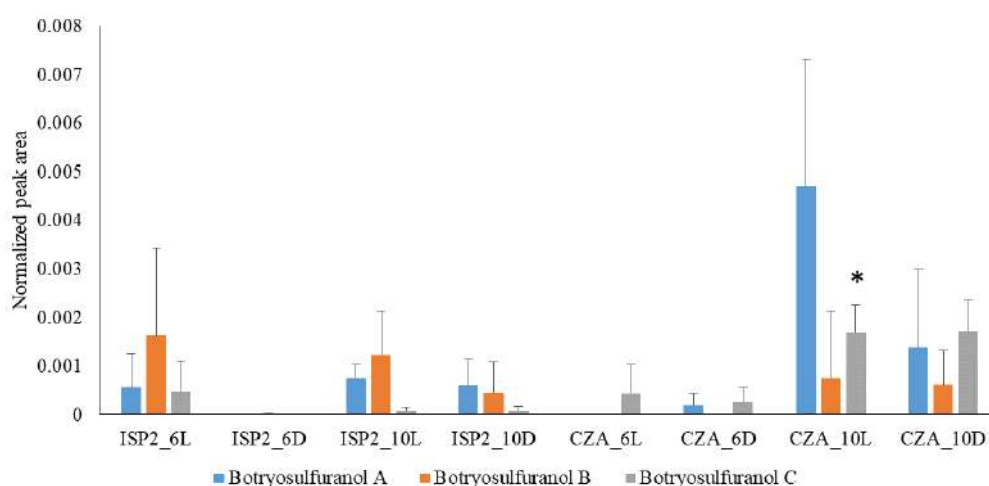


Figure 4. Aires de pic normalisées des TDKP botryosulfuranols A, B et C lors de la culture de *C. mamane* dans les milieux ISP2 et CZA pendant 6 et 10 jours dans des conditions de lumière (L) et d'obscurité (D). Les changements significatifs (test T avec $p < 0,05$) sont indiqués (*).

En ce qui concerne l'ajout de modulateurs chimiques, le DNMTi AZA a régulé négativement la production des botryosulfuranols A, B et C (**Figure 5**), indiquant que l'inhibition de la méthylation de l'ADN a un effet négatif sur leur production, soit directement, soit indirectement sur leur voie de biosynthèse. Cependant, l'ajout de HSL, qui est une molécule de quorum-sensing impliquée dans la communication des bactéries gram-négatives avec d'autres espèces, a induit de manière significative la production de botryosulfuranol C.

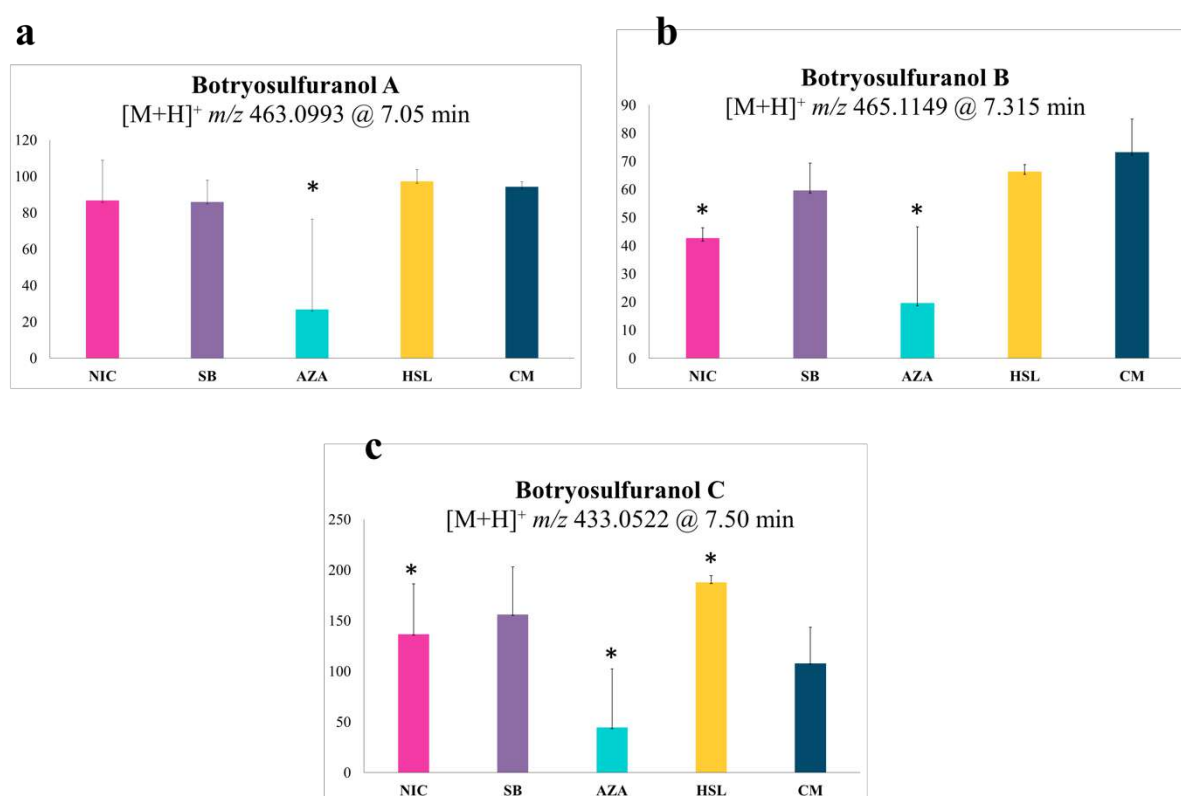


Figure 5. Aire de pic normalisée des TDKP (a) botryosulfuranol A, (b) botryosulfuranol B et (c) botryosulfuranol C détectés sous le traitement avec les modificateurs épigénétiques nicotinamide (NIC), butyrate de sodium (SB), 5-azacytidine (AZA) et homosérine lactone (HSL) en comparaison avec la culture non traitée de *C. mamane* (CM). Les changements significatifs (test T avec $p < 0,05$) sont indiqués (*).

Il est important de mentionner que HSL a non seulement induit de manière significative la production de ce TDKP mais également la production d'autres DKPs qui ont pu être annotées, dont la diphenylalazine B et la bisdethiobis(méthylthio)gliotoxine, soulignant que ces types de métabolites pourraient être impliqués dans la communication inter-espèces. Leur rôle en tant que molécules du QS avait été suggéré pour des DKPs produites lors d'interactions bactériennes entre *Cronobacter sakazakii* et *Bacillus cereus* (Bofinger *et al.* 2017) mais aussi pour les DKPs, emestrines A et B, produites par *Aspergillus fumigatus* en présence de HSL et lors de la co-culture avec *Streptomyces bulii* (Rateb *et al.* 2013).

Afin de mieux comprendre le rôle de ces composés dans les interactions entre microorganismes, la souche *C. mamane* E224 a été cultivée en présence de bactéries vivantes, et en présence de parasites inactivés. L'ajout de leishmanies inactivées par la chaleur à la culture de *C. mamane* a considérablement réduit la production de botryosulfuranol C (**Figure 6**), tandis que l'ajout de bactéries vivantes n'a pas affecté de manière significative sa production dans les conditions testées.

Au vu des résultats encourageants obtenus avec l'ajout de la molécule de QS, HSL dans le milieu de culture, des périodes de fermentation plus longues et des co-cultures avec d'autres espèces bactériennes et fongiques seront réalisées afin de vérifier l'influence des microorganismes vivants sur la production de TDKPs, et le rôle potentiel des TDKPs dans la communication microbienne.

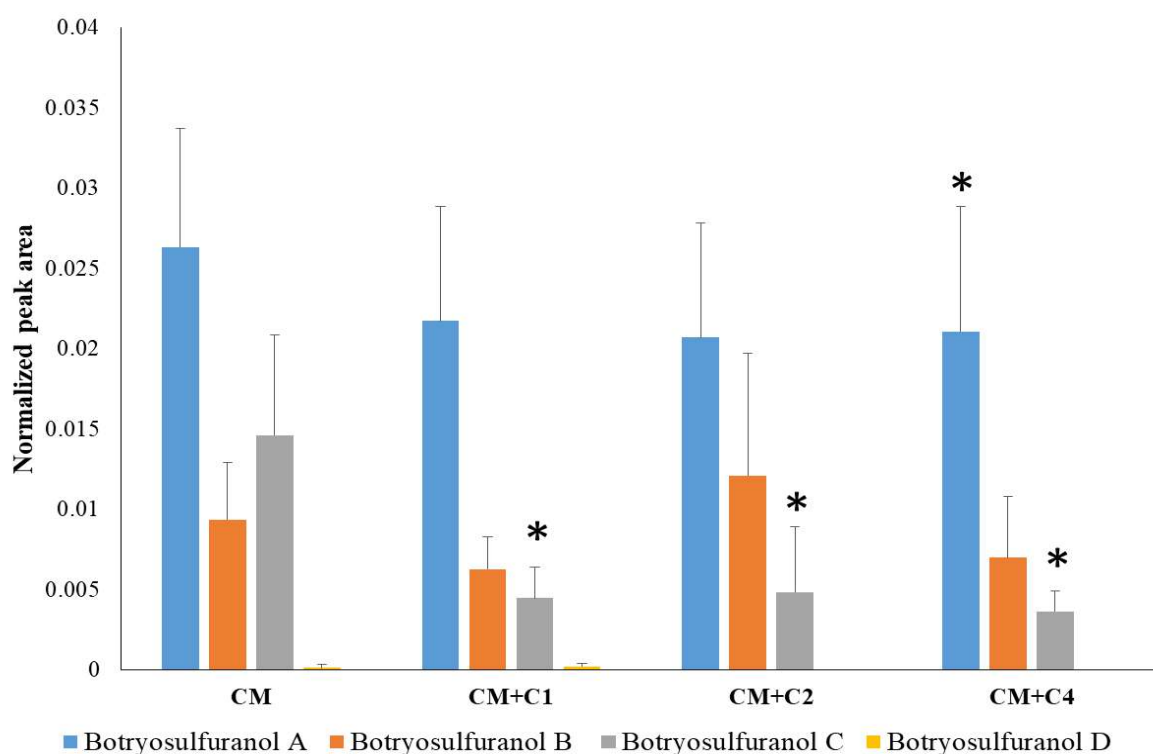


Figure 6. Aire de pic normalisée des TDKP botryosulfuranols A, B, C et D détectée sous l'addition d'amastigotes de *Leishmania* inactivés par la chaleur à 3 concentrations différentes (C1, C2 et C4) en comparaison avec la culture non traitée de *C. mamane* (CM). Les changements significatifs (test T avec $p < 0,05$) sont indiqués (*).

Par ailleurs, contrairement à ce qui était attendu avec l'ajout de deux bioprécurseurs de DKP, L-phe et L-try, la production de botryosulfuranols A-D a été régulée négativement par rapport à la culture non traitée (**Figure 7**), alors que ces métabolites sont biogénétiquement dérivés de deux résidus de phénylalanine (Barakat *et al.* 2019b). Plus généralement, l'ajout de ces deux acides aminés n'a pas semblé induire d'autres DKPs dérivées d'au moins une phénylalanine, tyrosine ou tryptophane (Welch & Williams 2014).

Des investigations supplémentaires sont nécessaires afin de comprendre les effets négatifs que ces acides aminés exercent sur la production de TDKP tandis que l'ajout de différents acides aminés à différentes concentrations sera également testé.

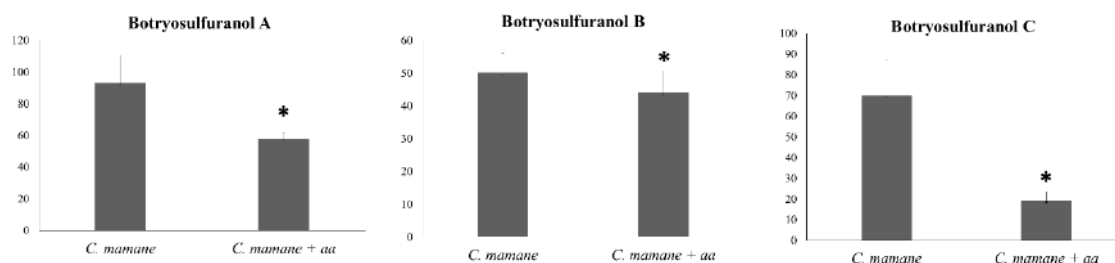


Figure 7. Aire de pic normalisée des botryosulfuranols A, B et C détectés sous l'addition d'acides aminés (*C. mamane* + aa) en comparaison avec la culture non traitée de *C. mamane*. Les changements significatifs (test T avec $p < 0,05$) sont indiqués (*).

En ce qui concerne le botryosulfuranol D, qui est l'un des TDKP les plus actifs contre *Leishmania infantum* par rapport aux botryosulfuranols A-C (données non publiées), il a présenté une faible production dans les différentes cultures réalisées au cours de ce travail et n'a pas été détecté dans la majorité des cas. Sa production semble donc suivre un modèle différent avec une étape cruciale sur sa voie de biosynthèse influencée par les conditions de culture et autres facteurs. Sa très faible détection n'a pas permis de l'inclure dans les analyses par réseau moléculaire (absence de données de fragmentation).

c) Induction de la production de métabolites anti-infectieux

L'ajout de parasites inactivés dans le milieu de culture ainsi que la co-culture avec des bactéries pathogènes ont modifié le métabolome global de *C. mamane* en régulant significativement un certain nombre de métabolites. Néanmoins, aucune famille chimique de métabolites n'a pu être identifiée comme étant spécifiquement influencée par chacune des conditions. Cette modification du métabolome lors de co-cultures a déjà été démontré que ce soit dans le cadre de co-culture champignon-champignon ou de co-cultures champignon-bactérie où des métabolites non produits dans des conditions axéniques ou faiblement détectés, ont été induits (Kamdem *et al.* 2018). Ceci peut s'expliquer par diverses interactions complexes concernant la compétition pour les nutriments ou les interactions antagonistes dans des environnements partagés (Deshmukh *et al.* 2018).

Différentes souches bactériennes pathogènes avec différents profils de résistance ainsi que la co-culture avec des endophytes précédemment isolés de *Bixa orellana*, conservés dans la mycothèque de l'UMR 152, seront considérées pour de futures expériences de co-culture.

De plus, toujours dans cette optique de recherche de nouveaux anti-infectieux, nous envisageons l'ajout de leishmanies vivantes dans la culture de *C. mamane* afin d'évaluer la différence avec les parasites tués par la chaleur. Les principales limites de cette méthodologie sont le pH et les nutriments du milieu de culture, ainsi que la température et le temps d'incubation, ce qui pourrait nécessiter une chambre de culture plus appropriée ou un dispositif différent pour la réaliser.

La métabolomique, utilisée tout au long de ce travail, représente une stratégie qui facilite et accélère les processus laborieux d'isolement des produits naturels et permet d'éviter la ré-isolement de composés connus en fournissant des informations MS/MS utiles pour l'élucidation structurale et la comparaison avec les bases de données disponibles (Demarque *et al.* 2020). Cependant, elle présente certaines limites et défis en raison de sa complexité inhérente, de la préparation des échantillons et de l'acquisition des données aux problèmes de reproductibilité et d'interprétation (Marshall & Powers 2017). En outre, l'absence de consensus dans les données structurales provenant de différentes plateformes et la faible disponibilité des bases de données gratuites peuvent générer de faibles taux d'annotation putative (Van Santen *et al.* 2019). Par conséquent, les études de déréplication manuelle continuent d'être une partie très importante pour compléter ce type de travail. En dépit de ses limites, la métabolomique nous a permis d'accéder à des informations qui n'auraient pu être dévoilées, notamment concernant les métabolites minoritaires. Cette approche relativement nouvelle utilisée dans la découverte de produits naturels s'est ainsi souvent avérée efficace pour réduire de grands ensembles de données en des ensembles beaucoup plus petits qui contiennent potentiellement des métabolites nouveaux ou actifs (Caesar *et al.* 2019). Une optimisation de cet outil, un focus sur certains métabolites spécifiques, ainsi qu'une augmentation du nombre de réplicats seront envisagées pour les futures expériences avec *C. mamane* et les métabolites produits par cette souche endophyte.

IV. Conclusions et perspectives

Les résultats obtenus dans le cadre d'une analyse métabolomique non ciblée nous fournissent des informations directes et utiles sur ce qui est produit par les cellules au cours de leur métabolisme, dans ce cas, ce que la souche endophyte *C. mamane* E224 produit à un moment précis dans des conditions de culture spécifiques, ce qui nous donne un aperçu de son état physiologique et de son activité cellulaire.

L'intégration de ces informations avec d'autres techniques -omiques telles que la génomique, la protéomique et la transcriptomique, qui relèvent du domaine de recherche de la biologie des systèmes, permettra d'obtenir une compréhension plus holistique des micro-organismes et même des communautés, en particulier pour les micro-organismes endophytes, ce qui représente en même temps un défi permanent (Pinu *et al.* 2019). En ce sens, la compréhension des réponses physiologiques de *C. mamane* aux changements environnementaux et aux différentes conditions de culture peut conduire à l'activation ou à la manipulation de voies spécifiques. Ceci est utile pour la recherche de métabolites

spécifiques, principalement pour déterminer les conditions optimales de cultures, y compris le temps d'incubation optimal pour leur production et leur extraction, comme c'est le cas dans ce travail avec les TDKP.

En plus de cela et des expériences supplémentaires nécessaires citées précédemment, l'isolement des métabolites induits les plus significatifs ainsi que des métabolites chimiquement liés aux TDKPs annotés et révélés par les réseaux moléculaires réalisés, sera également au centre des travaux futurs. La compréhension de leurs mécanismes biologiques et de leurs rôles écologiques potentiels dans la nature fera également l'objet de notre attention.

Par ailleurs, il est important de noter que grâce au projet de pré-maturation soutenu financièrement par la région Occitanie, et à la collaboration avec le Critt Bioindustries, une fraction enrichie en TDKPs a été obtenue à partir d'extraits de cultures de *C. mamane* en fermenteurs à l'échelle semie-industrielle. Ceci a permis de commencer les tests *in vivo* sur souris et permettra d'approfondir l'étude de l'activité biologique *in vivo* des botryosulfuranols A-D.

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INTRODUCTION

Natural products have been historically used for the development of drugs and drug leads. Currently, nearly 50% of the total small molecules approved as drugs, between 1981 and 2019, are derived from natural products or from their synthetic variations (Newman & Cragg 2020). Most of the clinically relevant drugs are derived from microorganisms, including bacteria, actinomycetes and fungi (Hutchings *et al.* 2019) with fungal species accounting for 45% of the microbial metabolite production (Bérdy 2012). This highlights the importance of microbial natural products even nowadays in 2021.

However, the arsenal of drugs with antimicrobial activity are becoming very limited while the increasing emergence of microbial resistant strains lead us to alarming predictions of 10 million of deaths by 2050 along with an enormous economic burden for the health care systems around the world (Browne *et al.* 2020). In addition to this, the World Health Organization (WHO), reported only 6 drugs in clinical development, addressed to the list of priority pathogens that were considered innovative. In order to face this problematic, new drugs with novel structural scaffolds and/or new mechanisms of action are needed, and they can potentially be found in unusual sources as in little explored or unexplored environments such as endophytes (Challinor & Bode 2015).

Endophytes are a group of microorganisms that successfully colonize internal plant tissues without generating visible harmful effects. This occurs as a result of multiple compatible interactions before, during and after invasion of the host plant, involving cross-talk signal molecules with the plant itself and its microbiome (Khare *et al.* 2018). It is considered that almost all plants on Earth are in constant and close interaction with endophytes (Arnold 2007). Despite of this, only 1-2% of all the known plants have been studied for their endophyte composition (Strobel 2018).

Most of the isolated and studied endophytes correspond to endophytic fungi which are capable of producing chemically diverse compounds (Gao *et al.* 2018; Zheng *et al.* 2021) with antibacterial, antifungal, anti-parasitic, antiviral, anticancer, antioxidant, immunosuppressive and antidiabetic properties (Manganyi & Ateba 2020; Adeleke & Babalola 2021). Many of these compounds represent potential applications in various areas such as public health, agriculture and pharmaceutical industries (Gupta *et al.* 2020a) with a total of 245 patent applications reported, between 2001 and 2019, involving secondary metabolites production and biotransformation processes (Torres-Mendoza *et al.* 2020). In this regard, endophytic fungi represent a renewable and effective source of natural products.

In this study, we focused on an endophytic fungal strain of *Cophinforma mamane* (D.E. Gardner) A. J. L. Phillips & A. Alves (Botryosphaeriaceae) isolated from the leaves of the medicinal plant *Bixa orellana* (Bixaceae). The collection of the leaves samples was carried out in the Amazonian forest of Peru allowing the isolation of 17 endophytic fungal strains belonging to the following genera: *Diaporthe*, *Nigrospora*, *Phyllosticta*, *Endomelanconiopsis*, *Sporothrix*, *Xylaria*, and *Cophinforma*

(unpublished data). A crude extract of the strain E224 (*Cophinforma mamane*) showed *in vitro* antileishmanial activity against *Leishmania infantum* (IC₅₀ value of 1.96 µg/mL) and led to the isolation of four novel thiodiketopiperazines (TDKPs) alkaloids, botryosulfuranols A, B, C and D (Barakat 2018). These compounds present sulfur atoms on α- and β-positions of the phenylalanine residues and two unprecedented spirocyclic centers at C-4 and C-2 (Figure 1). Antileishmanial activity of all the TDKPs were evaluated *in vitro* against the axenic amastigotes of *Leishmania infantum*. Botryosulfuranol D exhibited the strongest antileishmanial activity with an IC₅₀ value of 0.03 µM and a selective index (SI) of 190 followed by botryosulfuranol C (IC₅₀ = 0.44 µM); botryosulfuranol A (IC₅₀ = 0.69 µM) and botryosulfuranol B (IC₅₀ = 3.87 µM) (unpublished data). Botryosulfuranol A, B and C exhibited weak to moderate cytotoxic activity (GI₅₀ = 8.0 – 115.7 µM) against 4 cancer lines (Barakat *et al.* 2019). Although these new compounds presented interesting antileishmanial activity, the small isolated quantities did not allow a deeper investigation of their biological activity.

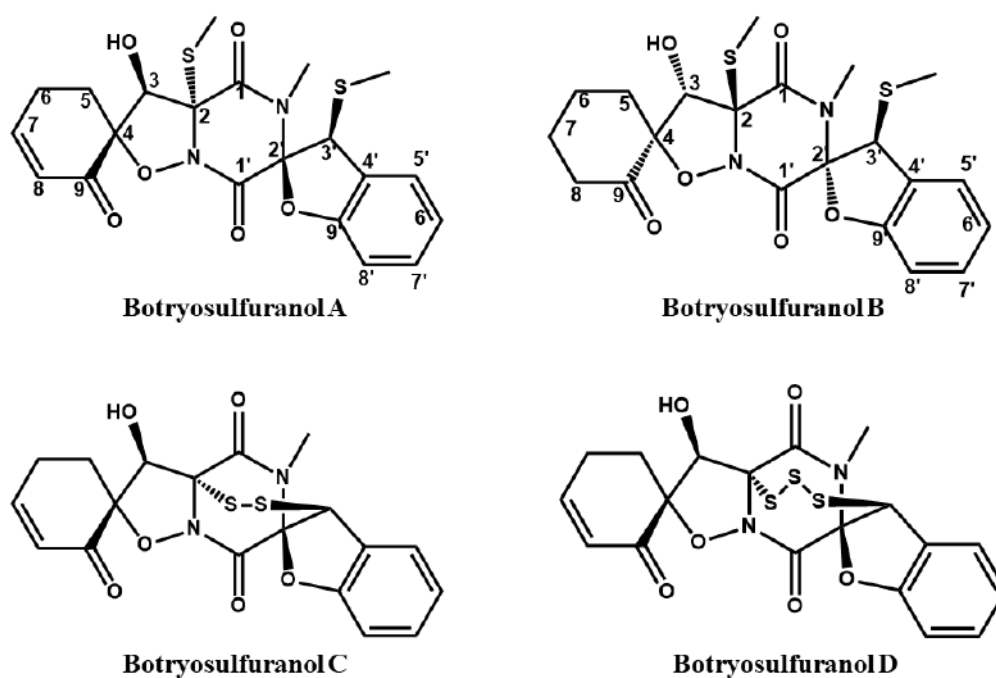


Figure 1. Chemical structure of thiodiketopiperazines, botryosulfuranols A - D

A pre-maturation project, financially supported by the Occitanie region, has been launched with the objective to continue on further research on biological activity through *in vivo* assays. This is performed through a collaboration with Critt Bioindustries who will take over the fermentation of the fungus on a large scale. In parallel to this project in which I took part, especially for the extraction and isolation of

the botryosulfuranols, a study on the metabolism changes under different culture conditions of this fungal endophyte has been performed as my doctoral research work.

Although the isolated compounds mentioned above have been obtained by cultivating the E224 strain of *C. mamane* under standard culture conditions, it is known that in fungi, the number of gene clusters is greatly superior to the number of gene clusters known from the isolated secondary metabolites (Pettit 2011) due to the presence of silent or lowly-expressed genes (Brakhage & Schroeckh 2011). Considering this, different approaches have been applied for the induction of the secondary metabolite in fungi, such as cultivation-based methodologies including OSMAC approach, co-culture with other microorganisms and the addition of chemical elicitors to the culture media, including epigenetic modifiers. In this regard, the metabolism of *C. mamane* E224 has been previously studied under the influence of two epigenetic modifiers (suberoylanilide hydroxamic acid and sodium valproate) (Triastuti *et al.* 2019) and in co-culture with *Fusarium solani* (Triastuti, 2021), identifying in both cases metabolites induced *de novo*.

In order to avoid the bottleneck of re-isolation of known compounds, metabolomics is a powerful tool for the analysis, identification and quantification of small metabolites (Zhao *et al.* 2018). Mass Spectrometry (MS) is the most widely used technique due to its high sensibility, small samples volume, the possibility of coupling with chromatographic techniques, acquisition of MS/MS fragmentation data for structural elucidation followed by a comparison with specific databases. Additionally, the association with computational and statistical tools can offer a better view that simplifies the vast data analyses (Demarque *et al.* 2020). Specifically, Untargeted Metabolomics, focuses on the detection of as many metabolites as possible to elaborate a comprehensive data set in order to better understand the metabolome of the object under study.

After a literature review prepared for the present doctoral thesis in **Chapter 1**, we applied different culture-based and epigenetic approaches (detailed in **Chapter 2**) to alter the secondary metabolism of an endophytic fungal strain of *Cophinforma mamane* (Botryosphaeriaceae) isolated from the medicinal plant *Bixa orellana* (Bixaceae). Special importance has been given to the 4 bioactive thiodiketopiperazines previously isolated in our team by F. Barakat (Barakat *et al.* 2019). The two main goals of this study are:

1. A better understanding of the production of the botryosulfuranols previously isolated and more generally, the induction of diketopiperazines and other alkaloids through kinetic and OSMAC approach including the use of epigenetic modifiers and biosynthetic precursors (**Chapter 3** and **Chapter 4**).
2. The production of new compounds, especially anti-infective metabolites through the co-culture of *C. mamane* with pathogenic bacterial strains, and the culture with inactivated parasites (**Chapter 5**).

UHPLC – Mass Spectrometry analysis and untargeted metabolomics tools have been used in order to reach our goals and they will be detailed and explained in **Chapter 2**, corresponding to materials and methods.

In order to respond to our first objective, a time-variations study of secondary metabolite production was carried out. This analysis was followed by the application of the OSMAC approach consisting in using different culture media and different light conditions as factors that may induce the secondary metabolism of this endophytic fungus. These results will be detailed and discussed in **Chapter 3**. Moreover, the induction of secondary metabolism under the treatment with chemical elicitors such as epigenetic modifiers to stimulate the transcription of lowly-expressed or silenced genes has been performed, as well as the addition of amino acids in the culture to stimulate specific biosynthetic pathways that utilize these building blocks for the production of secondary metabolites. These results will be detailed and discussed in **Chapter 4**.

Finally, to respond our second objective, heat-killed leishmanial parasites were added to the culture of the endophytic fungus in an attempt to simulate the presence of other microorganism that might induce the production of antiparasitic secondary metabolites. Co-cultures of *C. mamane* with living pathogenic bacterial strains have also been performed in order to induce the production of antibacterial metabolites. These results will be detailed and discussed in **Chapter 5**.

CHAPTER 1 LITERATURE REVIEW

1.1 Natural product research

1.1.1 Implications of antibiotic resistance in natural product research

The use of natural products as medicinal agents can be traced back to the records found in Mesopotamia (2600 BCE) where several substances derived from plants, used even today for many ailments, were described. The best known Egyptian record, the Ebers papyrus (1500 BCE), describes also several natural drugs and their usage (Newman *et al.* 2000) while traditional medicine from China (1100 BCE) (Petrovska 2012) and India (1000 BCE) (Dev 1999) are the best documented records, mentioning also hundreds of herbal drugs and prescriptions. Anthropological studies have proven that antibiotic exposure started before the antibiotic era due to the findings of tetracycline traces in human skeletal remains of ancient populations from Sudanese Nubia (350 – 550 CE) (Nelson *et al.* 2010). This was possible because tetracyclines, naturally produced by *Streptomyces* spp. (Actinomycetes), are potent chelators. Therefore they can bind to the bones and teeth and act as permanent markers, unlike other antibiotics that are difficult to trace (Aminov 2010).

Another known event is the first hospital use of a drug called Pyocyanase, prepared as an extract of *Pseudomonas aeruginosa*, active against different pathogenic bacteria but with inconsistent results and toxic effects (Emmerich & Löw 1899). In the early 1900s, Paul Ehrlich began to screen systematically a treatment for syphilis and several years later, a drug sold under the name of Salvarsan (later Neosalvarsan) showed great success treating this disease and it became frequently prescribed (Ehrlich & Hata 1910). Close to this date and thanks to the systematic approach introduced by Ehrlich, a new drug called Prontosil (sulfa drug) possessing a sulfanilamide active moiety, already used in the dye industry, was synthesized by Bayer chemists. The massive production of sulfonamide derivatives made these drugs the oldest antibiotics on the market (Domagk 1935). In 1928, the discovery of penicillin took place, considering that the antimicrobial activity of the mold was previously observed (Fleming 1929). Despite the difficulties that Alexander Flemming found in the purification and stability of the active substance, its production and distribution was finally achieved in 1945. Thanks to the sufficient quantities of penicillin obtained by Howard Florey and Ernest Chain and to the screening method using inhibition zones applied by Flemming, this antibiotic became widely used in academia and industry (Chain *et al.* 2005).

Between the early 1930s and late 1960s, most of the new classes of drugs were discovered, corresponding to the golden era of antibiotics (**Figure 1.1**). This period was followed by an important decline in drug discovery rate along with the increase and emergence of resistant pathogens (Aminov 2009). Four years before the introduction of penicillin as a therapeutic agent, a bacterial enzyme called

penicillinase (β -lactamases) was identified (Abraham & Chain 1988), an event that led the resistant strains to become prevalent by the time. In 1960, this situation became a pandemic problem and despite the introduction of new drugs, new resistant strains continued to appear, in addition to the first reported cases of methicillin-resistant *Staphylococcus aureus* (MRSA) in 1961 in UK and in 1968 in US (Johnson 2011). The antibiotic vancomycin was introduced in 1958 but the first cases of resistance to this antibiotic were reported for the first time in 1979 and in 1983 (Srinivasan *et al.* 2002). Similarly, antibiotic resistance to other antimicrobials was detected for the first time for enteric bacteria such as *Salmonella*, *Shigella* and *Escherichia coli*, as well as for the strains of *Mycobacterium tuberculosis* resistant to streptomycin, strains of *Neisseria gonorrhoeae* and *Haemophilus influenzae* resistant to ampicillin, tetracycline and chloramphenicol, among many other cases (Aslam *et al.* 2018).

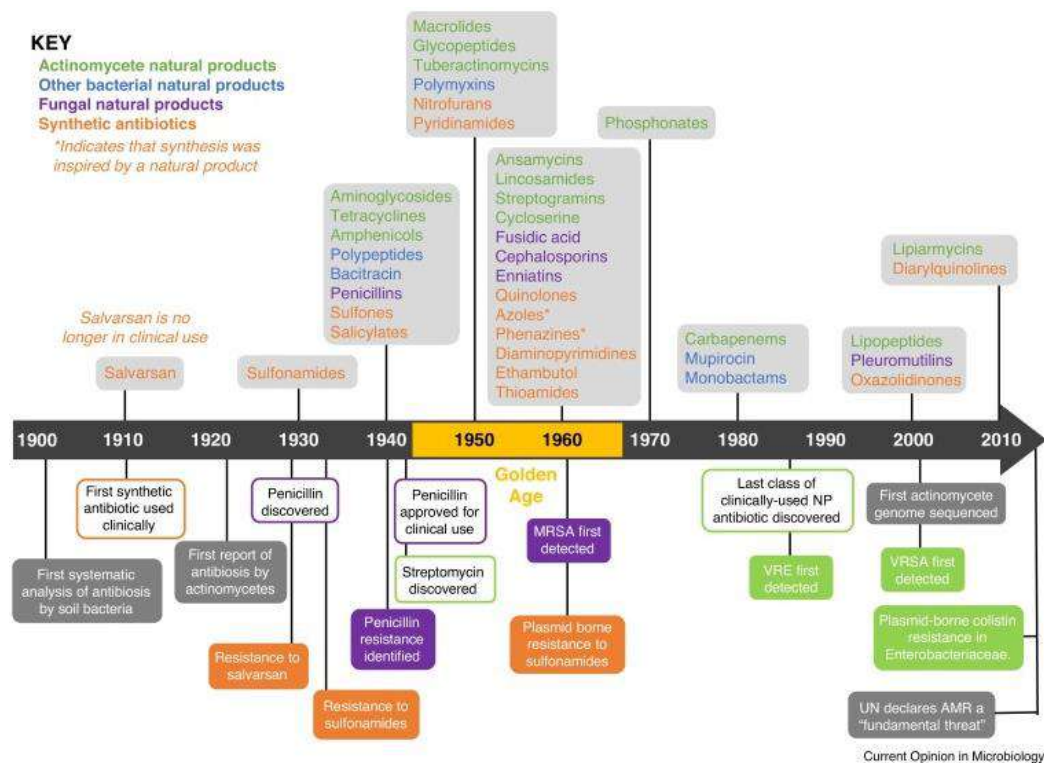


Figure 1.1 Timeline of antibiotics discovery and antimicrobial resistance reports, classified by their source in colors: bacterial, fungi and synthetic (Hutchings *et al.*, 2019)

Resistant phenotypes existed before the introduction of antibiotics in clinical treatments due to the finding that most of the antibiotic-producing strains carry the genes encoding resistance to the antibiotic produced (Hopwood 2007). This means that most of the microbial strains able to produce antibiotics, possess genes that confers them the resistance to the produced antibiotic. However, anthropogenic activity, such as agriculture and industry, caused the massive use of lethal concentrations of antibiotics and contributed to the rapid dissemination of resistant pathogens in a shorter period of time (**Figure 1.2**)

(Davies & Davies 2010). It is important to mention that antibiotic resistance occurring in nature and that one inflicted by human activity possess different dynamics due to the multiple microbial interactions occurring in natural microenvironments (Chait *et al.* 2012). On the other hand, pathogens are also exposed to sub lethal concentrations of antibiotics in clinical situations when the treatment has not been completed or due to low drug availability in tissues while during human activity, the use of animal manure containing sub lethal concentrations of antibiotics are released to the soil and water. Low levels of antibiotics also contribute to the dissemination of resistance, demonstrated by different studies carried out in bacteria (Sengupta *et al.* 2013).

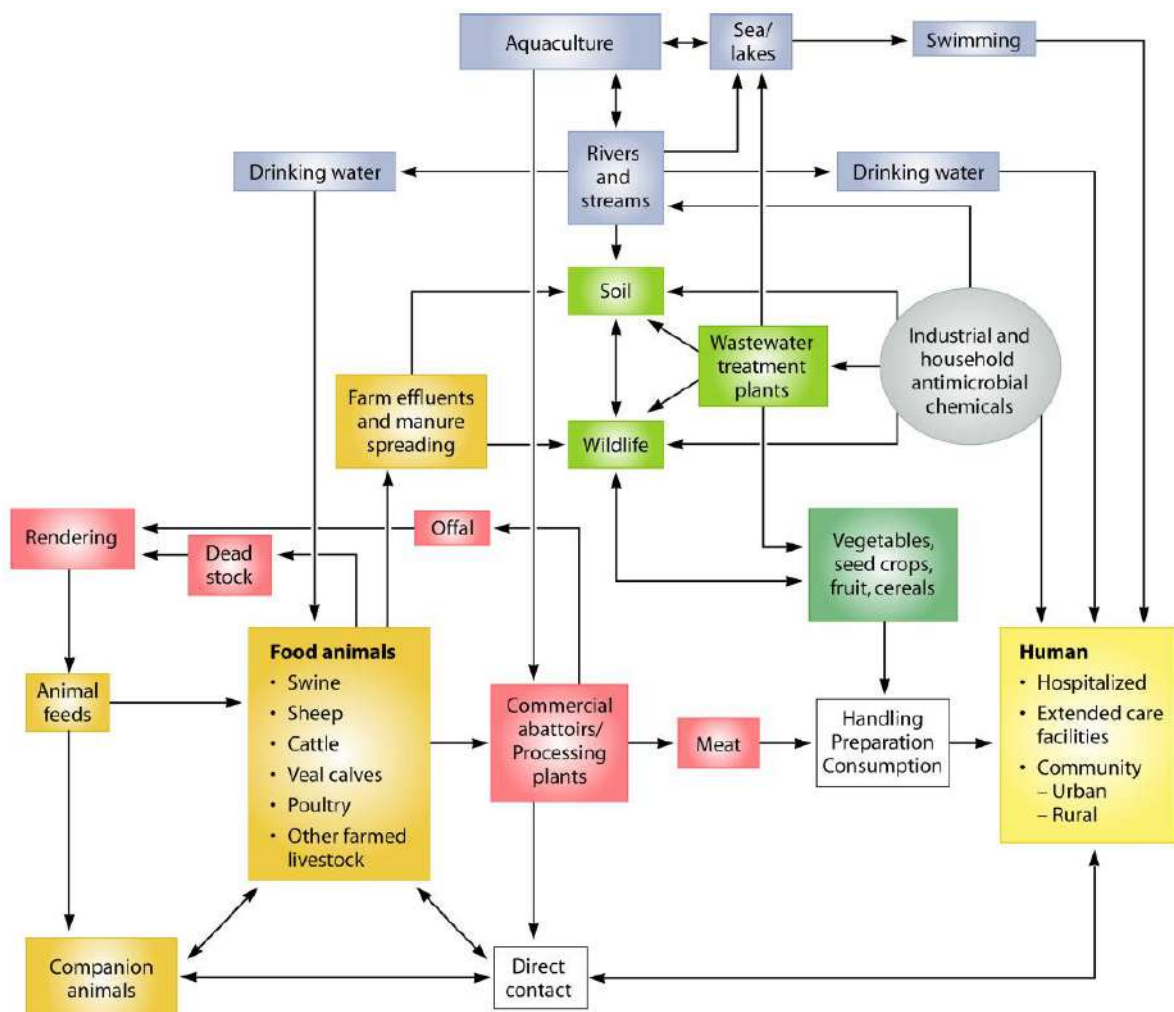


Figure 1.2. Human dissemination pathways of antibiotics contributing to antibiotic resistance (Davies et al., 2010)

Several investigations have shown us the biological properties that natural products possess and the useful applications in health and industry they can confer (Khan 2018). As these compounds are mainly produced in the late stationary growth phases, they have been called secondary metabolites, however the investigations about their different roles in biology and ecology are rare and remain largely unknown

(Davies & Davies 2010). In addition to their inhibitory activity, antibiotics might also act as signaling molecules in interspecies competition or in host-parasite interactions (Aminov 2009) and therefore they might help us to understand the complex interactions occurring in different ecological niches.

The discovery of antibiotics unquestionably revolutionized human history saving countless lives, allowing also its current use in animals and plants. Human exposure to antibiotics through history added to the extensive use of antimicrobials and the intrinsic resistance found in microbial genomes, lead us to alarming predictions of 10 million of deaths by 2050 with an enormous economic burden caused by antibiotic resistance (Browne *et al.* 2020). Most of the current new drugs for clinical use are based on semisynthetic modifications of existing chemical scaffolds due to the decline of drug development (Genilloud 2014). The urgent need for new antibiotics with novel structural scaffolds can be found in natural products produced by unusual sources in little explored or unexplored environments (Challinor & Bode 2015).

It is known that the major examples of antimicrobial resistance concern bacterial infections, but, what about the resistance cases occurring in protozoal diseases? Protozoal diseases such as malaria (caused by *Plasmodium* spp.) (**Figure 1.3**), leishmaniasis (caused by *Leishmania* spp.) (**Figure 1.4**), and trypanosomiasis (caused by *Trypanosoma* spp.) (**Figure 1.5**) affect mainly tropical and subtropical regions with great health and economic impacts worldwide. Antibiotic resistance involves natural environmental changes and proliferation of infections occurs mainly due to human activities. The absence of effective vaccines and the development of resistance to the current drugs situates these infections as major problems in endemic countries and highlight the need of novel molecules acting on new targets.

In the case of malaria, *Plasmodium falciparum* and *Plasmodium vivax* are responsible for most severe infections and most cases of antibiotic resistance (Tjitra *et al.* 2008). The first line recommended by the World Health Organization (WHO) is the Artemisinin Combination Therapy (ACT), which acts against the intraerythrocytic stages of *Plasmodium* parasites however it presents most of the cases of antibiotic resistance. Other drugs corresponding to the classes of naphthoquinones (atovaquone), quinolines (chloroquine), endoperoxides (artemisinin) and antifolates (pyrimethamine, sulfadoxine) present mutations in specific genes of the parasite that are responsible for antibiotic resistance (Capela *et al.* 2019).

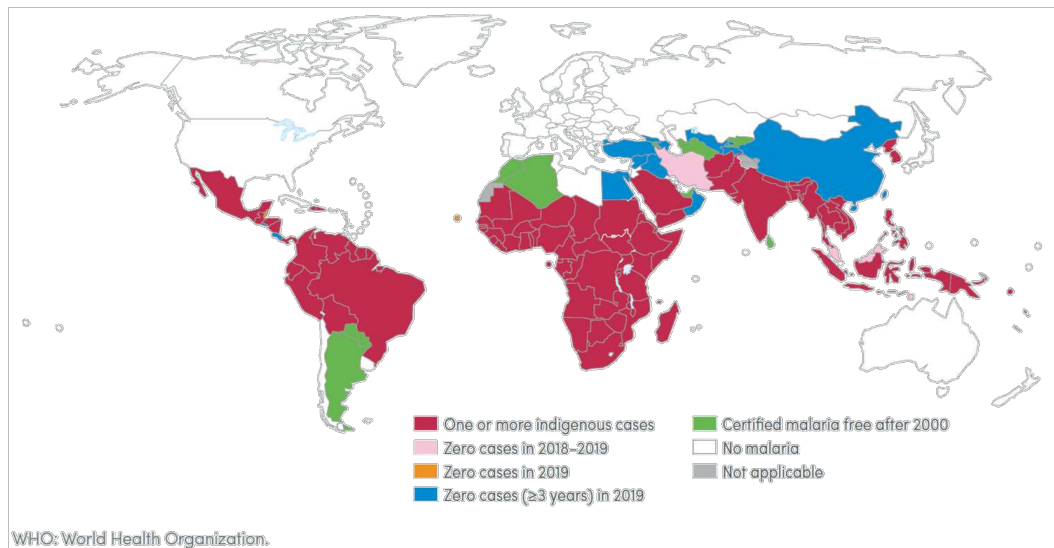


Figure 1.3. Countries with indigenous cases of malaria in 2000 and their status by 2019 (World Malaria Report 2020)

Leishmaniasis is a complex disease endemic in more than 98 countries (**Figure 1.4**) caused by more than 50 species from the *Leishmania* genus, causing visceral (VL), cutaneous (CL) or mucocutaneous leishmaniasis (MCL). VL is a form of infection that can remain in the skin for a lifetime and therefore act as a reservoir for a potential relapse. If this occurs in a person with a compromised immune system, the reactivation can be fulminant (Burza *et al.* 2018). For this disease, pentavalent antimonials are the standard treatment, currently compromised by antibiotic resistance while second-line (pentamidine and amphotericine B) and third-line (miltefosine) drugs are showing emerging resistance and toxicity (Capela *et al.* 2019).

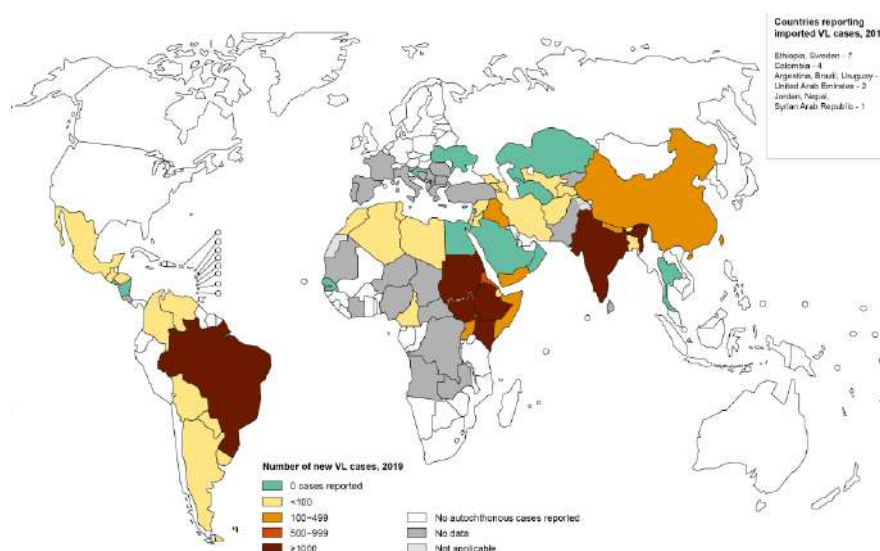


Figure 1.4. Status of endemicity of visceral leishmaniasis worldwide in 2019 (WHO Control of Neglected Tropical Diseases, 2021)

African trypanosomiasis is caused by two clinical variants *Trypanosoma brucei gambiense* (West and Central Africa) and *Trypanosoma brucei rhodesiense* (Eastern and Southern Africa) whose burden has currently decreased due to the efforts of various organizations (Barrett 2018). Parasite *T. b. rhodesiense* causes a more aggressive disease while *T. b. gambiense* develops a slower disease however, both parasites develop bloodstream and lymphatic stages and they are able to cross the blood-brain barrier into the central nervous system. For this reason, pentamidine and suramin (low toxicity) are drugs recommended on the first stage of infection while melarsoprol (high toxicity) is recommended for the second stage of infection due to its capacity to cross the blood-brain barrier. Despite the low rate of drug resistance in trypanosomiasis, compared to other infectious diseases, pentamidine and melarsoprol have shown increased emergence resistance and there is an urgent need for new treatments.

American trypanosomiasis or Chagas disease, on the other side, is caused by *Trypanosoma cruzi*, being endemic in Latin America (Figure 1.5) but infecting animals from central Argentina to the southern United States. In this case, most mechanisms of resistance of *T. cruzi* involve the immune system evasion, establishing a persistent infection with low parasite levels in the host referred as disease tolerance (Chevillard *et al.* 2018). In addition to this, resistance to nifurtimox has been also observed for Chagas' disease while the effects of fexinidazole as a new treatment have been recently evaluated (Deeks 2019).

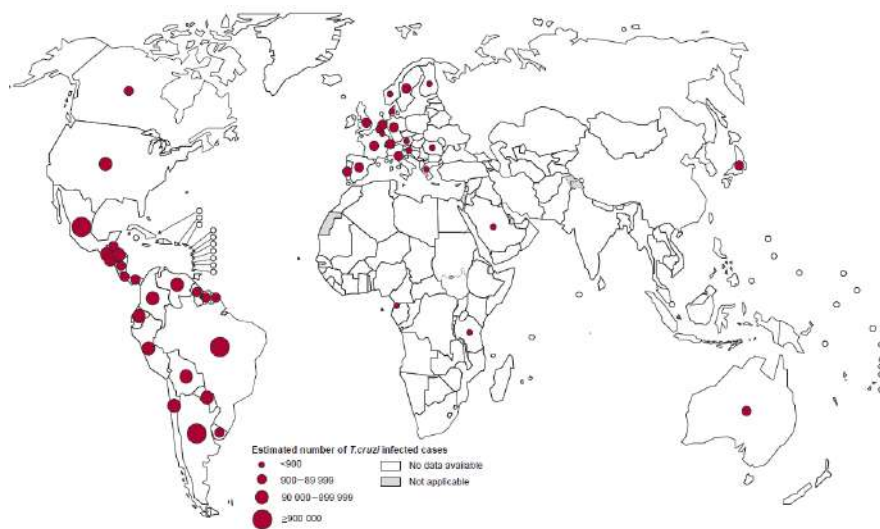


Figure 1.5. Global distribution of cases of Chagas disease worldwide in 2018 (WHO Control of Neglected Tropical Diseases, 2018)

To overcome the resistance problem for tropical parasitic diseases, especially in endemic countries but also for looking for eradication, different approaches are needed. Besides better understanding the mechanisms of action and resistance of drugs in order to design more appropriate alternatives, new

molecules that focus on new targets have to be investigated in order to increase the chemotherapeutic options with less undesirable effects.

1.1.2 Natural products as the main source of novel chemical structure

Natural products have been traditionally used throughout human history for treating illnesses and diseases and even nowadays, they continue to be lead compounds for drug discovery despite the fewer interest shown by the pharmaceutical industry due to the challenges found on their development, the increasing rediscovery of compounds, among other reasons (Molinski 2014). Some specific attributes of the chemical structure of natural products are the higher number of stereochemical centers and the fewer aromatic rings, compared to the drug leads provided by robotic and combinatorial chemistry providing structural analogues. In addition to this, more than 80% of the core ring scaffolds present in natural products were not present in molecules deriving from screening libraries or in those commercially active (Hert *et al.* 2009). These attributes of natural products provide the molecular complexity that has shown to be more successful in clinical trials, specifically with anticancer and antimicrobials properties (Dias *et al.* 2012). The latter may be also due to their capacity of acting as substrates or transporters therefore, inherently falling in the biological relevant space compared to the libraries of synthetic compounds that may possess appropriate physicochemical properties, such as permeability and bioavailability, but not necessarily be biologically active over the target (Kellenberger *et al.* 2011).

To have a better visualization of the importance of natural products in the development of new drugs, a follow-up study has shown the main sources of the approved drugs since 1981 until 2019 (Newman & Cragg 2020). The most interesting result is that the use of natural products or synthetic variations using their novel structures represent 50% of the total small molecules approved as drugs ($n=1394$), corresponding to: N: unaltered natural product (5.1%); NB: botanical drug (1%); ND: natural product derivative (27.5%); S*: synthetic with natural product pharmacophore (4.7%) and S* with NM: mimic of natural product (14.8%) (**Figure 1.6**).

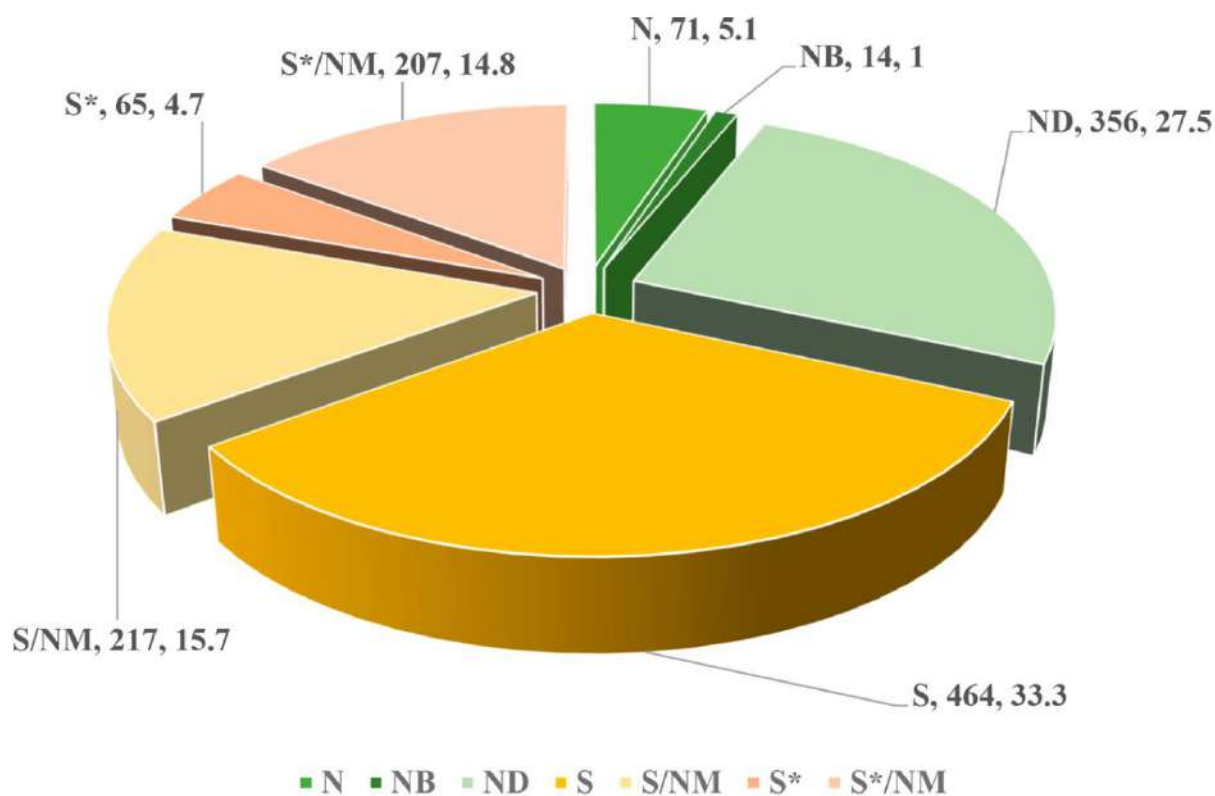


Figure 1.6. Small molecules approved as drugs from 1981 until 2019 from all source categories, n=1394. N: unaltered natural product; NB: botanical drug; ND: natural product derivative; S: synthetic; S*: synthetic with natural product pharmacophore; NM: mimic of natural product (Newman & Cragg, 2020)

As a result of the use and research of natural products, there are also important contributions in different aspects and disciplines such as the development of economies from resource-rich nations and the implementation of alternative medicines, elaboration of consumables (dyes, essential oils), production of fibers and polymers and the development of different other areas of health sciences (Khan 2018). As a consequence, human activities have endangered natural resources due to the ecological contamination and environmental alterations, which at the same time, have increased the concerns about resources protection from different points of view. In this regard, it is worth mentioning the regulations to international access to natural products arising from the United Nations Convention on Biological Diversity (CBD) and the Nagoya Protocol. Together, they seek to implement suitable laws and regulations and to suggest the practices to adopt in regards to the biodiversity and the traditional knowledge of the countries that are involved (Harvey *et al.* 2015).

1.1.3 Medicinal plants as a source of natural products

The most documented traditional medicine is the one from China which has been used for thousands of years and it remains as part of the current healthcare in China. Studying and isolating secondary

metabolites from medicinal plants have shown the enormous potential of finding drug-like or lead-like compounds for modern medicinal chemistry (Boufridi & Quinn 2018). For instance, the medicinal plant *Artemisia annua* (Asteraceae), used for treating malaria, led to the isolation of artemisinin; the potent anti-cancer drug paclitaxel was discovered thanks to the plant *Taxus brevifolia*¹ (Taxaceae) while the widely used drug aspirin was obtained from the medicinal plant *Filipendula ulmaria*² (Rosaceae) (Anand *et al.* 2019). Approximately only 15 % of the species from the plant kingdom have been investigated from which only 6% have been screened for biological activity. Around 80% of the population, particularly those in developing countries, continue using plant extracts as traditional medicine or even as the most affordable treatment available (Alvin *et al.* 2014). In developed countries, such as Australia, Canada or France, 48%, 70% and 75% of their population, respectively, have used the medicinal plants as alternative medicine at least once (Bussmann 2013).

Besides all the benefits, important uses and applications of medicinal plants, they have also shown some limitations such as the large quantities of plant needed to obtain sufficient bioactive compounds for clinical use. Another limitation are the compounds with biological activity isolated from plants that are endangered or highly endemic, raising concerns about their conservation. Although culture of plant tissue is an alternative, their costs are still elevated. For these reasons microorganisms, including micromycetes and bacteria might represent an easier way of scaling-up the production process of active compounds (Alvin *et al.* 2014).

¹ Also known as the Western yew whose principal toxic constituents are the diterpenoid derivatives termed taxanes

² Also known as Meadowsweet

1.2 Fungal secondary metabolites

Microorganisms produce compounds as a response to environmental factors or to stressful situations which provides them better survival mechanisms. This response will also depend on the genetic diversity of these microbial strains and therefore, this will maximize and diversify the natural products produced. Samples collected from diverse environments for the isolation of novel microorganisms, especially those with high biodiversity (hot spots) (**Figure 1.7**) or under-represented ecosystems, will potentially provide novel compounds (Knight *et al.* 2003). Collaborative work of microbiologists, ecologists and botanists to collect as many samples as possible from one hot-spot area will maximize the probabilities of finding novel species with unique metabolic pathways.



Figure 1.7. Biodiversity map representing 34 “hot-spot” areas of plant diversity and medicinal plants for microorganisms’ isolation and diversity studies (numbered from 1 to 34) as recognized by Conservation International (Nalini & Prakash 2017)

Plants represent niches for microorganism interaction and many natural products isolated from plants were, in fact, produced by bacteria or fungi symbionts living within their tissues (Aly *et al.* 2013). Prokaryotes and eukaryotes have existed on earth for over billions of years but only around 1% of them have been cultured under laboratory conditions and studied for their secondary metabolite production (Demain 2014). However, massive whole-genome sequencing has exposed the great capacity of microbes to produce secondary metabolites (Genilloud 2014). The challenge is to access the biosynthetic pathways that are not expressed under standard laboratory conditions from culturable microorganisms and from those that are difficult to culture, called also ‘unculturable’ (Challinor & Bode 2015). Natural products obtained from bacteria and fungi have shown to have diverse activities such as antibacterial, antiviral, antiprotozoal, antifungal, anticancer, anti-inflammatory and many other biological activities

important for the pharmaceutical industry (Pham *et al.* 2019), corresponding to different chemical classes. As they play a significant role in human activities, research initiatives are focusing towards fungi and bacteria isolated from high biodiversity areas or from marine ecosystems using novel approaches of culture to activate cryptic metabolic pathways (Abdel-Razek *et al.* 2020).

1.2.1 Biosynthesis and classification of fungal secondary metabolites

The systematic study of secondary metabolites started with the characterization of over 200 mold metabolites by Harold Raistrick in 1922 but it was not until the discovery of penicillin that this area of investigation gained more attention. In the case of fungi, secondary metabolites are low molecular compounds usually correlated with morphological differentiation stages. It is also known that synthesizer strains can grow without producing them, unlike primary metabolites, and they have restricted taxonomic distribution (Keller *et al.* 2005). Species from fungal kingdom are responsible for 45% of the production of microbial metabolites, belonging mainly to the species of *Penicillium*, *Aspergillus* and *Trichoderma*, while yeasts produce around 1% of all microbial metabolites (Bérdy 2012).

Although secondary metabolites are chemically very diverse, they are synthesized from a few building blocks or precursors derived from primary metabolism (**Figure 1.8**) (King *et al.* 2016). Most of the genes involved in the biosynthesis of secondary metabolites in fungi are organized in clusters and they encode multi-domain and multi-modular enzymes such as the polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs), besides other additional enzymes needed for transportation, regulation or used for specific modifications (Keller 2019). According to the class of enzyme that is involved in their biosynthesis, fungal secondary metabolites can be classified as polyketides, non-ribosomal peptides, terpenes or hybrid products (Keller *et al.* 2005).

Generally in fungi, polyketides are synthesized by type I PKSs, condensing acetyl coenzyme A and malonyl coenzyme A to form carbon chains of varying lengths. Non-ribosomal peptides are produced from amino acids (proteinogenic or non-proteinogenic) and synthesized by NRPSs. Terpenes are produced from activated isoprene units and synthesized by terpene cyclases and terpene synthases. Dimethylallyl diphosphate and isopentenyl diphosphate, products of the mevalonate pathway, act as building blocks for the production of indole alkaloids, monoterpenes, sesquiterpenes and diterpenes. Some secondary metabolites are synthesized by other enzymes encoded by different genes such as for the biosynthesis of gibberellin in *Fusarium fujikuroi* (Tudzynski *et al.* 1998) or they are hybrid products from NRPS-PKS systems and many other hybrid pathways, such as the case of aspyridones produced

by *Aspergillus* spp. (Theobald *et al.* 2019). Fungal secondary metabolites can be synthesized by diverse and complex pathways different from those mentioned here, which are not yet well elucidated.

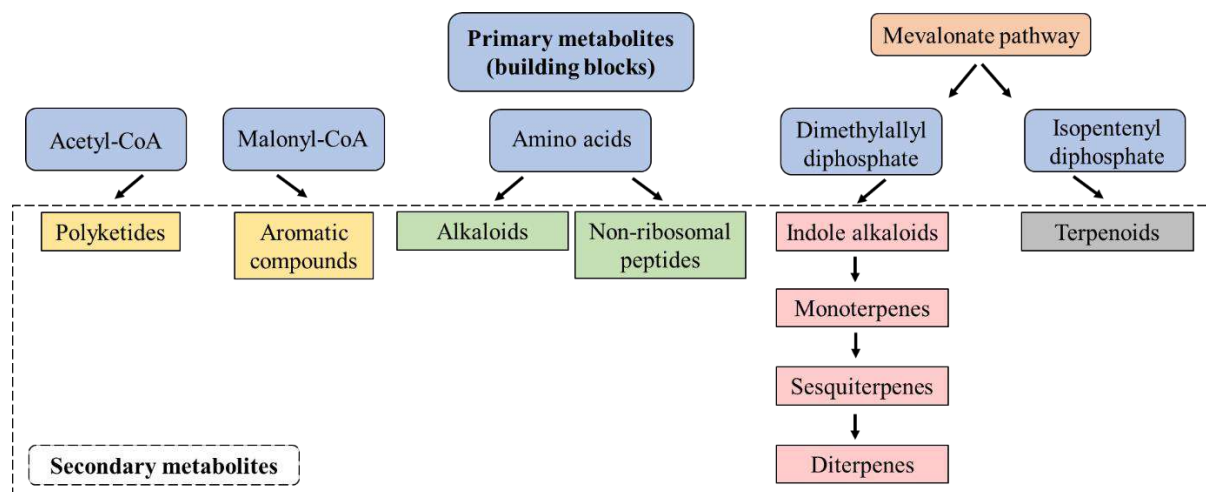


Figure 1.8. Main building blocks of fungal secondary metabolites (own diagram with information obtained from (King *et al.* 2016))

Some initial limitations for the study of the biochemical pathways in fungi were the short presence (minute scale) of enzymes involved in the biosynthesis of secondary metabolites and the lack of sexual stages in many species, needed for genetic studies. Later, thanks to gene cloning studies, many fungal gene clusters were characterized, such as for the aflatoxins, cephalosporin, penicillin, gibberellins, etc. (Keller *et al.* 2005).

Fungal secondary metabolites can also be grouped by chemical relationships (**Table 1.1**), as it is shown in the handbook elaborated by Cole and colleagues (2003) that summarizes and classifies most of the fungal secondary metabolites, including their biological activity information. Fungal natural products have exhibited a broad spectrum of biological activities such as antibiotic, anticancer, immunosuppressant drugs, hypocholesterolemic agents, mycotoxins, enzyme inhibitors, pigments, natural sweeteners, industrial enzymes and biofuels (Sanchez & Demain 2017). As previously mentioned, natural products from fungi possess high chemical originality, especially from those species isolated from high biodiversity areas (endophytes or marine organisms). A review covering 230 fungal compounds published between 2009 and 2013, highlighted novel structures and important bioactivities, showing the increasing interest of fungal natural products in drug discovery considering also innovative approaches (Schueffler & Anke 2014). Among these, cytospolides A-Q, decytospolides A and B, benzophomopsin A, xanalteric acids I and II, epicoccolides A and B and trichodermanin A were new compounds isolated from endophytic fungi.

Table 1.1 Main chemical groups of fungal secondary metabolites according to Cole *et al.* 2003*Classification of fungal secondary metabolites*

| | | | |
|----|---------------------------------------|----|--|
| 1 | Aflatoxins | 34 | Hebevinosides and Hebelomic acids |
| 2 | Aflavinines and related indoles | 35 | Helminthosporols |
| 3 | Alliacolide and related metabolites | 36 | Herbarin and related metabolites |
| 4 | Altenuene and related metabolites | 37 | Hericenens and Hericenones |
| 5 | Altertoxins | 38 | Indole alkaloids |
| 6 | Altranones | 39 | Indole metabolites |
| 7 | Antheridiols and Oogoniols | 40 | Janthitrems |
| 8 | Aphidicolins | 41 | LL-Z1271 antibiotics and related metabolites |
| 9 | Arcyroxepin A and related Bisindoles | 42 | LL-Z1272 antibiotics and related metabolites |
| 10 | Arugosins | 43 | Loline alkaloids |
| 11 | Austalides | 44 | Lucidenic acids |
| 12 | Azasterols | 45 | Lucidones |
| 13 | Baccharinoids | 46 | Macrocyclic trichothecenes and related metabolites |
| 14 | Botrydial and related metabolites | 47 | Memnobotrins |
| 15 | Boviquinones and related metabolites | 48 | Miotoxins |
| 16 | Cercophorins | 49 | Myrotoxins and Myotoxins |
| 17 | Cercosporin and related metabolites | 50 | Neovasinins |
| 18 | Chaetoglobosins/Cytochalasins | 51 | Ochratoxins and related metabolites |
| 19 | Chokols | 52 | Palmarumycins |
| 20 | Curvularins | 53 | Paspaline and related metabolites |
| 21 | Cyathanes | 54 | Penitrems/Lolitrems |
| 22 | Diketopiperazines | 55 | Phomosines |
| 23 | Enaminomycins and related metabolites | 56 | Radicinins |
| 24 | Fasciculols | 57 | Roritoxins |
| 25 | Fiscalins | 58 | Siccanochromenes and Grifolins |
| 26 | Fumiquinazolines | 59 | Stachybotrylactone and related metabolites |
| 27 | Fumonisin and related metabolites | 60 | Sterigmatocystin and related metabolites |
| 28 | Fusarochromanones | 61 | Sterols |
| 29 | Fusicoccins | 62 | Trichothecenes and related metabolites |
| 30 | Fusidanes and Protostanes | 63 | Tryptoquivalines |
| 31 | Ganoderic acids | 64 | Tsugicolines |
| 32 | Ganoderiols | 65 | Versicolorins |
| 33 | Ganomastenols | 66 | Viridin and related metabolites |

Among the fungal metabolites, the smallest cyclic peptides that are built from two amino acids are named diketopiperazines (DPKs), where the thiodiketopiperazines (TDKPs) are higher functionalized analogs that possess a broad bioactivity such as cytotoxic, antibacterial, antifungal or anti-inflammatory properties. They have been isolated from fungi species corresponding mainly to the genera *Aspergillus*, *Candida*, *Chaetonium*, *Gliocladium*, *Penicillium* and *Verticillium* (Bräse *et al.* 2013), but also from the species *Cophinforma mamane*, which is the object of study of this thesis. Natural thiodiketopiperazines

alkaloids are characterized by the presence of a diketopiperazine core with thiomethyl groups and/or sulfide bridges. According to the position of the sulfur linkages, they can be classified in 1,4-bridged epipolythioxopiperazines (ETPs), derivatives with sulfur bridge outside the 2,5-DKP ring, nonbridged dimethylthio derivatives and others cyclodipeptides containing sulfur (Wang *et al.* 2017a). Biogenetically, DKPs are derived from at least one aromatic amino acid and in fungi, the diverse scaffolds arise from the formation of a wide variety of amino acids and different sulfur bridges which directly affect their bioactivity (Niu *et al.* 2017). The major catalysts of the peptide bond formations are the nonribosomal peptides synthetases (NRPS) and cyclodipeptide synthases (CDP) (Borgman *et al.* 2019).

Regarding the 1,4-ETPs, their observed toxicity is due to the presence of a disulfide bridge, which inactivates proteins through the reaction with thiol groups and by the generation of reactive oxygen species (Borthwick 2012). The most known example is gliotoxin, which in the case of the marine-derived fungus *Penicillium* sp., seven gliotoxin analogues with cytotoxic effects were isolated while compounds with a disulfide bridge exhibited histone methyltransferase inhibitory activities (Sun *et al.* 2012). Recently, 4 new thiodiketopiperazines alkaloids were isolated from the endophytic fungus *Phaeosphaeria fuckelii* called phaeosphaeones A – D, while phaeosphaeone D exhibited mushroom tyrosinase inhibitory activity (Zhai *et al.* 2020). Regarding the sulfur-bridged derivatives outside the 2,5-DKP ring, epicoccins A – F have been isolated from fungus *Epicoccum nigrum* and they present unusual sulfur bridges and one of these compounds exhibited moderate antimicrobial activity (Zhang *et al.* 2007).

1.2.2 What are the roles of fungal secondary metabolites?

Metabolites produced during the secondary metabolism of fungi, called also specialized metabolism, are not essential for their growth or survival but they have been widely used for different human activities, as it has been previously mentioned. However, the role of these metabolites in their natural environment during their interaction with other microorganisms or during their own development are still under investigation with some findings that help us to understand their ecological functions besides their utility. Some secondary metabolites might be useful for their interaction with other microorganisms in their ecological niches, for protection or defense against competitors or against other environmental factors such as light. They can also serve as virulence factors during infections or during symbiotic interactions with plants and pathogens.

Interaction with other microorganisms

The role of secondary metabolites during microbial interactions is still not well understood, however some studies have shown their importance during these interactions. Quorum sensing (QS) is the communication process between bacteria largely studied in gram-positive and gram-negative bacteria. This process occurs mainly during biofilm formation, regulation of cell densities or secretion of virulence factors (Grandclément *et al.* 2015), while this communication process is less known in eukariotes. For example, it has been recently reported the production of a small peptide Qsp1 by *Cryptococcus neoformans* that acts as a quorum sensing regulator involved in its own virulence and morphology (Homer *et al.* 2016). Additionally, *Penicillium sclerotiorum* produces the molecule sclerotiorin, whose production is related to derivatives of a QS molecule that contains the signaling molecule gamma-butyrolactone. While in *Aspergillus terreus*, butyrolactone I might act in the regulation of lovastatin production as a QS molecule, stimulating its biosynthesis (Macheleidt *et al.* 2016). Regarding microbial communication, induction of new secondary metabolites due to intimate or physical interaction between fungi and bacteria might indicate the use of compounds to communicate that they are sharing the same habitat and nutrients, which will trigger different signaling pathways and responses.

Virulence factors during interactions with plants and pathogens

These types of interactions occur during symbiotic interactions with the host plant and the pathogens harbored within its tissues such as insects, other fungi and bacteria, parasites, etc. Some species are host-specific and they produce different types of infection and therefore different secondary metabolites. In the case of *Alternaria alternata*, while the AM toxin is produced during the infection of apples (*Alternaria mali*), the AF toxin is produced during infection of strawberries (*Alternaria alternata* f. sp. *Fragariae*), causing black spots and therefore showing their host and target site specificity (Tsuge *et al.* 2013). Additionally, *Botrytis cinerea* produces botrydial and botcinic acid on plants, which play a role in its pathogenicity. If one of the genes that encodes for these compounds is deleted, the fungal pathogenicity does not change but when both genes are deleted, the infection continues but with no visible symptoms of infection (Dalmais *et al.* 2011).

Another situation is for example the entomopathogenic fungus *Beauveria bassiana*, which expresses beauvericin and other secondary metabolites as virulence factors during the infection of the insect *Triatoma infestans*. Interestingly, this entomopathogenic fungus also secretes antimicrobial metabolites to the dead insect to limit competition for nutrients. Similarly, the fungus *Metarhizium robertsii* produces the secondary metabolite destruxin as a virulence factor during infection of the insect larva of *Spodoptera exigua* (Macheleidt *et al.* 2016).

Protection from toxic natural products and environmental conditions

Among all the bioactivities of fungal natural products, several possess antifungal activity. Fungi protect themselves from these toxic compounds using different mechanisms encoded in their biosynthetic gene clusters. It should be noted that the toxic concentrations under laboratory conditions are not the same to the physiologically relevant concentrations, making secondary metabolites more of a signal rather than a toxin in natural conditions. It is known that it exists mainly 3 fungal strategies to avoid toxicity from natural products: efflux transporters or pumps, intermediate transporters or translocators, detoxifying enzymes that reduce the target binding properties and duplicated copies of the target protein to recover from the toxic effect (Keller 2019).

Regarding environmental conditions, the production of melanin in fungi represents an evolutionary and important mechanism of adaptation against difficult conditions. This metabolite is a hydrophobic pigment formed by the oxidative polymerization of indolic or phenolic compounds produced in plants, animals and fungi. In fungi, melanin is produced after the active growth stage and therefore it is considered as a secondary metabolite that confers UV light protection thanks to its photochemical properties, transforming UV radiation into harmless amounts of energy. Moreover, it has been postulated that melanin possesses other roles such as fungal protection against toxins, hydrolytic enzymes, redox buffering, among others functions, providing them defense mechanisms and helping fungi to survive (Cordero & Casadevall 2017).

Fungal development processes

Fungal secondary metabolites are also produced as signals to initiate developmental programs or morphological changes such as protection of sexual structures, formation of fruiting bodies, spore germination or pigmentation. In *Aspergillus nidulans* the production of linoleic acid is associated with the sporulation induction, as it occurs in *Fusarium graminearum* and in *Aspergillus terreus* with the production of zearalenone and butyrolactone I, respectively. Also, the production of mycotoxins in *Aspergillus* spp. and the production of patulin in *Penicillium urticae* are induced after the sporulation process (Calvo *et al.* 2002).

1.2.3 Regulation of secondary metabolism in fungi

Interactions of microorganisms in natural environments are very complex due to the multiple chemical signals used for communication, competition or defense between fungi, bacteria, plants, protozoa or even metazoa (Brakhage 2013). Secondary metabolite production can also respond to various environmental signals such as nutritional sources, temperature, light, pH or the presence of other microorganisms (**Figure 1.9**). This process does not follow a hierarchical scheme as it is regulated at different levels within different cellular processes, including development and transcription and it can vary between different fungal species.

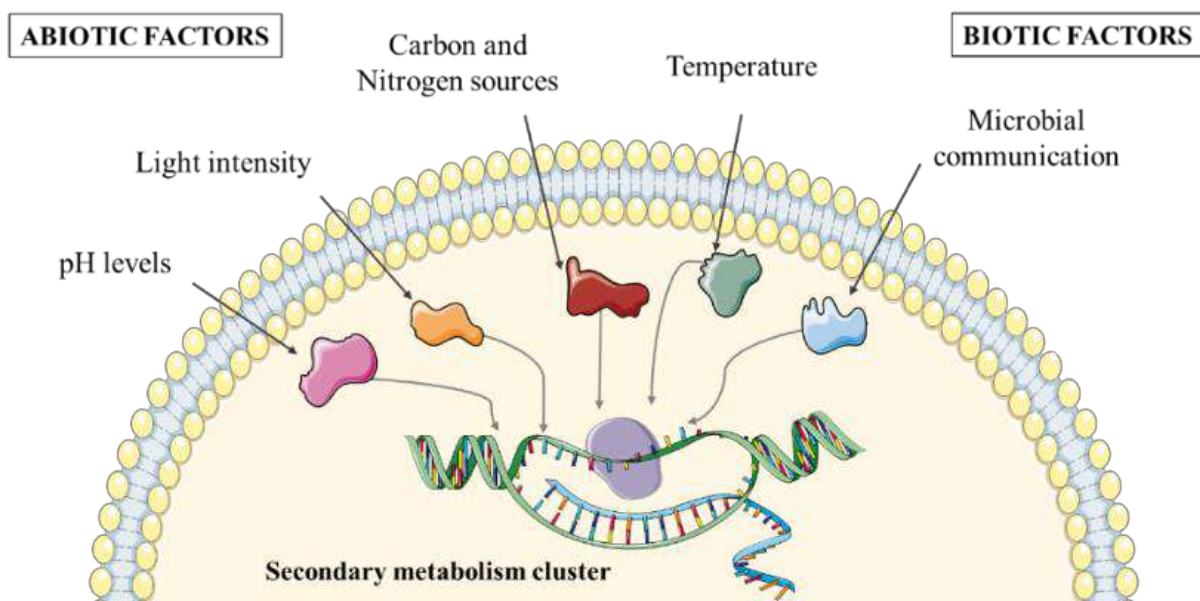


Figure 1.9. Environmental signals (biotic and abiotic) that influence the regulation of secondary metabolism clusters in fungi (Own diagram using smart.servier.com)

Transcriptional regulation

The regulation of a specific gene cluster by transcription factors occurs for instance in the biosynthesis of the mycotoxin gliotoxin regulated by the Zn-finger. This regulation has shown to be affected by pH, temperature, aeration or even the presence of gliotoxin itself in *Aspergillus fumigatus* (Scharf *et al.* 2012). In this case, if the unique gene of the cluster encoding for the Zn-finger is deleted, the gliotoxin production does not occur. Specific regulators can be encoded by genes found inside or outside the cluster they regulate. Interaction between gene clusters through cross-talk regulation may highlight the need of a deeper understanding of these regulatory networks.

On the other hand, different stimuli can induce distinct global regulatory proteins, showing a greater level of regulatory networks. Approximately, half of the currently known secondary metabolite gene clusters in fungi do not encode regulatory proteins, therefore they respond to a global regulation. Some of these global regulators have been identified: PacC for pH levels, AreA for nitrogen sources, CreA for carbon sources and the velvet complex for light intensity (Macheleidt *et al.* 2016). Light stimuli is involved in secondary metabolism, sporulation and development in fungi. For instance, the velvet complex coordinates filamentous fungal development in response to light and the nuclear protein LaeA, which is part of this complex, acts as a regulator at the chromatin level (Bayram & Braus 2012). Another example is the transcription factor PacC that activates penicillin biosynthesis in pH alkaline but represses aflatoxin production in *Aspergillus parasiticus* (Keller & Hohn 1997). Temperature also influences metabolite production as in *Aspergillus flavus*, whose optimal growth is 37°C but aflatoxin production occurs only at 30°C yet no global regulator has been identified.

Signaling pathways

In addition to this, highly conserved signaling pathways are also involved in the activation of secondary metabolism in fungi through regulation of gene expression. Fungal development and secondary metabolite production have been associated mainly during sporulation, pathogenicity or pigmentation (Keller *et al.* 2005). Cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) and mitogen-activated protein kinase (MAPK) are among the most investigated signaling pathways associated with the production of secondary metabolites. For instance, overexpression of one catalytic subunit of PKA induced the expression of a melanin gene cluster in *Aspergillus fumigatus* while PKA phosphorylation led to the enhanced production of the polyketide sterigmatocystin in *Aspergillus nidulans*. MAPK pathways possess 3 signaling cascades which are involved in cell wall repair (CW1), in the osmolarity stress (HOG) and in the sexual crossing and pseudohyphal formation (pheromone pathway). Inhibition of the pheromone pathway and repression of CW1 pathway generated lower levels of sterigmatocystin and penicillin production in *Aspergillus nidulans* (Macheleidt *et al.* 2016).

Epigenetic regulation

In eukaryotic cells, transcription is performed by RNA polymerase II and its access to the DNA depends on the chromatin state, composed by DNA and histone protein complexes assembled in nucleosomes. Biosynthetic gene clusters in fungi are often found in the distal telomeric regions of the chromosome (Williams *et al.* 2008), which are transcriptionally controlled by epigenetic regulation, mainly histone acetylation and methylation (Brakhage 2013). When fungal DNA is organized into histone proteins and is densely packed into heterochromatin, it becomes transcriptionally silent (Okada & Seyedsayamdost

2017). When DNA is loosely packed into euchromatin, it becomes transcriptionally active mainly due to the presence of highly acetylated histones (Cichewicz 2010). By contrast, when histones are methylated, genes are found to be silenced (Suzuki & Bird 2008) (**Figure 1.10**).

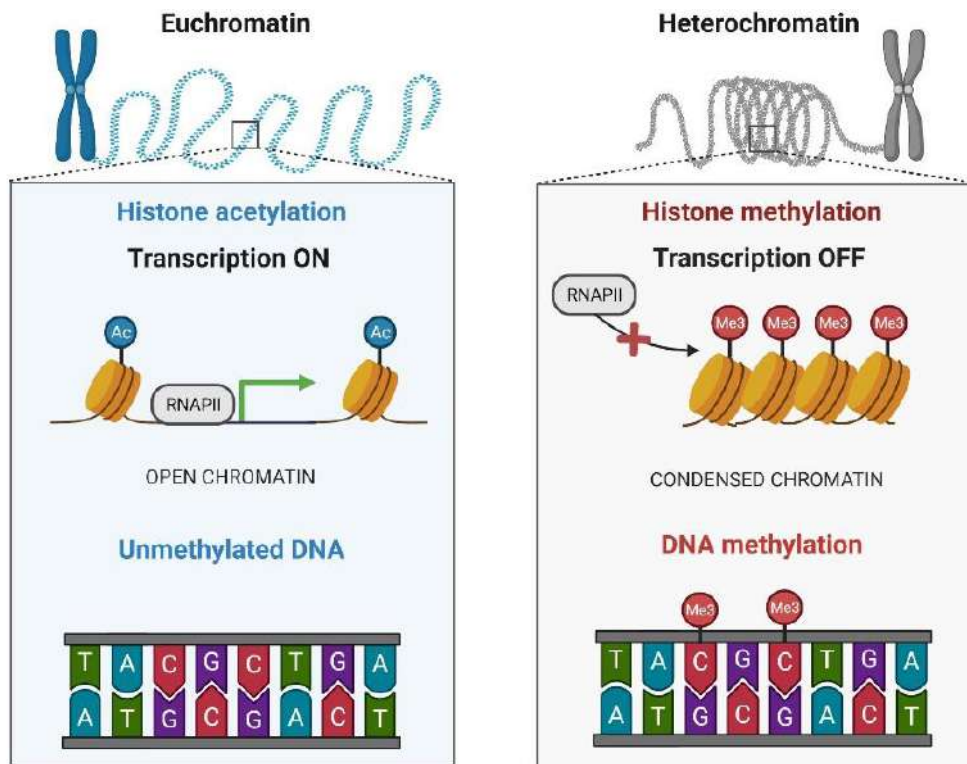


Figure 1.10. Epigenetic modifications of chromatin by DNA methylation and histone acetylation. RNAPII: RNA polymerase II (own creation using biorender.com)

Histone acetylation and methylation are catalyzed by histone acetyl transferases (HATs) and by histone methyltransferases (HMTs), respectively while the removal of acetyl and methyl groups are catalyzed by histone deacetylases (HDACs) and histone demethylases (HDMs), respectively. Similarly, DNA methylation also plays an important role in the regulation of gene expression, despite the low abundance of this modification found in fungal genomes. In this case, the transfer of the methyl group to the DNA is catalyzed by DNA methyltransferases (DNMTs).

It was observed that during the early-phase growth of *Aspergillus nidulans*, some methylation marks on histone protein H3 were reversed by the nuclear protein LaeA when the fungus changed to a stationary phase of growth. Additionally, when studying the effects of epigenetic manipulation by deleting a histone deacetylase gene (*hdaA*) in *Aspergillus nidulans*, it resulted in a transcriptional activation of multiple biosynthetic gene clusters (Shwab *et al.* 2007). Moreover, production of aflatoxin was reduced when a DNA methyltransferase gene (*dmtA*) was deleted in *Aspergillus flavus*, indicating its role in gene

activation rather than in gene repression. These findings allowed the expansion of additional studies on this subject, by either activating or silencing chromatin regions or by treating fungi with epigenetic modifier chemical inhibitors (Keller 2019).

However, besides histone acetylation and methylation, other post-transcriptional modifications have been observed in fungi such as butyrylation, propionylation, phosphorylation, sumoylation and ubiquitination. The relative number of modifications predicted in *Aspergillus nidulans* based on similarity shows that 46% correspond to methylation, 36% to acetylation, 10% to sumoylation, 5% to phosphorylation and 3% to ubiquitination (Collemare & Seidl 2019), indicating the potential influence of additional transcriptional regulations involved in secondary metabolism in fungi.

1.3 Endophytes as a source of new drugs

The term “endophyte” was first described in 1886 by Anton de Bary, and was broadly used to refer to any organism living within plant tissues however, this term has undergone various redefinitions through time. A widely accepted definition describes endophytes as any organism that colonizes internal plant tissues such as branches, stems, roots, petioles, leaves, fruits or seeds, without generally producing signs of its presence or showing harmful effects to the host plant. These microorganisms can spend all their life cycle or part of it within inter or intracellular spaces of the plant cells and the symbiotic interactions can vary from mutualist, antagonist or even parasitism, corresponding to obligate or facultative endophytes (Gouda *et al.* 2016). The latter also depends on the environmental and physiological conditions of the host plant.

Endophytic microorganisms have been descr in almost all plant families found in different environments and climates around the world. Most of the endophytic microorganisms isolated from plants correspond to fungal species from the Ascomycota and Basidiomycota phyla and to bacterial species from the Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria phyla (Martinez-Klimova *et al.* 2017). Only 1-2% of all the known plants have been studied for their endophyte composition (Strobel 2018), of which more than 34 plant families have been used for the isolation of endophytes, corresponding, for instance, to the Araceae, Asteraceae, Fabaceae or Lamiaceae families from different collection sites (**Figure 1.11**). Considering current environmental problems such as contamination, deforestation or biodiversity losses, many of these endophytic species will be lost before their value is explored. Therefore, the need to preserve and explore these natural reservoirs of biological agents is urgent.

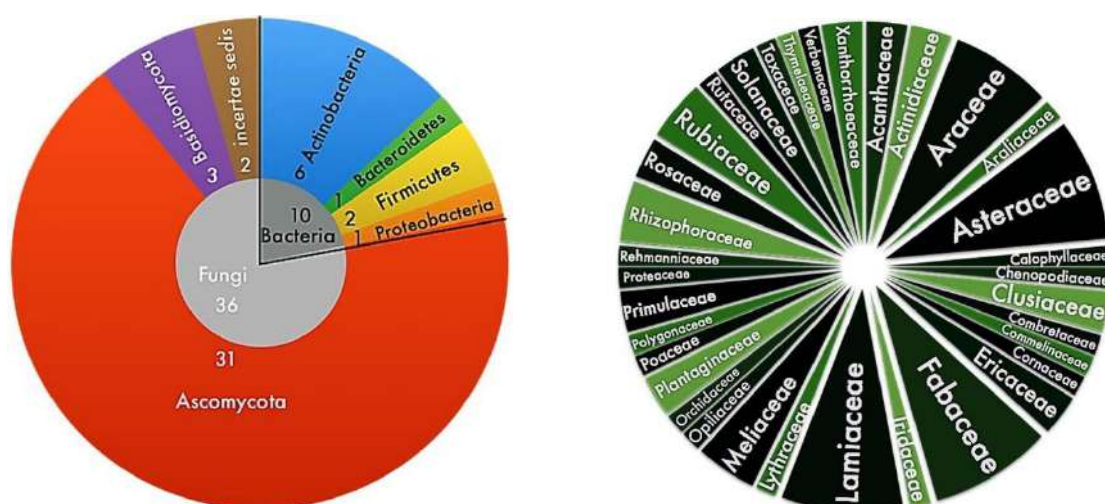


Figure 1.11. Taxonomic classification of isolated endophytes (left) from different plant families (right) (Martinez-Klimova *et al.* 2017)

Although endophytes were first described in 1904 from *Lolium temulentum* (Poaceae) grains, it was not until the isolation of paclitaxel in 1993 from the fungal endophyte *Taxomyces andreanae* that these microorganisms started to gain more attention for the study of new potential sources of novel natural products. This discovery also prompted the study of other plants able to host different endophytes capable of producing secondary metabolites with important pharmacological uses (Strobel & Daisy 2003).

1.3.1 Bacterial endophytes

Bacterial endophytes include more than 200 genera from 16 phyla of gram-positive and gram-negative bacterial species, considering actinobacteria and mycoplasma. They possess a capacity for colonizing root surfaces in the rhizosphere that actively switches to endophytic lifestyles to colonize stems, leaves and other host tissues. The diversity of bacterial endophytes include species from *Achromobacter*, *Acinetobacter*, *Agrobacterium*, *Bacillus*, *Brevibacterium*, *Pseudomonas* genera, being *Streptomyces* one of the most promising and dominant genera in endophytic actinomycetes (Gouda *et al.* 2016). Additionally, the most characterized genera are *Bacillus* and *Pseudomonas* while the less studied are the mycoplasma species, known to have a symbiotic relationship with some red algae.

In the context of plant-bacteria interactions, production of toxins, virulence factors, biofilm formation agents and plant hormones or their structural analogues have been reported. The endophytic actinobacterium *Pseudonocardia* sp., for instance, enhanced artemisinin production in the host plant *Artemisia annua* while the production of the cardiotoxin pavettamine is due to a bilateral biosynthesis of South African Rubiaceae and their bacterial endophytes (Brader *et al.* 2014). However, a large plethora of metabolites found in endophytic actinobacteria and in other phyla remain to be discovered.

1.3.2 Fungal Endophytes

Fungal endophytes represent the majority of endophytes isolated from different plant families. Similar to the results found by Martinez-Klimova and colleagues (Martinez-Klimova *et al.* 2017), a more recent analysis of diversity and distribution of endophytes including almost 600 published studies between 2016 - 2019, revealed that fungal endophyte diversity has been characterized in at least one host plant of the embryophyte families (**Figure 1.12**) however, most the host phylogeny remains unsampled (Harrison & Griffin 2020).

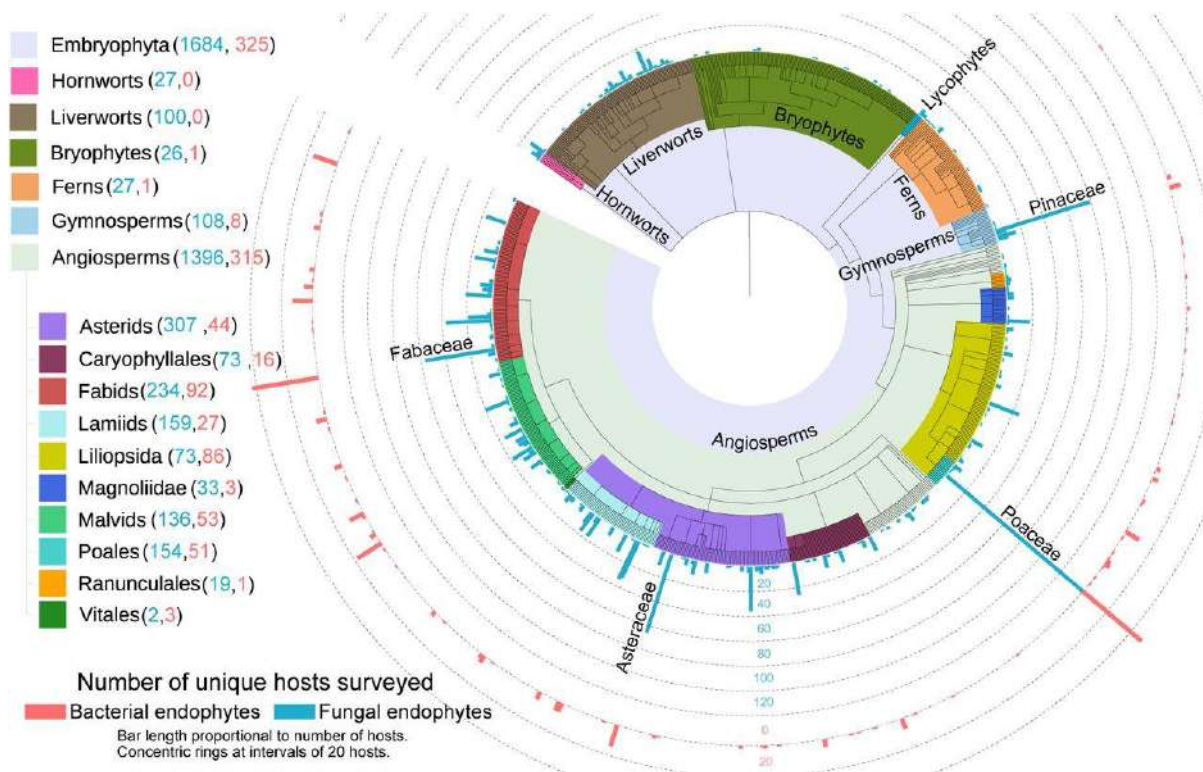


Figure 1.12. Number of studies between 2016-2019 surveying fungal (blue bars) and bacterial (red bars) endophytes from Embryophyta (Land plants) with main taxa labeled on the left including the number of unique host surveyed in parentheses (Harrison & Griffin 2020)

Taxonomic diversity of endophytic fungi is very vast and therefore these communities can be grouped under different criteria, based on their ecological or symbiotic function in relationship with the host plants. They can be classified based on the mode of transmission, mode of reproduction, expression of the infection, source of nutrition or type of tissue colonized within the plant (Bamisile *et al.* 2018). Besides this, two major groups of fungal endophytes are also distinguished: clavicipitaceous endophytes that occupy grasses as host plants while non-clavicipitaceous colonize vascular and nonvascular plants, among other considerations based on their phylogeny and life history traits (Rodriguez *et al.* 2009).

On the other hand, a total of 185 patents related to endophytic fungi applications were recorded for the period 1988-2019, of which 88 correspond to stress tolerance and growth promoters, 90 patents correspond to biocontrol of plant pathogens and 7 patents correspond to bioremediation and phytoremediation and the most representative fungi belong to the genera *Alternaria*, *Aspergillus*, *Chaetomium*, *Fusarium*, *Penicillium* and *Muscodor* (Ortega *et al.* 2020). However, commercial exploitation and application remains a big challenge due to the low biomass generated by fermentation or the low stability of axenic endophytes culture over time (Venugopalan & Srivastava 2015). Many strategies are being developed in order to explore the potential of endophytic fungi and enhance their productivity of bioactive secondary metabolites.

Considering the importance of fungal endophytes, being the representative group of endophytes in comparison to the bacterial endophytes, the following sections will be focused on fungal endophytes, regarding their relationship with their hosts, their isolation for laboratory studies, and the novel bioactive natural products obtained from these microorganisms.

1.3.3 The special relationship between endophytic fungi and their host plants

Over long periods of evolution, endophytes have formed a special symbiotic relationship with their host plant. They can act as a biological defense for the plant against phytopathogens and produce the same or similar secondary metabolites as their host (Alvin *et al.* 2014), with approximately 18% of plant metabolites that can be produced by associated fungi. Endophytes can alter the quantity and quality of drugs derived from medicinal plants due to their capacity to increase their fitness and their growth, improving their tolerance to biotic or abiotic stress factors as well as inducing the accumulation of bioactive secondary metabolites. In this regard, the endophyte population is also indirectly affected by environmental conditions such as temperature, humidity, soil nutrients, sunlight hours, etc., therefore the same host plant species from different regions can exhibit different endophytic populations (Jia *et al.* 2016).

In general, the interactions between endophytic fungi and their host plant occurs in different forms at a metabolic level: endophytes can induce plant metabolism, plants can induce endophyte metabolism, both contribute and share metabolic pathways, plants metabolize products from their endophytes or endophytes synthesize secondary metabolites (or similar) of the plant host (Cabezas Gómez & Hortolan Luiz 2018).

The study of plants for the isolation of fungal endophytes and further research of novel natural products represents a promising approach that has already shown positive results. A review study analyzed several investigations of endophytic fungi and medicinal plants covering a period of 30 years (1986 - 2016). A total of 46 families and 96 species of plants showed a mutualistic relationship with their endophytes, used either as part of traditional medicine or through the consumption of the extracted bioactive compound. Additionally, they were distributed in temperate, tropical, subtropical or extreme environments (savanna deserts and high altitude conditions) (Jia *et al.* 2016). Of the multiple ecosystems on Earth, those having the greatest biodiversity can also hold the greatest number of biodiverse endophytes, including also high endemic plants which harbor several species of endophytes that have evolved along with their hosts.

A study on the identification of endophytic fungi communities from the medicinal plant *Withania somnifera* (Solanaceae), led to the isolation of 33 fungal strains corresponding to 24 species from the Ascomycota and Deuteromycetes classes. Another study of the endophytic fungi residing in the endemic

medicinal plants from India *Artocarpus hirsutus* (Moraceae) and *Vateria indica* (Dipterocarpaceae), recovered a total of 106 strains belonging to diverse genera such as *Coniothyrium*, *Trichoderma*, *Mortierella*, *Phyllostica* and *Acremonium* (Nisa *et al.* 2015). Endophytic fungi associated with the medicinal Amazonian plant *Carapa guianensis* (Meliaceae) were classified into 35 different taxa in the genera *Colletotrichum*, *Diaporthe* and *Pestalopsis* as the most frequent endophytes while the rest correspond to the genera *Aspergillus*, *Botryosphaeria*, *Fusarium*, *Phomopsis*, among others. Many of these fungi presented antibacterial, antiparasitic and antiviral activities (Ferreira *et al.* 2015).

Potential role of fungal endophytes in agriculture

The relationship between host plants and their endophytes are very complex due to all the biotic and abiotic factors that can influence (**Figure 1.13**). Generally, the presence of endophytes is beneficial for the host plant since they positively influence plant growth under different active or passive mechanisms, which might have useful applications for sustainable agriculture (Fadiji & Babalola 2020). In this sense, endophytes could help cultivate crops with less fertilizers or pesticides and chemicals, making crop plant management a more parsimonious process with the environment (White *et al.* 2019).

Endophytes enhance a plant's ability to survive under stress for example by increasing nutrient availability. The endophytic fungus *Cladosporium sphaerospermum* isolated from the roots of *Glycin max* (*Fabaceae*) improved the growth of its host by the production of bioactive compounds gibberellin (GA) GA4 and GA7 (Hamayun *et al.* 2009). Also root growth and elongation were observed under the presence of endophytic fungi *Penicillium chrysogenum* and *Alternaria alternata* isolated from *Asclepias sinaica* (*Apocynaceae*) due to the production of ammonia and indole acetic acid (IAA) (Fouda *et al.* 2015). They have also shown to enhance plant tolerance against cold or drought stress through the induction of photosynthesis by enhancing phytohormones, chlorophyll and gaseous exchange, maintenance of osmosis, reduction of reactive oxygen species generation and increasing the production of IAA and GA.

As part of their capacity to produce bioactive natural products against microorganisms, endophytic fungi can be used as potential biocontrol agents for plant diseases. They can suppress plant pathogens by releasing toxins or antibiotics, competing for nutrients and space or inducing plant defense systems. Endophytic fungi *Penicillium* sp. and *Hypocrea* sp., isolated from different healthy chinese plants, could effectively inhibit mycelial colony growth of *Fusarium oxysporum* f. sp. *cucumerinum* causing wilt in cucumber (Abro *et al.* 2019).

In addition to this, endophytic fungi play an important role in the production of volatile organic compounds (VOCs) which can be bioactive against pathogenic microbes such as those produced by the endophytic fungus *Muscodor* sp. considered as a biological control agent (Zhang *et al.* 2010) or by

Nodulisporium sp. isolated from *Myroxylon balsamum* (Fabaceae) producing VOCs with also biofuel potential (Tess Mends & Yu 2012).

It is known that endophytic fungi have the potential to decompose organic matter such as lignin or cellulose, helping in the balance of nutrients present in the ecosystem and making them available. However, they can also be involved in bioremediation processes to remove waste and pollutants from the environment. For instance, two strains of the endophytic fungus *Pestalotiopsis microspora* were able to use polymer polyester polyurethane as their only source of carbon under aerobic and anaerobic conditions probably due to the enzyme serine hydrolase found in a molecular characterization analysis (Russell *et al.* 2011).

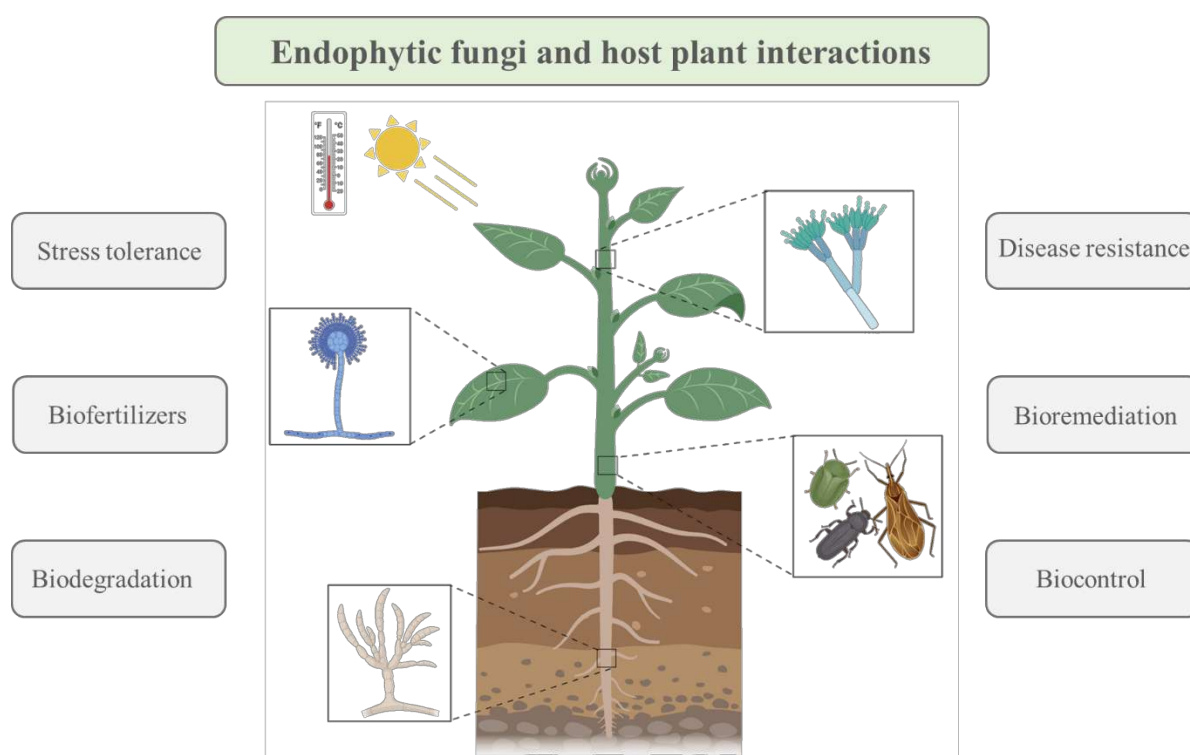


Figure 1.13. Plant growth promotion by endophytic fungi through different mechanisms (own creation using biorender.com)

Plant secondary metabolites produced by endophytic fungi

Some bioactive secondary metabolites produced by plants can also be produced by endophytic fungi besides all the interactions between the host plant and their endophytic fungi mentioned before. The most known example is the production of the anticancer compound paclitaxel by *Taxus brevifolia* (Taxaceae) but also produced by its endophytic fungus *Taxomyces andreanae*. Moreover, at least 20 genera of endophytic fungi have been screened for their ability to produce paclitaxel or its analogues,

isolated from different plant families such as *Spindaceae*, *Rutaceae*, *Cupressaceae*, *Ginkgoaceae*, *Malvaceae*, *Podocarpaceae*, *Taxodiaceae* and *Combretaceae* (Zhao *et al.* 2011). Another example is the production of the anticancer compound camptothecin by *Camptotheca acuminata* (Nyssaceae) and *Nothapodytes foetida* (Icacinaceae) but also by different endophytic fungi belonging to the genera *Botryosphaeria*, *Entrophospora*, *Fusarium*, *Phomopsis*, *Neurospora*, *Trichoderma* and *Alternaria* (Uzma *et al.* 2018).

In addition to these compounds, there are many other plant-derived bioactive secondary metabolites that are produced by endophytic fungi such as piperine (alkaloid), marmesin (coumarin), kaempferol (flavonoid), pinselin (xanthone) and many others, including lignans and saponins (Singh *et al.* 2021).

1.3.4 Procedures for the isolation and identification of fungal endophytes

There are mainly two methodologies for studying endophytes, cultivation-based and cultivation-independent techniques. For recovering isolates on solid culture media, there are no standardized steps for surface sterilization because the solutions and the time of exposure might vary depending on the type of the sampled tissue. In addition to this, the endophyte community present on the plant can be altered depending on the sampling season, the environmental conditions, the growth stage and physiological state of the plant and the genotype of the plant, besides all the culture conditions applied for their recovery (Gouda *et al.* 2016). In this regard, there is a set of steps with important considerations to be taken into account. For instance, it is essential to select a healthy plant tissue to avoid the isolation of pathogen microorganisms and to collect fresh plant material to be used within 24 hours of harvesting to prevent death of endophytes. The surface sterilization is necessary to ensure the isolation of endophytes and not of external contaminants, such as epiphytes or roots actinomycetes.

Washing plant tissues under tap water before sterilization will help to reduce soil particles but also Tween 20 (0.1%) has been used for rinsing. For surface sterilization, strong oxidant or general disinfectant followed by a sterile rinses is the most common procedure. Among the most used solutions for surface sterilization are sodium hypochlorite solution between 2-10%, hydrogen peroxide at 3% or potassium permanganate at 2%, combined with ethanol (70-95%) washing steps and rinses with sterile distilled water (Martinez-Klimova *et al.* 2017). Sterilized surface is aseptically cut into small fragments, between 3 to 5 mm long, to maximize surface exposure to the solid media. Different tissue segments are cut, rinsed and dried under a laminar flow hood and placed over the agar, incubated during 2-4 weeks for posterior isolation (**Figure 1.14**). Generally, plates are discarded after 4 weeks since contaminants are more likely to grow after this time, however certain endophytic fungi might take longer incubation times thus it is important to corroborate this using the control plates containing culture media with water samples.

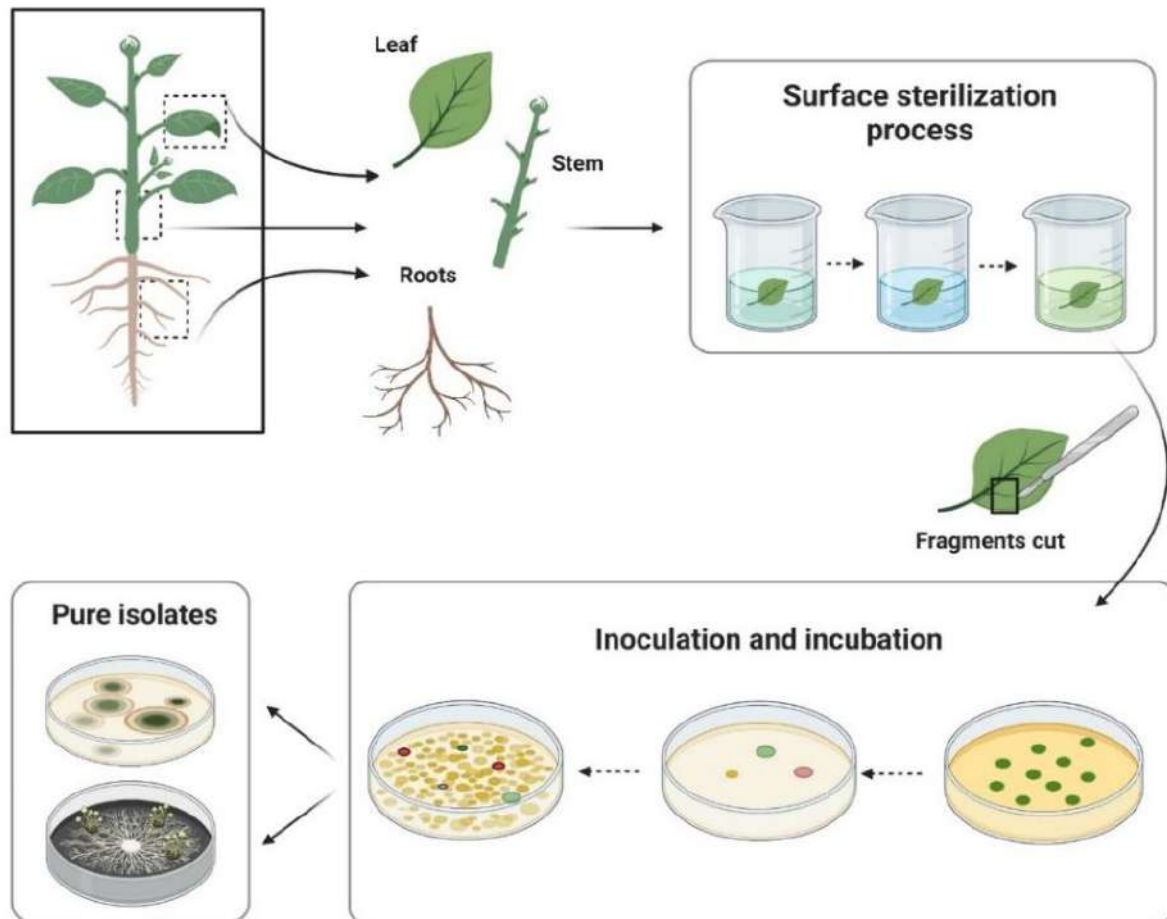


Figure 1.14. Sample preparation for endophyte isolation (own creation using biorender.com)

It is important to mention that several endophytes are not readily cultivable, go undetected or are under-represented. Faddetta and colleagues have observed the occurrence of endophytes through laser scanning microscopy with fluorescence *in situ* (Faddetta *et al.* 2021). For identification of isolates, combinations of microscopic and molecular methods are recommended. For fungi, after the morphology identification, sequence comparisons of 18S rDNA, internal transcriber spacer (ITS1 and ITS2), 5.8S rDNA or 28S subunit of rDNA are done (Zhang *et al.* 2006). Almost 70% of the currently isolated endophytes from different plant tissues are fungi of which 81% are culturable and 19% are considered unculturable. The other 30% of endophytes correspond to bacteria of which 65% are culturable and 45% are considered unculturable, according to the nucleotide database from the National Center for Biotechnology Information (NCBI) (Manias *et al.* 2020).

1.3.5 Novel bioactive compounds from fungal endophytes

As previously mentioned, fungal species have been one of the main sources of bioactive natural products while endophytic fungi represent a promising source of novel and different bioactive secondary metabolites due to their physiological and ecological roles as part of the complex interaction with the host plant and with other microorganisms and pathogens that harbor these types of unexplored niche areas. Recently, 449 new secondary metabolites obtained from endophytic fungi were reported between 2017 and 2019 from 134 journal articles described in a review study (**Figure 1.15**), highlighting that a large percentage correspond to terpenoids and polyketones, followed by alkaloids, steroids and other compounds (Zheng *et al.* 2021). Additionally, many of these secondary metabolites possess structurally diverse new carbon skeletons, unusual ring systems or rare and unique structural moieties biosynthesized by different pathways corresponding to the polyketides, non-ribosomal peptides, and isoprenoids and hybrid products. The last originating from the PKS-NRPS, NRPS-Terpene, PKS-Terpene or PKS-NRPS-Terpene pathways (Gao *et al.* 2018).

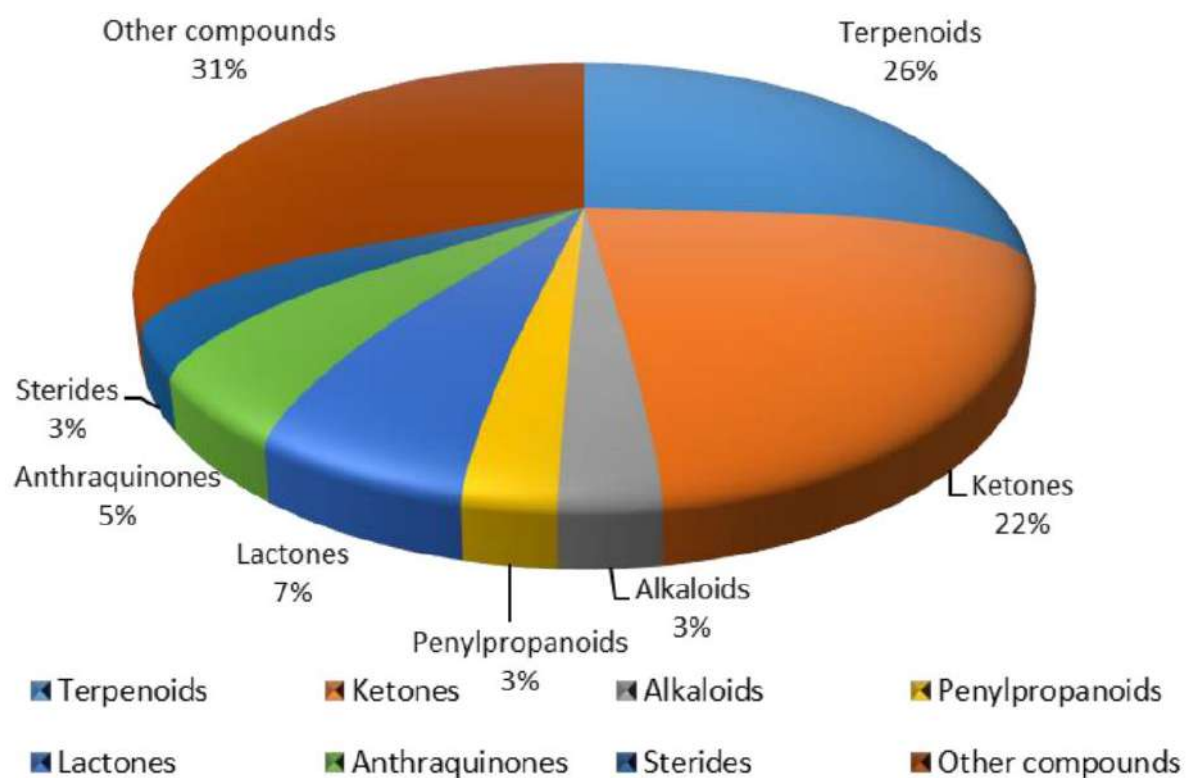


Figure 1.15. Percentage of new secondary metabolites synthesized by endophytic fungi reported from 2017-2019 ($n=449$) (Zheng *et al.*, 2021)

A recent review study analyzed the patent applications made between 2001 and 2019 that were related to the production of secondary metabolites and biotransformation processes by using endophytic fungi. Interestingly, 224 patents correspond to secondary metabolite production while 21 are related to the biotechnological processes such as biotransformation, being *Aspergillus*, *Fusarium*, *Penicillium*, *Trichoderma* and *Phomopsis* the most patented fungi genera (Torres-Mendoza *et al.* 2020).

Endophytic fungi are able to produce chemically diverse bioactive compounds (**Figure 1.16**) that can be renewable and more affordable than the current antimicrobials. Many of these compounds have shown antibacterial, antifungal, anti-parasitic, antiviral, anticancer, antioxidant, immunosuppressive and antidiabetic properties (Manganyi & Ateba 2020; Adeleke & Babalola 2021) important for public health, agriculture, pharmaceutical industries and many other areas (Gupta *et al.* 2020a) and some of them will be mentioned in the following paragraphs.

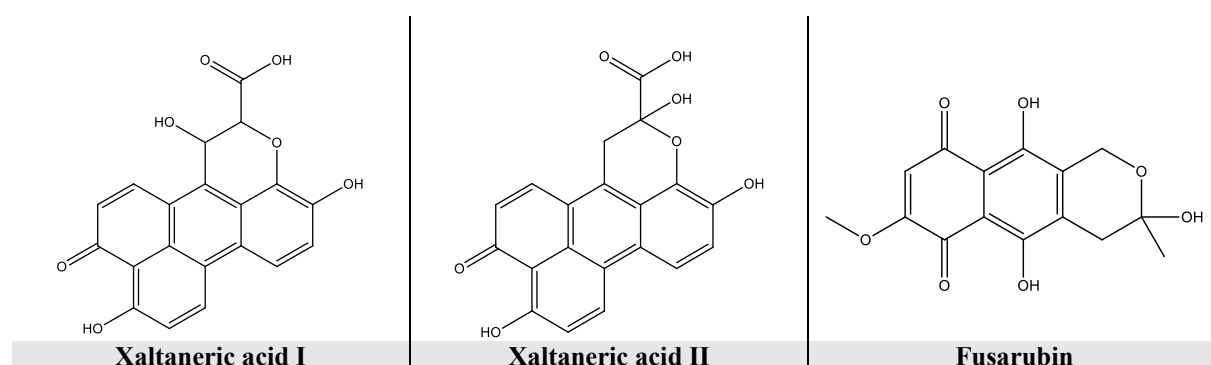
| | | | | |
|-----------------------------|--|--|--|---|
| Bioactivity | <ul style="list-style-type: none"> • Antibacterial • Antifungal • Antiviral | <ul style="list-style-type: none"> • Anticancer • Antioxidant • Antiparasitic | <ul style="list-style-type: none"> • Immunosuppressive • Antidiabetic | |
| Biosynthesis pathway | <ul style="list-style-type: none"> • Polyketides • Non-ribosomal peptides | <ul style="list-style-type: none"> • Isoprenoids • Hybrid compounds | | |
| Chemical groups | <ul style="list-style-type: none"> • Alkaloids • Terpenoids • Flavonoids | <ul style="list-style-type: none"> • Ketones • Phenols • Peptides | <ul style="list-style-type: none"> • Lactones • Quinones • Steroids | <ul style="list-style-type: none"> • Anthraquinones • Penylpropanoids • Sterides |

Figure 1.16. Classification of secondary metabolites from fungal endophytes by bioactivity, biosynthetic pathway and chemical group (own creation)

The book chapter entitled “Fungal endophytes: a source of anti-infectives”, wrote with my colleagues (Pacheco *et al.*, in Press) summarize the antibacterial and antiparasitic compounds isolated from endophytic fungi are largely discussed, specifically those isolated from medicinal plants from areas with great biodiversity (**Annexes**). In the following, some examples of bioactive compounds isolated from endophytic fungi will be mentioned.

Antibacterial

According to the World Health Organization (WHO), antibiotic resistance is one of the major global health problems with increasing cases of resistance in bacteria such as *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus* (MRSA), *Neisseria gonorrhoeae* and *Mycobacterium tuberculosis*. Two new compounds, xaltaneric acid I and II, were isolated from *Alternaria* sp., an endophytic fungus isolated from chinese mangrove plant *Sonneratia alba* (Lythraceae). The 10-oxo-10H-phenalenon[1,2,3-*de*]chromene skeleton of these compounds was for the first time reported in fungi. These new carboxylic acids exhibited antibacterial activity against multidrug-resistant *Staphylococcus aureus* (Kjer *et al.* 2009). Another life-threatening disease is tuberculosis caused by *Mycobacterium tuberculosis* and commonly associated with compromised human immune systems. Several antitubercular compounds corresponding to different chemical classes such as amides, peptides, polyketides and terpenes have been obtained from endophytic fungi (Alvin *et al.* 2014). For example, endophytic fungus *Fusarium solani* isolated from the medicinal plant *Glycyrrhiza glabra* (Fabaceae) was able to produce a new compound, fusarubin and other known compounds with antimicrobial activity, being fusarubin the compound that showed a good activity against *M. tuberculosis* (Shah *et al.* 2017). In addition to these compounds, three novel aminolipopeptides, trichoderins A, A1 and B were obtained from the endophytic fungus *Trichoderma* sp. isolated from an unidentified marine sponge. These compounds exhibited potent activity against *M. smegmatis*, *M. bovis* and *M. tuberculosis* (Pruksakorn *et al.* 2010). The structures of these compounds are shown in **Figure 1.17**.



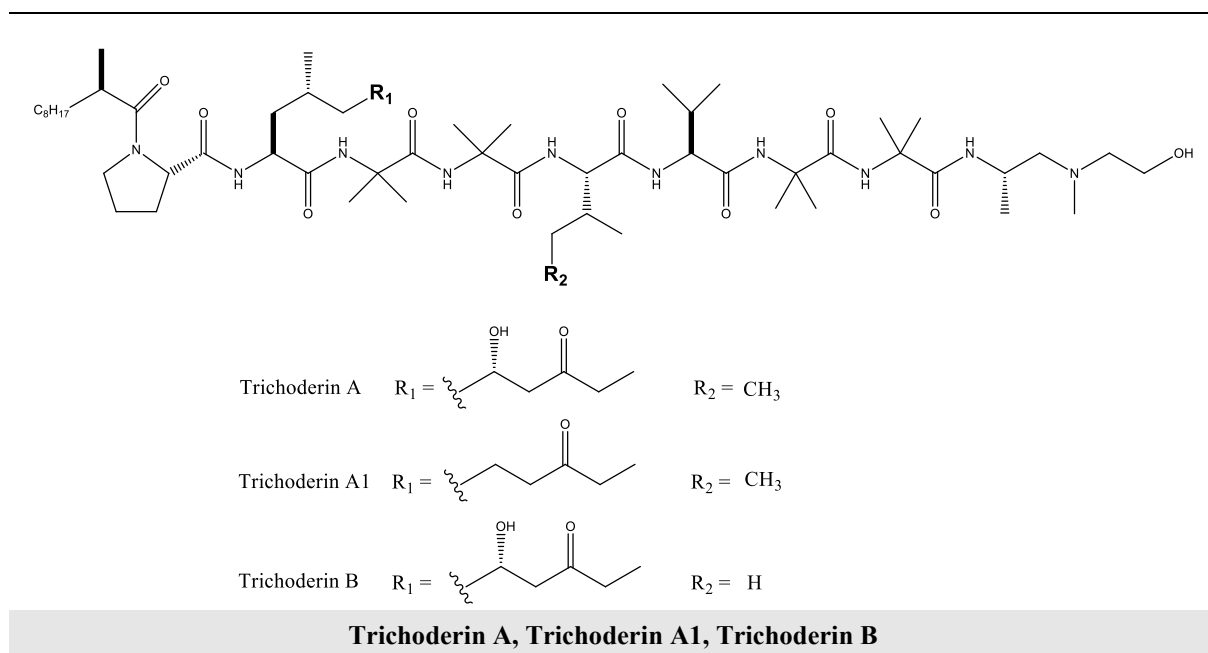


Figure 1.17 Examples of novel compounds isolated from endophytic fungi with antibacterial activity

Antifungal

Fungal diseases cause 6 millions of human deaths per year while in the agricultural sector, they generate many economic losses, threatening the food security and production. Antibiotic resistance in fungi are dramatically increasing mainly in immunocompromised individuals, posing a serious burden on healthcare systems. Endophytic fungi isolated from medicinal plants have shown to produce chemically diverse secondary metabolites with antifungal activity (Deshmukh *et al.* 2018). Endophytic fungus *Pestalotiopsis adusta*, isolated from the stem of an unidentified chinese tree, produced three new chlorinated benzophenone derivatives, pestalachlorides A-C, which exhibited significant antifungal activity against 3 plant pathogenic fungi, *Fusarium culmorum*, *Gibberella zae* and *Verticillium albo-atrum* (Li *et al.* 2008). The structures of these compounds are shown in **Figure 1.18**.

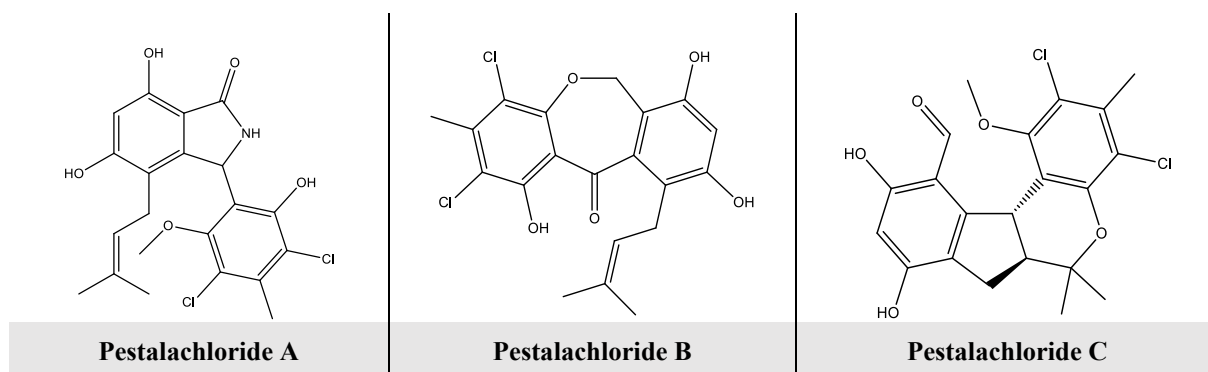


Figure 1.18. Examples of novel compounds isolated from endophytic fungi with antifungal activity

Antiparasitic

In the research of new drugs for treating tropical diseases such as leishmaniasis (caused by *Leishmania* spp.) or malaria (caused by *Plasmodium* spp.), endophytes have shown to harbor novel metabolites with biological activity. During a 15-year project, more than 2700 isolates were obtained from the fresh leaves of 3198 healthy plant samples (232 different genera) located in different National Parks of Panama possessing high biodiversity. The crude extracts of these endophytic fungi were tested for anti-parasitic activity showing that 16.6% of the isolates were highly active against *P. falciparum*, 15.4% were highly active against *L. donovani* while 4.1% of them were highly active against *T. cruzi* (Higginbotham *et al.* 2013).

The new ergochrome, purpleone was obtained from the endophytic fungus *Purpureocillium lilacinum* isolated from the roots of the medicinal plant *Rauvolfia macrophylla* (Apocynaceae). This compound exhibited a potent activity against the amastigotes of *Leishmania donovani* with a good selectivity index and antimicrobial activity (MIC=62.6 $\mu\text{g mL}^{-1}$), signaling the promising future investigations for drug development (Lenta *et al.* 2016). Additionally, two novel xanthone dimers, Phomoxanthone A and B, were obtained from the fungal endophyte *Phomopsis* sp. isolated from the leaves of *Tectona crispa* (Lamiaceae). They exhibited activity against *Plasmodium falciparum* K1 (IC₅₀=0.11 - 0.33 $\mu\text{g mL}^{-1}$) and cytotoxicity (IC₅₀=0.51 - 4.1 $\mu\text{g mL}^{-1}$) against 3 different cancer cell lines (Isaka *et al.* 2001). The structures of these compounds are shown in **Figure 1.19**.

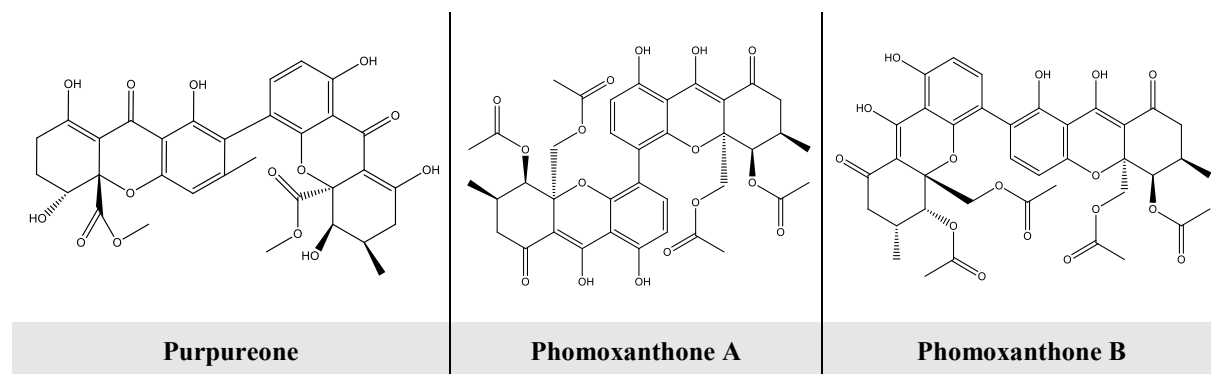


Figure 1.19. Examples of novel compounds isolated from endophytic fungi with antiparasitic activity

Antiviral

Viruses are a leading cause of mortality and morbidity. Current antivirals usually target viral proteins or host cellular factors used to reproduce and gain control of host cell processes, however the increasing emergence of resistance of viruses represents an important global public health problem. Some examples have shown that endophytic fungi are able to produce compounds active against some viruses. For

instance, a new rare sesquiterpenoid, phomanolide was recently isolated from the endophytic fungus *Phoma* sp. isolated from the roots of the plant *Aconitum vilmorinianum* (Ranunculaceae). This compound exhibited antiviral activities against influenza A virus (Liu *et al.* 2019). Also, a new hydroanthraquinone derivative, 6-O-demethyl-4-dehydroxyaltersolanol A, exhibited inhibitory effects on an influenza viral strain. This compound was isolated from the endophytic strain of *Nigrospora* sp. obtained from the roots of *Aconitum carmichaeli* (Ranunculaceae) (Zhang *et al.* 2016). The structures of these compounds are shown in **Figure 1.20**.

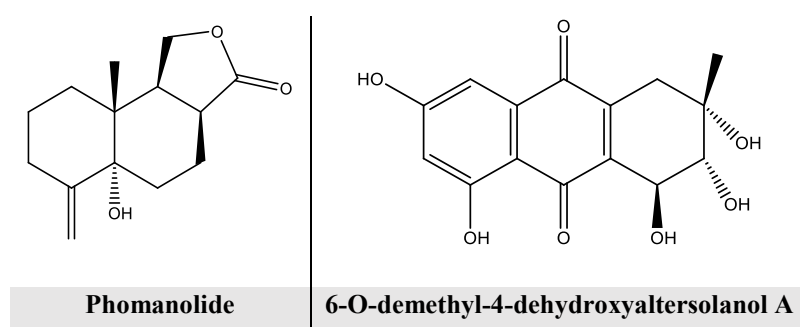


Figure 1.20. Examples of novel compounds isolated from endophytic fungi with antiviral activity

Anticancer

Despite all the efforts in developing new chemotherapies, cancer remains a major disease with high human mortality rates. Only in 2018, more than 18 million cancer incidences with almost 10 million of deaths were reported by the WHO. Considering the prolonged use of anticancer drugs and the several threatening side effects, many other sources were evaluated, including endophytic fungi (Li *et al.* 2018) after the discovery of taxol obtained from the endophytic fungus *Taxomyces andreanae*. Three novel azaphilone alkaloids, chaetomugilides A-C, produced by endophytic fungus *Chaetomium globosum*, isolated from the healthy barks of the medicinal plant *Ginkgo biloba* (Ginkgoaceae), exhibited high cytotoxicity against human cancer cell line HePG2 (hepatoblastoma) (Li *et al.* 2013). Other novel compounds were obtained from endophytic fungus *C. globosum* isolated from the fresh tissue of marine green alga *Ulva pectusa* (Ulvaceae). The novel alkaloids, cytoglobosins C and D exhibited cytotoxic activity against A-549 tumor cell line (adenocarcinoma human alveolar basal epithelial cells) (Cui *et al.* 2010). The structures of these compounds are shown in **Figure 1.21**.

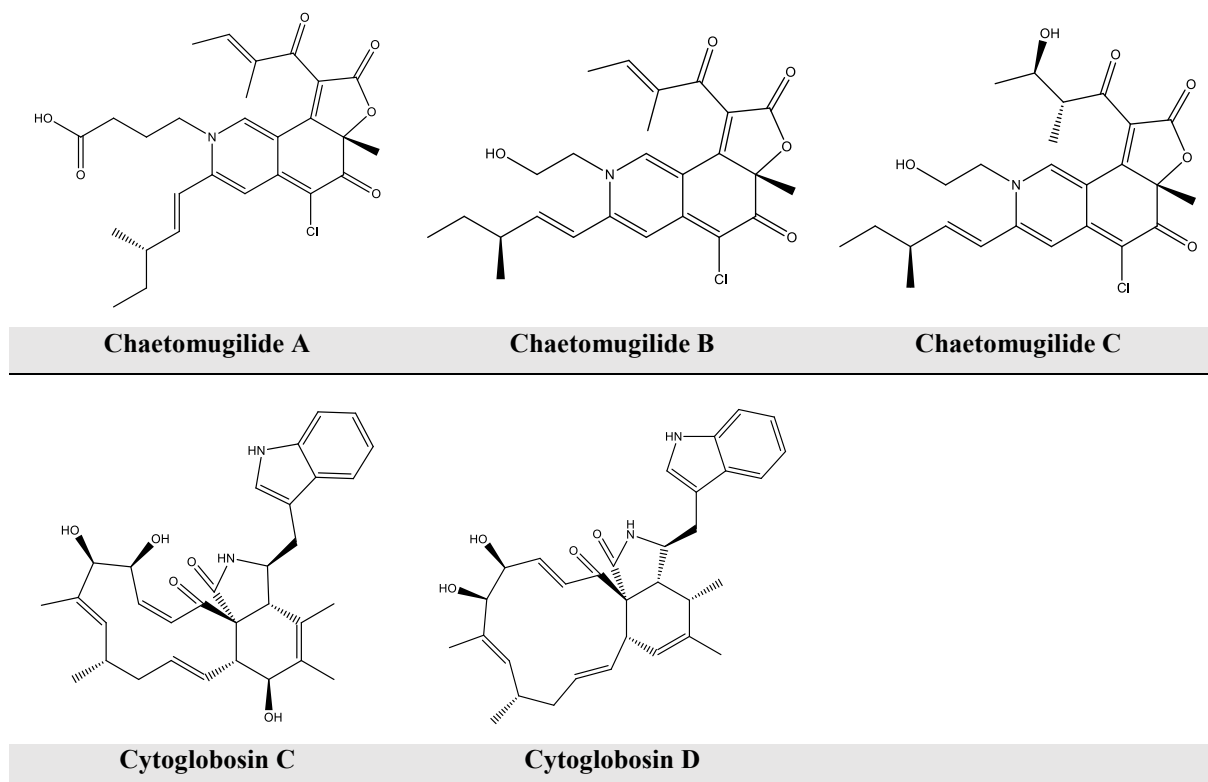
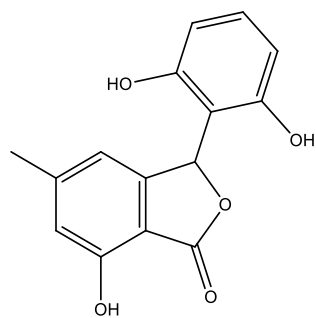


Figure 1.21. Examples of novel compounds isolated from endophytic fungi with anticancer activity

Antioxidant

Many studies have shown that phenolic acids, phenylpropanoids, flavonoids, melanin and tannins exhibit antioxidant activity. Endophytic fungi are known to produce these types of compounds to confer stress tolerance to the plant under oxidative stress. Additionally, in the human body, free radicals cause cell damage and contribute to cellular degeneration as in Alzheimer's disease, Parkinson's disease, Crohn's disease, atherosclerosis, hepatic and kidney damage, among other disorders influenced by oxidative stress. Extracts and compounds with strong antioxidant activities have been isolated from endophytic fungi colonizing medicinal plants from diverse environments (Kouipou Toghueo & Boyom 2019). Isopestacin is a new isobenzofuranone with a unique core, obtained from endophytic fungus *Pestalotiopsis microspora* isolated from the tropical plant *Terminalia morobensis* (Combretaceae). Besides its antifungal activity, it behaves as an antioxidant against superoxide and hydroxyl free radicals (Strobel *et al.* 2002). Moreover, a total of 42 endophytic fungi were isolated from *Nerium oleander* (Apocynaceae) and tested for their antioxidant activity, of which one strain of *Chaetomium* sp. showed the strongest antioxidant capacity, probably due to the high levels of phenolic acids and their derivatives and flavonoids detected in the fungal cultures (Huang *et al.* 2007). The structures of these compounds are shown in **Figure 1.22**.



Isopestacin

Figure 1.22. Example of a novel compound isolated from endophytic fungi with antioxidant activity

Immunosuppressive

Immunosuppressive drugs are important for treating autoimmune disorders or during organ transplants to suppress or reduce rejection. Fungal endophytes have shown to produce secondary metabolites with immunomodulatory activity and immunosuppressive action with low cytotoxicity and high selectivity. For instance, the endophytic fungus *Mycosphaerella nawae* isolated from the leaves of the medicinal plant *Smilax china* (Smilacaceae) produced a novel amide derivative, (-)-mycousnine enamine that selectively inhibited the proliferation of T cells through the inhibition of the expression of antigens CD25 and CD29 (Wang *et al.* 2017b). Moreover, the endophytic fungus *Penicillium* sp. isolated from the leaves of the mangrove plant *Sonneratia apetala* (Lythraceae), produced 9 polyketides including 2 novel benzophenone derivatives, peniphenone and methyl peniphenone, with immunosuppressive properties (Liu *et al.* 2016). The structures of these compounds are shown in **Figure 1.23**.

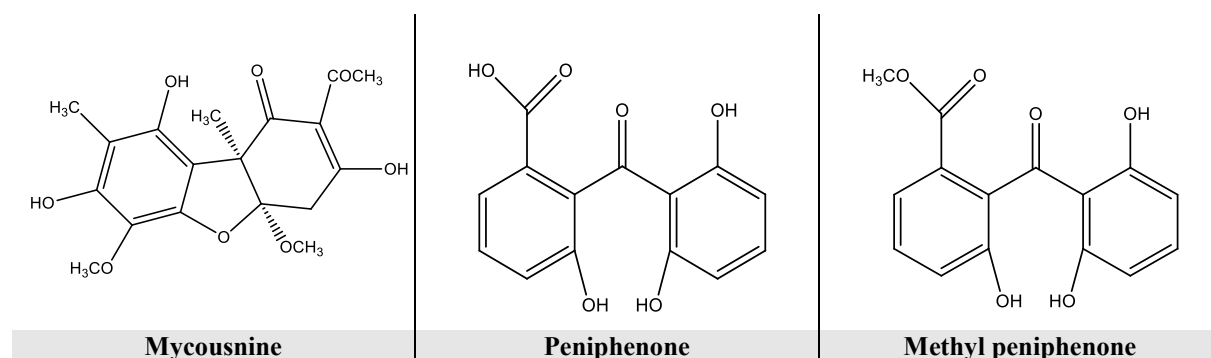


Figure 1.23. Examples of novel compounds isolated from endophytic fungi with immunosuppressive activity

Among the fungal endophytes, Dothideomycetes is one of the largest and most diverse class in Ascomycete fungi (Schoch *et al.* 2009). Among them, the Botryosphaeriales order contains the Botryosphaeria family and the species *Cophinforma mamane*, the object of this thesis. The following sections will give a special focus on this order and family, as well as the current knowledge about this fungus.

1.4 Botryosphaerales order (Dothideomycetes)

There is an increased interest in the study of the Botryosphaerales order and especially in the species within the Botryosphaeriaceae family which is evidenced by the number of publications found on SciFinder over the last years containing “Botryosphaerales” and “Botryosphaeriaceae” (**Figure 1.24**). This increase is related to the continuous efforts for a better taxonomic classification of the diverse fungal species but also due to their ecological importance as pathogens of important plant families and to their linkage to climate and anthropogenic changes to the environment (Slippers *et al.* 2017).

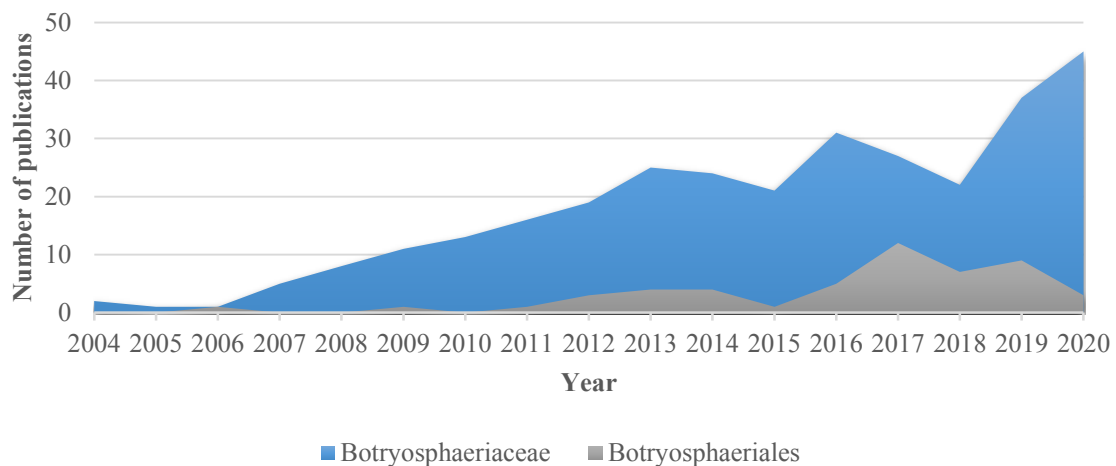


Figure 1.24. Number of Journal publications and patents including "Botryosphaerales" or "Botryosphaeriaceae" over the last years according to SciFinder

The Botryosphaerales order includes endophytic and saprophytic fungal species but most commonly pathogenic species associated with branches, leaves, fruits or seeds of woody plants distributed worldwide (Slippers *et al.* 2017). In 2006, the Botryosphaerales order was introduced for the Botryosphaeriaceae family and since then, the number of families residing in this order has been increasing (Slippers *et al.* 2013). Many of the re-assignments of the taxonomic groups based on DNA sequence data did not find a consensus due to the different genes or loci used for the phylogenetic reconstructions.

One of the last re-assessment of this order in terms of morphology, phylogenetic relationships based on ITS (Internal transcribed spacer) and LSU (large-subunit rRNA) sequences and evolutionary divergence allowed to consider 6 lineages that represent the families in the Botryosphaerales (**Table 1.2**): *Aplosporellaceae*, *Botryosphaeriaceae*, *Melanopsaceae*, *Phyllostictaceae*, *Planistromellaceae* and *Saccharataceae* (Phillips *et al.* 2019). However, in a precedent study, 8 families were recognized as part of the Botryosphaerales order in addition to the newly described families, *Endomelanconiopsisaceae* and *Pseudofusicoccumaceae* (Yang *et al.* 2017). This family went under dramatic taxonomic changes

mainly at the family level, besides the definition for the recognition of cryptic and hybrid species, essential for the understanding of the Botryosphaerales diversity (Slippers *et al.* 2017). More recently, a multigene phylogenetic analysis was performed using the sequences of ITS, LSU, *tefl* (translation elongation factor 1-alpha gene), *tub2* (beta-tubulin gene) and *rpb2* (RNA polymerase II second largest subunit gene) for 230 fungal isolates, where 8 novel species were described within a total of 33 genera recognized for the Botryosphaerales order, of which the majority belong to the Botryosphaeriaceae family (Zhang *et al.* 2021).

Table 1.2. Families and genera described in the Botryosphaerales by different authors

| Family | Genus | Author | | |
|---------------------------|---------------------------|-------------------|-----------------------|-----------------------|
| | | Yang et al., 2017 | Slippers et al., 2017 | Phillips et al., 2019 |
| Aplosporellaceae | <i>Aplosporella</i> | X | X | X |
| | <i>Bagnisiella</i> | X | X | X |
| | <i>Alanomyces</i> | - | - | X |
| Botryosphaeriaceae | <i>Alanphillipsia</i> | X | X | X |
| | <i>Bahusutrabeeja</i> | - | X | - |
| | <i>Barriopsis</i> | X | X | X |
| | <i>Botryobambusa</i> | - | X | X |
| | <i>Botryosphaeria</i> | X | X | X |
| | <i>Cophinforma</i> | X | X | X |
| | <i>Diplodia</i> | X | X | X |
| | <i>Dothiorella</i> | X | X | X |
| | <i>Endomelanconiopsis</i> | - | Other family | X |
| | <i>Eutiarospella</i> | X | X | - |
| | <i>Lasiodiplodia</i> | X | X | X |
| | <i>Macrophomina</i> | X | X | X |
| | <i>Marasasiomyces</i> | X | X | - |
| | <i>Mucoharknessia</i> | - | X | - |
| | <i>Neodeightonia</i> | X | X | X |
| | <i>Neofusicoccum</i> | X | X | X |
| | <i>Neoscytalidium</i> | X | X | X |
| | <i>Oblomgocollomyces</i> | X | X | X |
| | <i>Othia</i> | - | X | - |
| | <i>Phaeobotryon</i> | X | X | X |
| | <i>Pseudofusicoccum</i> | - | Other family | X |
| | <i>Sakireeta</i> | X | X | - |
| | <i>Sardiniella</i> | - | X | - |
| | <i>Spencermartinsia</i> | (=Dothiorella) | (=Dothiorella) | - |
| | <i>Sphaeropsis</i> | - | X | X |
| | <i>Tiarosporella</i> | X | X | X |

| | | | | |
|------------------------------|---------------------------|--------------|--------------|--------------|
| Endomelanopsisaceae | <i>Endomelanconiopsis</i> | X | X | Other family |
| Melanopsaceae | <i>Melanops</i> | X | X | X |
| Phyllostictaceae | <i>Phyllosticta</i> | - | X | X |
| | <i>Pseudofusicoccum</i> | Other family | Other family | X |
| Planistromellaceae | <i>Kellermania</i> | X | X | X |
| | <i>Umthunziomyces</i> | - | X | X |
| Pseudofusicoccumaceae | <i>Pseudofusicoccum</i> | X | X | Other family |
| | <i>Saccharata</i> | Other family | X | Other family |
| Saccharataceae | <i>Saccharata</i> | X | Other family | X |
| | <i>Neoseptorioides</i> | - | - | X |
| | <i>Septorioides</i> | Other family | Other family | X |
| Septorioideaceae | <i>Septorioides</i> | X | X | Other family |

1.4.1 Botryosphaeriaceae family

Botryosphaeriaceae is the largest family within the Botryosphaeriales order, generally existing as hemibiotrophic³ in healthy plant tissues but also as endophytes or saprobes. They possess a cosmopolitan distribution and a diverse morphology, where the conidial features are the most informative characters for differentiation such as pigmentation, wall thickness and septation. Despite the efforts for a better taxonomic classification and the gained knowledge about their phylogenetic divergence, there are still some differences in the consensus about the number of genera belonging to this family. Besides the genera shown in **Table 1.2** for Botryosphaeriaceae, two other studies recognize a total of 24 well-defined genera with more than 200 species (Burgess *et al.* 2019; Garcia *et al.* 2021). In general, the ecology of the taxa from Botryosphaeriaceae is complex added to the fact that some fungi have been considered as pathogenic species but later, they have been found during their endophytic phase and reported as such.

In the case of infection of horticultural crops, ecological genomic comparisons of isolates originating from different symptomatic crops showed that gene expansion falls mainly into lytic capabilities and putative transporters, which are related to pathogenicity. The genome sequences of 17 species representing 6 genera of these family, indicated that species with gene families in expansion, encoding for secreted cell wall degrading enzymes, secondary metabolism and transporters, are the most virulent ones such as species from the genera *Lasiodiplodia*, *Botryosphaeria* and *Neofusicoccum* (Garcia *et al.* 2021).

Considering climate change, mainly the increase of temperature, environmental conditions have shown to modify symbiotic or commensal interactions into pathogenic interactions. Under this scenario,

³ Hemibiotrophic: group of plant pathogens that require living plant tissue to survive and complete their life cycle

frequent extreme temperatures and prolonged droughts can make this transit of interactions easier. For example, filtrates of 5 species of Botryosphaeriaceae showed to be dramatically more phytotoxic and cytotoxic when the temperature of culture changed from 25 to 37°C, indicating that in during a global increase of temperature, this species might contribute to plant and even to human infections (Nazar Pour *et al.* 2020).

A diverse range of bioactive secondary metabolites are produced by members of the Botryosphaeriaceae family, of which most of them are associated with plant host diseases, however further studies are needed to understand whether or not these metabolites are related with their virulence. It is known that during their endophytic life, they do not cause apparent damage to the host cell and do not generate a host response. Despite this, when the host gets subjected to different stress factors, they might become latent pathogens. In this regard, it is suggested that climate change will continue to trigger stress on plant species and this will indirectly affect the interactions between the plant and their fungal communities.

Most of the bioactive secondary metabolites reported in the literature produced by members of the Botryosphaeriaceae family were isolated from species belonging to the genera *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neofusicoccum* and *Sphaeropsis* (Kumar 2020) and they are shown in **Table 1.3**.

Table 1.3. Main bioactive compounds from the Botryosphaeriaceae Family

| Genus | Species | Compound | Bioactivity | Reference |
|-----------------------|----------------|------------------------------|---|------------------------|
| <i>Botryosphaeria</i> | <i>rhodina</i> | Botryorhodine A-D | Antifungal, cytotoxic | Abdou (2010) |
| | <i>species</i> | Botryosphaerin H | Antifungal, nematocidal | Chen (2015) |
| | <i>obtusa</i> | (3R,4R)-4,7-dihydroxymellein | Phytotoxins | Djoukeng (2009) |
| | <i>species</i> | Botryoisocoumarin A | COX-2 inhibitor | Ju (2015) |
| | <i>species</i> | Botryosphaerin B | COX-2 inhibitor | Ju (2016) |
| | <i>rhodina</i> | Botryosphaerilactone A-C | Antibacterial | Rukachaisirikul (2009) |
| | <i>rhodina</i> | Botryosphaeridione | Antibacterial | Rukachaisirikul (2009) |
| | <i>rhodina</i> | Botryosphaerihydrofuran | Antibacterial | Rukachaisirikul (2009) |
| | <i>rhodina</i> | Botryosphaerinone | Antibacterial | Rukachaisirikul (2009) |
| | <i>rhodina</i> | Botryosphaeriodiplodin | Antibacterial | Rukachaisirikul (2009) |
| | <i>obtusa</i> | Melleins | Phytotoxins | Venkatasubbaiah (1991) |
| | <i>obtusa</i> | Tyrosol | Phytotoxins | Venkatasubbaiah (1991) |
| | <i>obtusa</i> | 4-hydroxymellein | Phytotoxins | Venkatasubbaiah (1991) |
| | <i>obtusa</i> | 5-hydroxymellein | Phytotoxins | Venkatasubbaiah (1991) |
| | <i>obtusa</i> | 4-hydroxybenzaldehyde | Phytotoxins | Venkatasubbaiah (1991) |
| | <i>rhodina</i> | Botryosphaeran | Immunomodulatory, antioxidant, antitumor, hypoglycemic, hypocholesterolemic | Weng (2011) |

| | | | | |
|-------------------------|-------------------------|---|--|------------------|
| | <i>dothidea</i> | 3-Hydroxy-2-methoxy-5-methylpyridin-2(1H)-one | Antimicrobial, antioxidant, cytotoxic | Xiao (2014) |
| | <i>dothidea</i> | Pycnophorin | Antimicrobial | Xiao (2014) |
| | <i>dothidea</i> | Stemphyperylenol | Antifungal | Xiao (2014) |
| | <i>dothidea</i> | Altenuene | Cytotoxic | Xiao (2014) |
| | <i>dothidea</i> | Altenusin | Antioxidant | Xiao (2014) |
| | <i>dothidea</i> | Djalonsone | Antioxidant | Xiao (2014) |
| | <i>australis</i> | 6-Ethyl-2,7-dimethoxyjuglon | Phytotoxic | Xu (2011) |
| | <i>australis</i> | 6-Ethyl-2,7-dimethoxyjuglon derivative | Cytotoxic, antimicrobial | Xu (2011) |
| | <i>species</i> | Botryosphaerin A-E | Antibacterial | Yuan (2009) |
| <i>Diplodia</i> | <i>quercivora</i> | Diplopimarane | Phytotoxic | Andolfi (2014b) |
| | <i>quercivora</i> | Sphaeropsidin A | Antifungal | Andolfi (2014b) |
| | <i>corticola</i> | Diorcinol | Phytotoxic | Cimmino (2016b) |
| | <i>mutila</i> | Diplopyrone | Phytotoxic | Evidente (2003) |
| | <i>corticola</i> | Diplofuranone A | Phytotoxic | Evidente (2007) |
| | <i>cupressi</i> | Sphaeropsidone | Phytotoxic | Evidente (2011a) |
| | <i>cupressi</i> | Sphaeropsidin A | Phytotoxic | Evidente (2011b) |
| | <i>africana</i> | Afritoxinone A | Phytotoxic | Evidente (2012) |
| | <i>mutila</i> | Diploquinone A-B | Phytotoxic | Reveglia (2018a) |
| | <i>mutila</i> | Vanillic acid | Phytotoxic | Reveglia (2018a) |
| <i>Dothiorella</i> | <i>vidmadera</i> | Resorcinol | Phytotoxic | Reveglia (2018b) |
| | <i>vidmadera</i> | Protocatechuic alcohol isopropyl ether | Phytotoxic | Reveglia (2018b) |
| | <i>vidmadera</i> | Benzene-1,2,4-triol | Phytotoxic | Reveglia (2018b) |
| | <i>vidmadera</i> | 3-Hydroxymethyl phenol | Phytotoxic | Reveglia (2018b) |
| | <i>vidmadera</i> | Protocatechuic alcohol | Phytotoxic | Reveglia (2018b) |
| | <i>vidmadera</i> | Tyrosol | Phytotoxic | Reveglia (2020) |
| <i>Lasioidiplodia</i> | <i>pseudotheobromae</i> | Mellein | Bioherbicidal | Adetunji (2018) |
| | <i>theobromae</i> | Jasmonic acid | Plant growth inhibitor | Alridge (1971) |
| | <i>Species</i> | Lasiojasmonate A | Phytotoxic | Andolfi (2014a) |
| | <i>Mediterranea</i> | Lasiolactol A-B | Phytotoxic | Andolfi (2016) |
| | <i>species</i> | Lasicicol | Alfa-glucosidase inhibitory activity | Chen (2015) |
| | <i>theobromae</i> | Chloropreussomerin A-B | Cytotoxic, Antibacterial | Chen (2016b) |
| | <i>theobromae</i> | Preussomerin A, F, G, H | Antibacterial | Chen (2016b) |
| | <i>brasiliense</i> | (3R,4S)-(-)-4-Hydroxymellein | Phytotoxic | Cimmino (2017) |
| | <i>theobromae</i> | 3-Indolecarboxylic acid | Phytotoxic | Felix (2018b) |
| | <i>species</i> | Adeninealkylresorcinol | Antioxidant | Gao (2016) |
| | <i>species</i> | Lasiosan | Antimicrobial, anti-biofilm, antioxidant, immunomodulatory | Kumar (2018) |
| | <i>species</i> | 7-Oxolasioidiplodin | Cytotoxic | Li (2016) |
| | <i>species</i> | Ethyl-2,4-Dihydroxy-6-nonylbenzoate | Cytotoxic | Li (2016) |
| <i>pseudotheobromae</i> | Palmarumycin LP1 | Cytotoxic | Lü (2014) | |

| | | | | |
|-------------------------|-------------------------|-------------------------------------|------------------------|---------------------------|
| | <i>pseudotheobromae</i> | Cladospirone B | Cytotoxic | Lü (2014) |
| | <i>theobromae</i> | (R)-(-)-2-Octeno- δ -lactone | Insect attractant | Matsumoto and Nago (1994) |
| | <i>theobromae</i> | Cyclohexenone derivatives | Phytotoxic | Matsuura (1998b) |
| | <i>theobromae</i> | Lasiodiplodin | Cytotoxic | Sultan (2014) |
| | <i>theobromae</i> | Ethyl linoleate | Plant growth regulator | Uranga (2016) |
| | <i>theobromae</i> | Ethyl palmitate | Plant growth regulator | Uranga (2016) |
| | <i>theobromae</i> | Ethyl stearate | Plant growth regulator | Uranga (2016) |
| | <i>pseudotheobromae</i> | Lasiodipline A-F | Antibacterial | Wei (2014) |
| | <i>theobromae</i> | Maculosin | Antibacterial | Zaher (2015) |
| <i>Neofusicoccum</i> | <i>parvum</i> | (+)-Epi-sphaeropsidone | Phytotoxic | Abou-Mansour (2015) |
| | <i>australe</i> | Cyclobotryoxide | Phytotoxic | Andolfi (2012) |
| | <i>parvum</i> | Botryosphaerone D | Phytotoxic | Burrano (2016) |
| | <i>parvum</i> | Exopolysaccharide | Phytotoxic | Cimmino (2016a) |
| | <i>luteum</i> | Exopolysaccharide | Phytotoxic | Martos (2008) |
| <i>Spenceriartinsia</i> | <i>viticola</i> | Spencertoxin | Phytotoxic | Reveglia (2020) |
| | <i>viticola</i> | p-Hydroxybenzaldehyde | Phytotoxic | Reveglia (2020) |
| | <i>viticola</i> | 2-(4-Hydroxyphenyl) acetic acid | Phytotoxic | Reveglia (2020) |
| <i>Sphaeropsis</i> | <i>sapinea</i> | R-(-)-Mellein | Phytotoxic | Cabras (2006) |
| | <i>sapinea</i> | (3R,4R)-4-hydroxymellein | Phytotoxic | Cabras (2006) |
| | <i>sapinea</i> | (3R,4S)-4-hydroxymellein | Phytotoxic | Cabras (2006) |
| | <i>sapinea</i> | Sphaeropsidin A - C | Phytotoxic | Evidente (1997) |

1.4.2 *Cophinforma mamane* (*Botryosphaeria mamane* D.E. Gardner) A. J. L. Phillips & A. Alves

Botryosphaeria mamane was first described in an endemic Hawaiian tree, *Sophora chrysophylla* (Fabaceae), associated with witches' broom disease (Gardner 1997). Unfortunately, the ex-type cultures of this strain no longer exist and no other sequences were generated after the ones published in Genbank under the codes AF246929 and AF246930. Later, this endophyte was described in other plants such in *Garcinia mangostana* (Clusiaceae) (Pongcharoen *et al.* 2007), in the medicinal Amazonian plants *Carapa guianensis* (Meliaceae) (Ferreira *et al.* 2015) and in *Bixa orellana* (Bixaceae) (Barakat *et al.* 2019). A similar species was also found in *Acacia*, *Eucalyptus* and *Pinus* species from Venezuela (Mohali *et al.* 2007) but it was later considered to represent *Cophinforma atrovirens* (Phillips *et al.* 2013). Additionally, the genus *Cophinforma* was introduced in 2012 to better accommodate the species

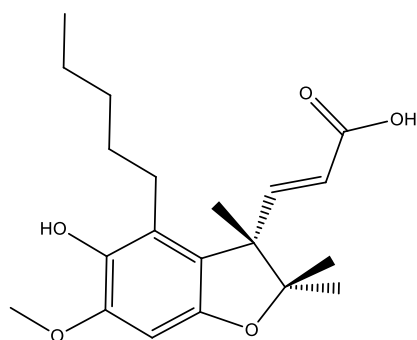
C. eucalypti (Liu *et al.* 2012) while in 2013, the species *Botryosphaeria mamane* was recognized as a basionym of *Cophinforma mamane* (Phillips *et al.* 2013).

Few chemical studies have been performed on *C. mamane*. Currently, only 17 compounds have been isolated from different strains of this species. One new dihydrobenzofuran derivative, botryomaman and six other known compounds were obtained from *Botryosphaeria mamane* isolated from *Garcinia mangostana* (Clusiaceae). All these compounds were tested for antibacterial activity, primin being the one that exhibited bioactivity (MIC 8 µg/mL) against two strains of *Staphylococcus aureus* (Pongcharoen *et al.* 2007). Moreover, volatile organic compounds (VOCs) were obtained from different endophytic fungi of the Botryosphaeriaceae family, including 6 VOCs isolated from one strain of *Botryosphaeria mamane* isolated from diverse brazilian plants (species not specified) (Oliveira *et al.* 2015). Also, three novel thiodiketopiperazines were isolated from the endophytic fungus *Botryosphaeria mamane* isolated from the healthy leaves of *Bixa orellana* (Bixaceae). These compounds present sulfur atoms on rarely described $-\alpha$ and $-\beta$ positions of phenylalanine derived residues and also two unprecedented spirocyclic centers at C4 and C2'. Botryosulfuranols A-C exhibited cytotoxic activity against 4 different cancer cell lines (Barakat *et al.* 2019). A fourth thiodiketopiperazine, botryosulfuranol D, has also been isolated from this strain (unpublished data). These compounds are listed in **Table 1.4** and their respective structures are shown in **Figure 1.25**.

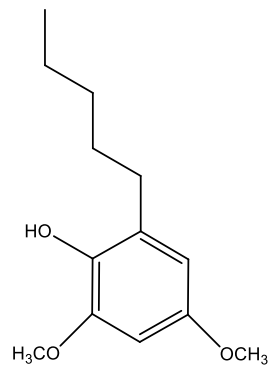
Table 1.4. Compounds isolated from *Cophinforma mamane* (*Botryosphaeria mamane*)

| Nº | Compound | Bioactivity | Reference |
|----|-------------------------------|--|--------------------|
| 1 | Botryomaman | >128µg/mL against SA and MRSA | Pongcharoen (2007) |
| 2 | 2,4-dimethoxy-6-pentylphenol | >128µg/mL against SA and MRSA | Pongcharoen (2007) |
| 3 | (R)-(-)-mellein | >128µg/mL against SA and MRSA | Pongcharoen (2007) |
| 4 | cis-4-hydroxymellein | >128µg/mL against SA and MRSA | Pongcharoen (2007) |
| 5 | trans-4-hydroxymellein | >128µg/mL against SA and MRSA | Pongcharoen (2007) |
| 6 | 4,5-dihydroxy-2-hexenoic acid | >128µg/mL against SA and MRSA | Pongcharoen (2007) |
| 7 | Primin | 8 µg/mL against SA and MRSA | Pongcharoen (2007) |
| 8 | Isobutanol | - | Oliveira (2015) |
| 9 | Isopentyl alcohol | - | Oliveira (2015) |
| 10 | 2-Methylbutan-1-ol | - | Oliveira (2015) |
| 11 | Phenylethyl alcohol | - | Oliveira (2015) |
| 12 | Aristolene | - | Oliveira (2015) |
| 13 | α -Selinene | - | Oliveira (2015) |
| 14 | Botryosulfuranol A | Antiparasitic (IC ₅₀ =0.69 µM) against <i>L. infantum</i> amastigotes; Cytotoxic (CC ₅₀ 8 - 23.5 µM) against 4 cancer cell lines | Barakat (2019) |
| 15 | Botryosulfuranol B | Antiparasitic (IC ₅₀ =3.87 µM) against <i>L. infantum</i> amastigotes Cytotoxic (CC ₅₀ 49.9 - >100 µM) against 4 cancer cell lines | Barakat (2019) |

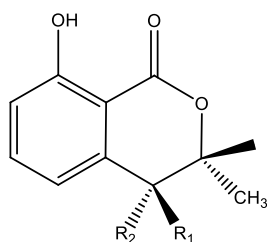
| | | | |
|----|--------------------|--|----------------|
| 16 | Botryosulfuranol C | Antiparasitic ($IC_{50}=0.44 \mu M$) against <i>L. infantum</i> amastigotes Cytotoxic ($CC_{50} 15.9 - >100 \mu M$) against 4 cancer cell lines | Barakat (2019) |
| 17 | Botryosulfuranol D | Antiparasitic ($IC_{50}=0.03 \mu M$) against <i>L. infantum</i> amastigotes | Barakat (2019) |



(1) Botryomaman



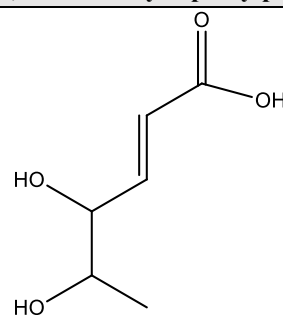
(2) 2,4-dimethoxy-6-pentylphenol



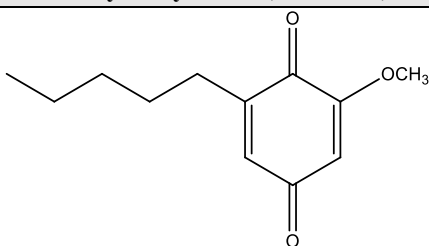
(3) (R)-(-)-mellein; $R_1 = R_2 = H$

(4) *cis*-4-hydroxymellein; $R_1 = H, R_2 = OH$

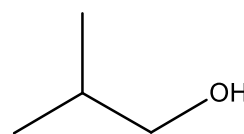
(5) *trans*-4-hydroxymellein; $R_1 = OH, R_2 = H$



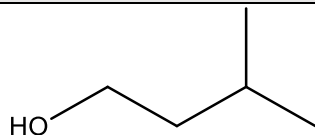
(6) 4,5-dihydroxy-2-hexenoic acid



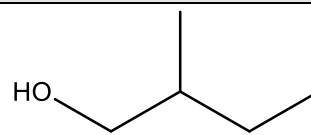
(7) Primin



(8) Isobutanol



(9) Isopentyl alcohol



(10) 2-Methylbutan-1-ol

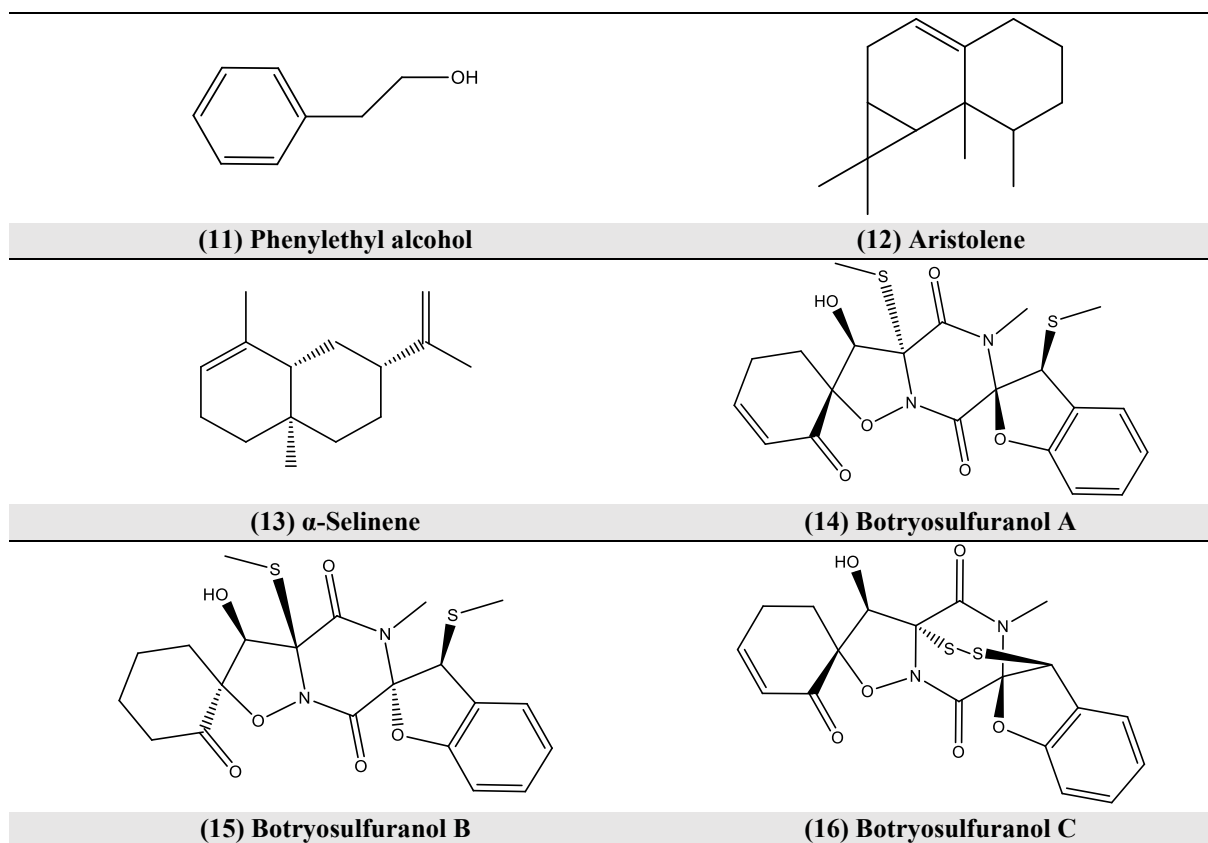


Figure 1.25. Structures of compounds isolated from *Cophinforma mamane* (*Botryosphaeria mamane*)

Regarding the plants used in traditional medicine and also used for the isolation of endophytic fungi, the following section will focus on the plant *Bixa orellana*, from which the fungus *Cophinforma mamane* E224 and other endophytes were isolated.

Bixa orellana L. (*Bixaceae*) as a source of endophytes

Bixa orellana L. is a tropical tree from the Bixaceae family (**Figure 1.26**), native from the Central and South American rainforests known under the common names of annatto, achiote, urucum, urucu or roucou. One of the main uses by the Amazonian tribes is the colored ink of the seeds for body painting. Traditionally, it is used to treat various diseases and ailments such as fevers, inflammatory conditions, parasitic diseases, dysentery, vomiting, heartburn, prostate, urinary difficulties and stomach problems (Giorgi *et al.* 2013). It also has a commercial use for food industry, textiles, dye manufacturing and printing industry due to its high content of the red-colored carotenoid, bixin (Vilar *et al.* 2014).



Figure 1.26. Plant of *Bixa orellana* (a) with their fruits (b, c) and seeds (d) (Raddatz-Mota *et al.*, 2017)

Several studies have shown the different biological activities of the leaves, seeds and roots of *B. orellana* while some the findings are in accordance with the traditional use. For instance, the ethanolic extract of the plant seeds showed significant activity against promastigotes and amastigotes of *Leishmania amazonensis* (García *et al.* 2011). Similarly, the hairy root culture of *B. orellana* was extracted with ethyl acetate for the isolation of major compounds. Both crude extract and isolated compounds exhibited activity against *Plasmodium falciparum* (Zhai *et al.* 2014). Regarding its antimicrobial activity, the methanolic extracts of the seeds and leaves of *B. orellana* showed significant activity against bacteria such as *Staphylococcus aureus*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Vibrio cholera*, *Moraxella catarrhalis*, *Acinetobacter sp.*, *Brucella sp.* and against fungi such as *Trichophyton mentagrophytes* and *Trichophyton rubrum* (Tamil Selvi *et al.* 2011). The hydroalcoholic and ethyl acetate fractions of the leaves exhibited also antimicrobial activity against *Mycobacterium abscessus* accompanied by anti-inflammatory activity in a peritonitis model induced in mice by *M. abscessus* (Lima Viana *et al.* 2018). Additionally, the aqueous extract of the seeds from a plant collected in the northeast of Brazil was capable of reversing the hypertriglyceridemia induced in mice (Ferreira *et al.* 2013) while the aqueous extract of the leaves from *B. orellana* collected in Malaysia showed anti-inflammatory activity produced by histamine in rats (Yong *et al.* 2013).

Compounds isolated from different parts of this plant are chemically diverse, such as carotenoids, apocarotenoids, sterols, aliphatic compounds, terpenoids and terpenes, volatile compounds (essential oils) and many others have been described from all the plant parts from *Bixa orellana* (Giorgi *et al.* 2013; Vilar *et al.* 2014; Shahid-ul-Islam *et al.* 2016).

Regarding the use of the plant for the isolation of endophytes, different parts of a healthy plant of *B. orellana* growing in an Indian forest were collected for the isolation of fungal endophytes (Kannan 2017). This was the first report on endophytic fungi documented from this medicinal plant, where 300 segments of stem cuttings, leaves and roots were used. The identification was based on morphology of the isolates. Isolates from the leaves were identified as *Pestalotiopsis* sp., *Phoma* sp., *Phyllosticta* sp. and *Alternaria* sp., *Colletotrichum* sp. and *Nigrospora* sp. Stem was dominated by *Pestalotiopsis* sp. *Chaetomium* sp. and *Nigrospora* sp. Recently, a novel lactone was obtained as a red pigment from the endophytic fungus *Fusarium verticillioides*, isolated from the leaves of *Bixa orellana* (Bixaceae) (Vijayakumar *et al.* 2021). Additionally, a harvest of *Bixa orellana* leaves from the Amazonian forest of Peru led to the isolation of 17 fungal endophytes including a strain of *Cophinforma mamane* (E224) from which the antileishmanial botryosulfuranols had been isolated (Triastuti *et al.* 2019; Barakat *et al.* 2019). Other strains were also isolated in this study belong to the genera *Diaporthe* spp., *Nigrospora* spp., *Phyllosticta* spp., *Endomelanconiopsis* spp., *Sporothrix* spp. and *Xylaria* spp. (unpublished data).

1.5 Different methodologies for metabolite induction in endophytic fungi

The number of gene clusters found in fungi is greatly superior to the number of gene clusters known from the isolated secondary metabolites (Pettit 2011). Conditions for gene expression are not fully understood and the presence of silent or lowly-expressed genes represent one of the main challenges for the isolation of novel secondary metabolites under laboratory conditions (Brakhage & Schroeckh 2011). Different approaches are proposed for the activation of cryptic gene clusters in endophytic fungi, broadly divided into methods based on genetic engineering techniques, in order to express biosynthetic gene clusters, and methods based on modifying the cultivation conditions to provide a better environment to trigger the expression of these gene clusters. The variations of the culture conditions known as OSMAC approach (Pan *et al.* 2019), the co-cultivation with other microorganisms (Chagas & Pupo 2018) and the addition of chemical elicitors and epigenetic modifiers, have shown to be rapid and useful techniques for the induction of cryptic genes in fungi (Zutz *et al.* 2014).

1.5.1 One Strain - Many Compounds (OSMAC)

The different methodologies that aim to modify the growth parameters are known as the OSMAC approach (One Strain - Many Compounds) which is based on the potential of one single strain to produce different compounds under small changes in the culture conditions (Bode *et al.* 2002; Romano *et al.* 2018). Under this approach, many endophytic fungi have been able to expand their chemical diversity in culture producing new metabolites with biological activity (Ariantari *et al.* 2019; Tran-Cong *et al.* 2019; Fan *et al.* 2019). Regarding the changes in nutrient regimes, the types of carbon, nitrogen, sulfur and phosphorus sources, the presence of trace elements and the culture on solid or liquid media can elicit a differential production of secondary metabolites besides its influence on growth. Also, the change of physical parameters such as temperature, aeration and shaking conditions, salinity, pH levels and osmotic stress are considered for the optimization of culture conditions as well as for the metabolite induction (Pan *et al.* 2019).

Fungi use light as a source of information to anticipate conditions for vegetative growth, conidia production, sexual development and circadian rhythms (Tisch & Schmoll 2010). Quantity, direction, wavelength and quality of light are used as a signal to induce these adaptive responses and to control development which can also affect their interaction with the plant hosts (Schumacher 2017). A large proportion of the fungal genome is regulated by light which encodes a photosensory system capable of responding to different intensities and wavelengths of light (Yu & Fischer 2019). For instance, aflatoxin production by *Aspergillus flavus* is influenced by light (Joffe & Lisker 1969) as well as the compounds ochratoxin 1 (Aziz & Moussa 1997), averufin or versicolorin A and C (Bennett *et al.* 1981). In addition

to this, bioactive compounds such as anthraquinone and griseofulvin have exhibited an enhanced production under red, blue or green light conditions compared to dark conditions, showing the importance of light in metabolite production (Zhang *et al.* 2017).

The time for growth and development of each fungal strain is another important factor to consider for the discovery of new metabolites or for monitoring specific compounds behavior (Peters *et al.* 2010). The metabolome of microorganisms is constantly changing due to all the dynamic processes involved during development (Roullier *et al.* 2016). Some studies have shown the potential novelty hidden when considering the time frame in fungi culture for natural product discovery (Choi *et al.* 2010; Bertrand *et al.* 2014). For example, the metabolome of the endophytic fungus *Cophinforma mamane* was studied in a time-scale revealing the presence of 3 *de novo* compounds with different emergence periods induced in co-culture with *Fusarium solani* (Triastuti *et al.* 2021).

1.5.2 Chemical elicitation

Several small molecules possessing diverse chemical structures are reported to act as histone deacetylase (HDAC) inhibitors or DNA methyltransferase (DNMT) inhibitors which possess diverse chemical structures and have been used for the induction of cryptic genes in fungal endophytes (**Figure 1.27**). The HDAC family can be divided into four major classes, I, II, III and IV. Among the HDAC inhibitors, the organic compounds suberoylanilide hydroxamic acid (SAHA), suberohydroxamic acid (SBHA), sodium butyrate, valproic acid and trichostatin A inhibit HDAC classes I and II, while nicotinamide and quercetin inhibit HDAC class III (Gupta *et al.* 2020b). Among the DNMT inhibitors, 5-azacytidine (AZA), hydralazine, procainamide and procaine have been reported (Toghueo *et al.* 2020). For instance, the compound piperine showed an enhanced production when the endophytic fungi, *Diaporthe* sp. was treated with suberohydroxamic acid and valproic acid (Jasim *et al.* 2019). Similarly, the addition of 5-azacytidine enhanced the production of camptothecin in *Botryosphaeria rhodina* (Vasanthakumari *et al.* 2015) while the addition of nicotinamide to the culture of the endophytic fungus *Graphiopsis chlorocephala*, led to a general enrichment in secondary metabolite production, allowing the isolation of new benzophenones and cephalanones (Asai *et al.* 2013). The treatment with epigenetic modifiers for the induction of secondary metabolites in endophytic fungi has the advantage of not requiring genetic manipulation, allowing the identification of new compounds and the enhancement of the synthesis of low-yield metabolites (Deshmukh *et al.* 2018).

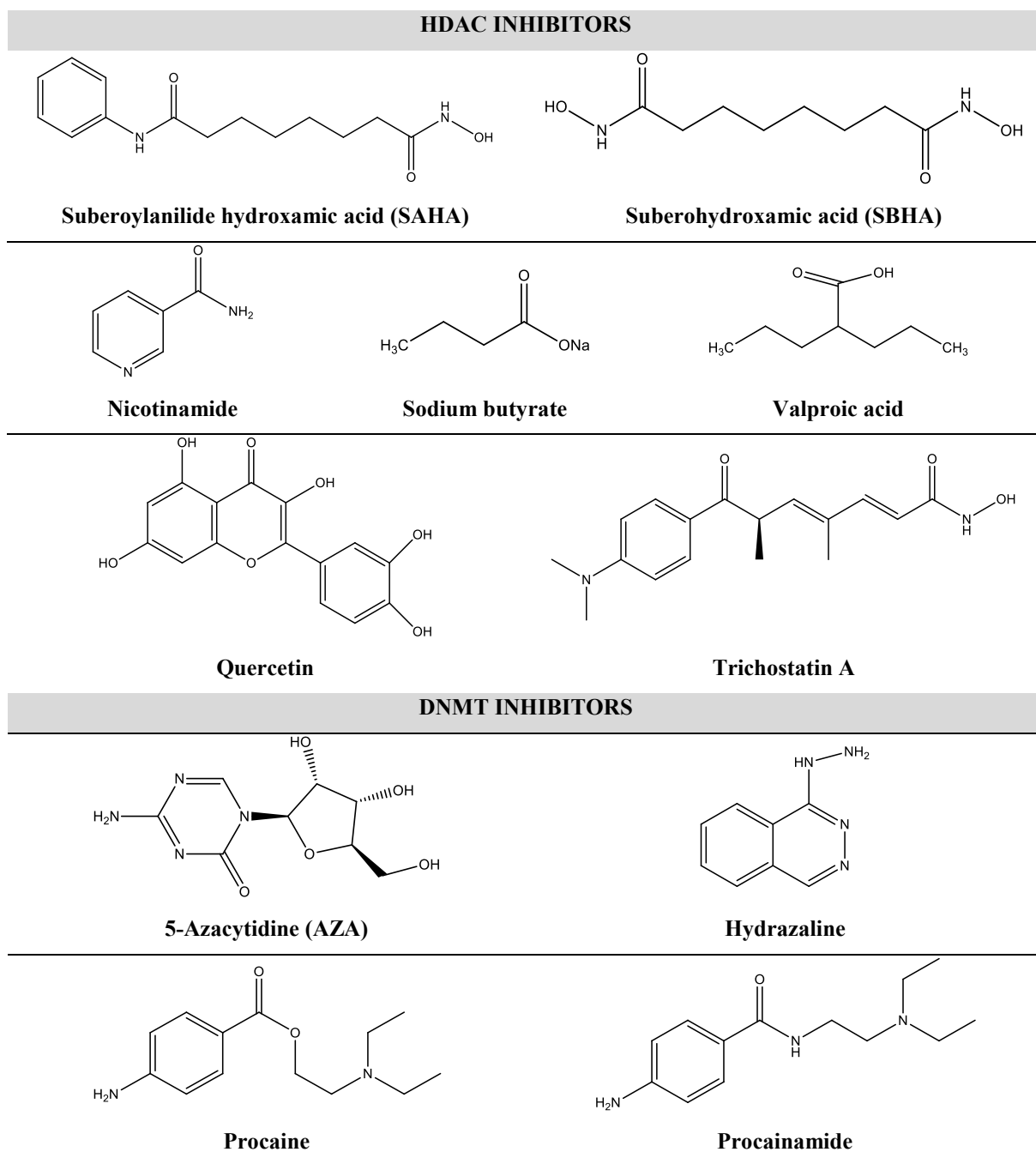


Figure 1.27. Structure of some HDAC and DNMT inhibitors used for metabolite induction

Solvents and heavy metals have also shown to disturb the secondary metabolism; however, the mechanisms are not well understood. For example, the addition of 6% of ethanol or 3% of dimethylsulfoxide (DMSO) to the culture might cause mistranslation and induction of the stress response (Pettit 2011). The addition of methanol, acetone, acetonitrile, 1-butanol and chloroform have also shown to change the profile of the metabolites produced by endophytic fungi (Toghueo *et al.* 2018). Similarly, fungi cultured in presence of enzyme inhibitors such as metyrapone (cytochrome P-450 inhibitor) or jasplakinolide (F-actin inhibitor) were able to synthesize new compounds (Pan *et al.* 2019).

Moreover, the addition of plant products in fungi culture have also been reported. For example, nitric oxide is a phytohormone that has shown to influence the secondary metabolite production in *Hypocrea koningii* (*Trichoderma koningii*) and *Penicillium miczynskii* (Zutz *et al.* 2014). In addition to this, the supplementation of the endophytic fungi culture medium with leaves, barks, roots and other plant parts or extracts can induce or maintain the production of secondary metabolites. Finally, metal ions such as Mn^{2+} , La^{3+} , Ce^{3+} and Nd^{3+} are involved in the stimulation of carotenoids synthesis while Cd^{2+} , Cu^{2+} and Cr^{3+} have been used for the induction of secondary metabolites in fungi (Toghueo *et al.* 2020).

1.5.3 Addition of biosynthetic precursors

Some studies have recently applied the amino-acid directed strategy for the induction of cryptic biosynthetic pathways in fungi. The addition of phenylalanine, methionine, tryptophan, lysine, and threonine to the culture of fungus *Pseudallescheria boydii* induced the production of 25 compounds corresponding to diverse chemical structures comprising alkaloids, diketopiperazines, sesquiterpenoids and polyketides (Lan *et al.* 2016). In another study, novel compounds corresponding to amides, polyketides and diketopiperazines were obtained from the marine-derived fungus *Dichotomomyces* when the culture was supplemented with L-tryptophan and L-phenylalanine (Chen *et al.* 2017). Continuing the research of this endophytic strain by the addition of the same amino acids, 7 additional compounds were discovered: 2 new polyketides, 2 new alkaloids and 3 known fumiquinazolines indicating the enhanced the production of nitrogen-containing compounds in this fungus under the addition of these amino acids (Wu *et al.* 2018). Likewise, other studies were able to isolate novel bioactive alkaloids from marine-derived fungi using this same strategy (Huang *et al.* 2017; Yuan *et al.* 2019; Qiu *et al.* 2020; Guo *et al.* 2020).

1.5.4 Co-cultivation

Endophytic fungi coexist with other microorganisms in their natural environment and co-culturing fungus-fungus, fungus-bacteria or bacteria-bacteria under laboratory conditions has shown to naturally enhance the production of cryptic compounds that were not detected in axenic culture. This increased production might be the result of competition for nutrients or antagonism in their development between microorganisms sharing the same niche (Deshmukh *et al.* 2018). The endophytic fungus *Paraconiothyrium variabile* was able to induce its metabolite production and to negatively modulate the production of the mycotoxin beauvericin of the phytopathogen *Fusarium oxysporum* when they were co-cultured in PDA medium (Combès *et al.* 2012). Also, endophytic fungus *Fusarium tricinctum* was co-cultured with *Bacillus subtilis*, leading to the accumulation of secondary metabolites and to the production of 3 new natural products including macrocarpon C, (-)-citreoisocoumarin and 2-

(carboxymethylamino) benzoic acid (Ola *et al.* 2013). Co-cultivation is an emerging tool and several studies have been applying this approach in terrestrial and marine-derived fungi and bacteria (Marmann *et al.* 2014) including many endophytic fungi (Chagas & Pupo 2018; Pan *et al.* 2019).

1.6 Applying Metabolomics to natural products research

1.6.1 Study of the Metabolome with Mass Spectrometry

The classical approach for the discovery of natural products with biological activity starts with the biological screening of crude extracts and the posterior fractionation, isolation and identification of the bioactive compounds. To complement this approach and to avoid the bottleneck of re-isolation of known compounds, metabolomics is a powerful tool for the analysis, identification and quantification of small metabolites, usually molecular weights less than 1000 Da, with biological activities. This approach studies a biological system at a specific time for measuring their changes that captures the final alteration of endogenous metabolites which at the same time facilitates the search for novel bioactive compounds (Zhao *et al.* 2018). The most common analytical techniques used for this purpose are Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR). The first technique is the most widely used and possess some advantages over NMR, including high sensibility, small samples volume, acquisition of MS/MS fragmentation data for structural elucidation followed by a comparison with databases. Additionally, the association with computational and statistical tools can offer a better view that simplifies the vast data analyses (Demarque *et al.* 2020).

Depending on the research discipline, the specific objectives and the equipment used, the general analytical strategies can be divided in four: Targeted metabolomics and Metabolic profiling, in order to perform validations (quantification of one or a few metabolites) and Metabolic Fingerprinting (identification of metabolite patterns) and Untargeted Metabolomics, in order to discover fingerprints or patterns in a biological process to identify metabolites of interest. Specifically, untargeted metabolomics focuses on the detection of as many metabolites as possible to elaborate a comprehensive data set due to the little or no information available about the metabolome under study. It is important to mention that any strategy covers only a part of the real metabolome (Uthe *et al.* 2021).

1.6.2 Data processing software

For data processing, there are many bioinformatics tools that enable the obtention of a data matrix with the intensities or abundances of peaks from raw data of each sample. For this purpose, bioinformatical operations such as peak detection, retention time shift correction and peak alignment are the typical steps performed by bioinformatics tools, including MZmine (Pluskal *et al.* 2010), MS-DIAL (Tsugawa *et al.* 2015), along with MS-FINDER (Tsugawa *et al.* 2016) and MS-CleanR package (Fraisier-Vannier *et al.* 2020), and XCMS (Domingo-Almenara & Siuzdak 2020) as the most popular open-source software.

1.6.3 Databases for annotation

Published chemical structures available in chemical databases indicate that the most abundant natural products are derived from plants (about 70%), followed by animal-derived, fungi-derived and bacteria-derived natural products (Ntie-Kang & Svozil 2020). Currently, there is no open database that gathers all natural products produced by microbes besides the fact that many of the predictions of fragmentation patterns from mass spectrometry depend on the availability of known compounds found in databases for comparison with the experimental data. Commercial databases, such as Antibase or The Dictionary of Natural Products, and open databases have some difficulties including limited information, not downloadable or overly specific compounds (**Figure 1.28**). Recently, a new automated curation platform followed by manually curation, was created to identify microbial-derived natural products found in scientific literature and patents. The Natural Products Atlas (<https://www.npatlas.org/>) currently contains more than 29, 000 natural product structures and is linked to the GNPS database (Van Santen *et al.* 2019).

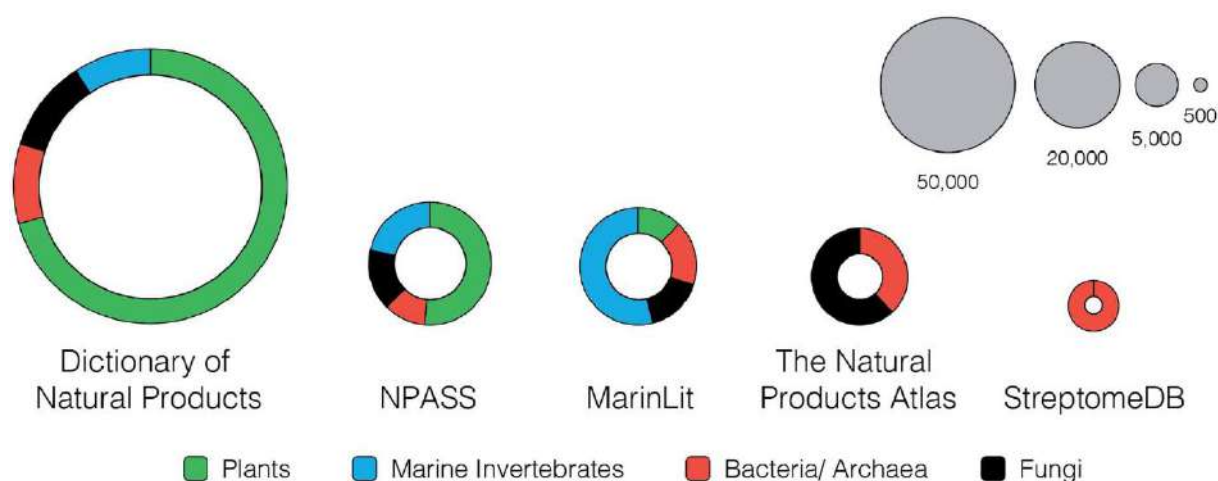


Figure 1.28. Distribution of compound source in some natural products databases (van Santen *et al.*, 2020)

1.6.4 Statistical analysis and data visualization for further interpretation

The data matrix obtained from the data processing software can be used for statistical analyses or uploaded to data repositories and libraries for the scientific community. Untargeted metabolomics usually provides thousands of features that require multivariate analysis such as unsupervised and supervised methods for pattern recognition among different groups. Online platforms, such as Workflow4Metabolomics and Galaxy workflow (<https://workflow4metabolomics.usegalaxy.fr/>) or

Metaboanalyst (<https://www.metaboanalyst.ca/>) can cover data processing entirely from the feature table to perform statistical analysis.

In addition to this, molecular networking is a tool that facilitates the mapping of chemical diversity observed in an untargeted mass spectrometry experiment, being the GNPS platform (<https://gnps.ucsd.edu/>) and MetGem software (Olivon *et al.* 2018) the main methodologies used for this purpose. Molecular networking compares and aligns ion fragmentation patterns and structurally related molecules are assigned with a cosine score. Molecules are represented by a node and can be related to others forming a cluster according to the similarity of the fragment ions. Cytoscape® (Shannon 2003) is another software that helps to better visualize the clustered data in the molecular network produced either by GNPS or MetGem. In the case of GNPS, public information provided by the scientific community is searched against spectral libraries allowing the annotation of high confidence spectral matches (Aron *et al.* 2020). To complement the information given by this online platform, it exists many other annotation and classification tools that either use fragmentation libraries or *in silico* tools that predict fragmentation (da Silva *et al.* 2018).

CHAPTER 2 MATERIALS AND METHODS

The general steps of the methodology, from fungal isolation to metabolomics analysis, performed during this doctoral work is summarized in the following schema (**Figure 2.1**).

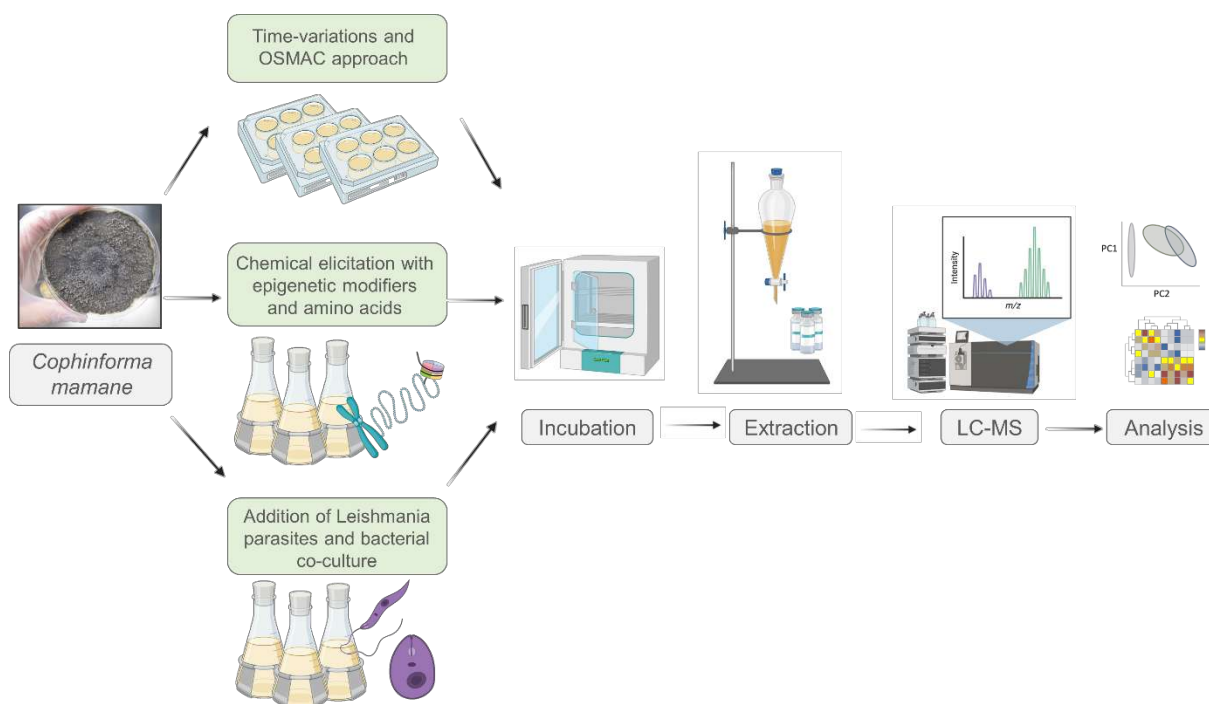


Figure 2.1. General methodology steps: the different approaches for secondary metabolite induction in *C. mamane* (own creation with biorender.com)

2.1 Materials and reagents

2.1.1 Culture media

The culture media used for the different experiments are the following:

- Trypticase Soy Agar (TSA, BD – BBL) containing 15 g of pancreatic digest of casein, 5 g of papaic digest of soybean meal and 5 g of sodium chloride for 1L of distilled water.
- Malt extract broth or agar (MEB / MEA) containing malt extract 20g, dextrose 20g, peptone 1g, copper sulfate pentahydrate 0.005g, zinc sulfate heptahydrate 0.01g in 1 L of distilled water. MEA contains 20 g/L of agar-agar.
- LB broth (BD – Difco™) containing 10 g of tryptone, 10 g of yeast extract and 10 g of sodium chloride for 1L of distilled water.

- Czapek solution agar (Difco™) containing saccharose 30 g, sodium nitrate 2 g, dipotassium phosphate 1 g, magnesium sulfate 0.5 g, potassium chloride 0.5 g, and ferrous sulfate 0.01 g for 1 L of distilled water.
- Yeast malt extract agar or ISP2 containing malt extract 10 g, dextrose 4 g, and yeast extract 4 g for 1L of distilled water (Shirling & Gottlieb 1966).
- Potato dextrose broth or agar (PDB / PDA) (Difco™) containing 4g of potato extract and 20g of glucose for 1L of distilled water. PDA contains 15 g/L of agar-agar.
- RPMI 1640 containing L-glutamine and phenol red.

2.1.2 Reagents and solvents

The different reagents and chemical solvents used for the experiments are the following:

- Dimethyl sulfoxide (DMSO, Sigma Aldrich)
- Glycerol 99% extra pure (ACROS Organics™)
- Nicotinamide (NIC, Sigma)
- Sodium butyrate (SB, Sigma)
- Butyryl-DL-homoserine (HSL, Sigma)
- 5-azacytidine (AZA, Sigma)
- L-Phenylalanine (L-Phe, Sigma)
- L-Tryptophan (L-Trp, Sigma)
- Formic acid (FA, Sigma)
- Methanol HPLC grade (Fisher chemical)
- Ethyl acetate HPLC grade (Fisher chemical)
- Acetonitrile HPLC grade (Fisher chemical)
- Dichloromethane HPLC grade (Fisher chemical)

2.1.3 Fungal material

The strain E224 of *Cophinforma mamane* (D.E. Gardner) A. J. L. Phillips & A. Alves was isolated from the fresh leaves of *Bixa orellana* L. collected in November 2013 by Carlos Amasifuen and Mohamed Haddad in the National Reserve of Allpahuayo Mishana in the Amazonian forest of Iquitos, Peru (GPS coordinates: 3°58'2.3'' S, 73°25'3.9'' W). The isolation, identification and storage processes performed for this strain have been previously described in the literature (Triastuti *et al.* 2019; Barakat *et al.* 2019).

Briefly, the collected tissues were sterilized with 5% sodium hypochlorite, 70% ethanol (2 times) and rinsed with distilled water. Sterilized surfaces were cut into small fragments (5x5 mm) and placed on a Petri dish containing MEA and chloramphenicol (100 mg/L). The pure isolates were identified using internal transcriber spacer (ITS1 and ITS2) sequences which are available in the GenBank database under the accession number MG457709.1. The cryotubes are conserved at -80 °C in 30% of glycerol in the fungal collection of the UMR 152 laboratory.

2.1.4 Bacterial material

For the co-culture experiments three different strains from the American Type Culture Collection (ATCC) were used: *Escherichia coli* ATCC® 25922™; *Pseudomonas aeruginosa* ATCC® 27853™ and *Staphylococcus aureus* ATCC® 25923™.

2.1.5 Leishmanial parasites

For the culture of *C. mamane* and for the biological tests of its crude extracts, *Leishmania infantum* parasites expressing luciferase activity (MHOM/MA/67/ITMAP-263, CNR Leishmania, Montpellier, France) were used.

2.2 Culture conditions and extraction procedures

2.2.1 Time-variations study and OSMAC approach

Culture media and conditions

For the time-variations study, *C. mamane* was incubated in 6-well plates containing 4 mL of MEA during 28 days at 27°C under natural light. At the end of each culture day, a culture plate and a control plate were placed at -80°C until extraction with solvents.

Concerning the OSMAC approach, *C. mamane* was incubated in 6-well plates containing CZA and ISP2 during 6 and 10 days at 27 °C under presence or absence of natural light in a 24 h dark regime. At the end of each incubation time, the culture plates and control plates were placed at -80 °C until extraction with solvents.

Extraction and preparation of crude extracts

For time-variations experiment, each culture (biomass and medium) was freeze-dried. The dry material was transferred into a glass tube with 10 mL of solvent. The extraction was performed using a freshly prepared dichloromethane-methanol-water mixture 64:36:8 (v/v/v) (Bertrand *et al.* 2014).

For OSMAC-based experiment, each culture was directly transferred to a glass tube containing 10 mL of ethyl acetate. The medium and mycelia were crushed in the solvent and taken under ultrasound treatment over 60 minutes.

In both experiments, the supernatants were filtered through glass cotton then dehydrated on magnesium sulfate and filtered using filter paper. Finally, all the extracts were evaporated to dryness under reduced pressure (KNF rotary evaporator RC 600) at 40°C.

2.2.2 Chemical elicitation with epigenetic modifiers and addition of amino acids

Culture media and conditions

The endophyte *C. mamane* was cultured in PDB and PDA. First, the fungus was grown on PDA during 7 days to inoculate the seed culture in 50 mL of PDB during 3 days at 27°C. Two mL of seed culture were used to inoculate 250mL Erlenmeyer flasks containing 50 mL of PDB. The fungus culture was treated with 4 different epigenetic modulators, NIC, SB, AZA and HSL, dissolved in DMSO and added to the 50 mL of PDB. NIC and SB were added at 1 µM, AZA was added at 25 µM and HSL was added at 100 µM as final concentrations. Fermentation was carried out during 2 weeks at 27°C under static conditions. All conditions, including negative controls, were done in triplicate.

For the addition of amino acids, *C. mamane* was cultured in MEB and supplemented MEB with 2 mg/L of L-Trp and L-Phe. Fermentation was carried out in 250mL Erlenmeyer flasks during 2 weeks at 27°C under constant agitation containing 50 mL of MEB and 50 mL of supplemented MEB. All conditions and negative controls were done in triplicate.

Extraction and preparation of crude extracts

After 2 weeks of incubation, the whole culture broths (including mycelium) were extracted with 50mL of ethyl acetate under 60 minutes of ultrasound treatment. Mycelia were removed from the culture supernatant through filtration with glass cotton. Organic phases were then dehydrated on magnesium

sulfate and filtered with filter paper. Finally, they were evaporated to dryness under reduced pressure (KNF rotary evaporator RC 600) at 40°C.

2.2.3 Addition of heat-killed leishmanial parasites

Culture media and conditions

Heat-killed *Leishmania* parasites were added into the culture of *C. mamane*. For this, fresh cultures of promastigotes and amastigotes of *Leishmania infantum* were first centrifuged at 2100 RPM during 10 minutes to remove the supernatant. Each pellet was resuspended in distilled water and autoclaved at 121°C during 15 min for parasite inactivation.

Following this procedure, *C. mamane* was cultured in MEB in the presence of three different concentrations of heat-killed amastigotes: C1=5.2 x 10⁵, C2=1.06 x 10⁶ and C3=2.12 x 10⁶ parasites / mL. Fermentation was carried out in 250mL Erlenmeyer flasks containing 50 mL of MEB during 2 weeks at 27 °C under constant agitation. All conditions, including negative controls, were done in triplicate.

Extraction and preparation of crude extracts

After 2 weeks of incubation under constant agitation, the whole culture broths (including mycelium) were extracted with 50mL of ethyl acetate under 60 minutes of ultrasound treatment. Mycelia were removed from the culture supernatant through filtration with glass cotton. Supernatant was filtered using regenerated cellulose acetate membranes (0.45 µm) to remove the cellular debris. Organic phases were then dehydrated on magnesium sulfate and filtered with filter paper. Finally, they were evaporated to dryness under reduced pressure (KNF rotary evaporator RC 600) at 40°C.

2.2.4 Co-culture of *C. mamane* with pathogenic bacteria

Culture media and conditions

Escherichia coli ATCC® 25922™; *Pseudomonas aeruginosa* ATCC® 27853™ and *Staphylococcus aureus* ATCC® 29923™ were first seeded in TSA and incubated at 30°C during 24 h. One colony of each strain was later cultivated in LB broth at 30°C during 48 h at 150 RPM.

C. mamane was grown on PDA during 7 days to inoculate the seed culture in 50 mL of PDB during 3 days at 27°C. 1 mL of seed culture were used to inoculate 250mL Erlenmeyer flasks containing 50 mL of PDB.

For the co-culture experiments, each flask containing 50 mL of PDB was inoculated with 1 mL of seed culture from *C. mamane* and 200 µL of the 48 h LB broth from each bacterial strain and then incubated at 27°C during 10 days.

Extraction and preparation of crude extracts

After incubation time, whole culture broths were extracted with 50mL of ethyl acetate under 60 minutes of ultrasound treatment. Mycelia were removed from the culture supernatant through filtration with glass cotton. Organic phases were then dehydrated on magnesium sulfate and filtered with filter paper. Finally, they were evaporated to dryness under reduced pressure.

The different culture conditions used in this doctoral thesis have been summarized in **Table 2.1**.

Table 2.1. Summary of culture conditions for the induction of *C. mamane* metabolites

| Study | Culture medium | Time of incubation | Other conditions |
|-------------------------------|-----------------------|---------------------------|---|
| Time variations | MEA | 28 days | 27°C |
| OSMAC | ISP2, CZA | 6 and 10 days | 27°C, 24h of natural light and 24 h of darkness |
| Epigenetic modifiers | PDB | 14 days | 27°C, no agitation |
| Amino acids | MEB | 14 days | 27°C, 120 RPM |
| Heat-killed <i>Leishmania</i> | MEB | 14 days | 27°C, 120 RPM |
| Co-culture | PDB | 10 days | 27°C, no agitation |

2.3 Evaluation of the biological activity

Anti-parasitic activity assay was performed for the crude extracts obtained from the culture of *C. mamane* in presence of the thermally inactivated amastigotes of *L. infantum*. For this, *L. infantum* promastigotes were cultivated in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) and harvested in logarithmic phase of growth by centrifugation at 900 g for 10 min. The supernatant was removed

carefully and was replaced by the same volume of RPMI 1640 complete medium at pH 5.4 and incubated for 24 h at 24°C. The acidified promastigotes were then incubated for 24 h at 37°C in a ventilated flask to transform acidified promastigotes into axenic amastigotes (Sereno & Lemesre 1997). The effects of the extracts on the growth of *L. infantum* axenic amastigotes were assessed as follows: *L. infantum* amastigotes were incubated (2×10^6 parasites/mL) in sterile 96-well plates with each samples dissolved in MeOH (final concentration 0.5% v/v), in duplicate. Appropriate controls, DMSO or MeOH and amphotericin B, were added to each set of experiments.

After a 48 h incubation period at 37°C, each plate-well was then microscopically-examined to detect any precipitate formation. To estimate the luciferase activity of axenic amastigotes, 80 µL of each well were transferred to white 96-well plates, Steady Glow® reagent (Promega) was added according to manufacturer's instructions, and plates were incubated for 2 min. The luminescence was measured in Microbeta Luminescence Counter (PerkinElmer). Efficient concentration 50% (EC₅₀) was defined as the concentration of drug required to inhibit by 50% the metabolic activity of *L. infantum* amastigotes compared to control. EC₅₀ values were calculated by non-linear regression analysis processed on dose response curves, using TableCurve 2D (version 5.0) software.

2.4 UHPLC-Mass spectrometry analysis

Methanolic solutions at 2 mg/mL of all extracts were prepared for Ultra-High Performance Liquid Chromatography - MS analysis. Quality control (QC) samples were prepared by pooling an aliquot of all extracts for each experiment. Analyses were performed on a UHPLC Ultimate 3000 system (Dionex) coupled with an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK). Samples were separated on a C18 Acquity column (100 × 2.1 mm i.d, 1.7 µm, Waters, MA, USA) equipped with a guard column. The mobile phase A was ultrapure water acidified with 0.1% FA and mobile phase B was acetonitrile acidified with 0.1% FA. The solvent gradient was: 0 min, 95% A; 10 min, 95% B; 12.5 min, 95% B. The flow rate was 0.45 mL/min, the column temperature was set to 40°C, the autosampler temperature was set to 15°C and injection volume fixed to 5 µL for extracts. Mass detection was performed using an electrospray source (ESI) in positive ionization (PI) mode at 15,000 resolving power [full width at half maximum (FWHM)] at 400 *m/z*. The mass scanning range was *m/z* 100-2000 for all samples. Ionization spray voltage was set to 3.5 kV and the capillary temperature was set to 300°C. Each full MS scan was followed by data-dependent acquisition of MS/MS spectra for the three most intense ions using stepped collision-induced dissociation (CID) at 35 arbitrary energy units.

2.5 Data treatment and statistical analysis

2.5.1 Data processing

LC-MS data from each experiment were separately processed with MS-DIAL version 4.7 (Tsugawa *et al.* 2015) for mass signal extraction between 100 and 1500 Da from 0.5 to 12.5 min. MS1 and MS2 tolerances were set to 0.01 and 0.025 Da, respectively, in centroid mode for each dataset. Peaks were aligned on a QC reference file with a retention time (RT) tolerance of 0.05 min and a mass tolerance of 0.025 Da. Minimum peak height was set to 70% below the observed total ion chromatogram (TIC) baseline for blank samples. MS-DIAL data was cleaned with MS-CleanR workflow using default parameters (Fraisier-Vannier *et al.* 2020): all filters with a minimum blank ratio set to 0.8, a maximum relative standard deviation (RSD) set to 30 and a relative mass defect (RMD) ranging from 50 - 3000. The maximum mass difference for feature relationships detection was set to 0.005 Da and maximum RT difference was set to 0.025 min. The Pearson correlation links were considered with correlation ≥ 0.8 and statistically significant $\alpha = 0.05$. Two peaks were kept in each cluster: the most intense and the most connected to other ions. The kept features were annotated with MS-FINDER version 3.5 (Tsugawa *et al.* 2016). The MS1 and MS2 tolerances were set to 0.005 and 0.05 Da, respectively. Formula finder were processed with C, H, O, N, P and S atoms. Annotation was done with the use of internal generic databases from MS-FINDER: LipidMaps, YMDB, ChEBI, NPA, NANPDB, COCONUT, KNAPSACK and UNPD. Only annotations with a score greater than 5 were retained throughout the analysis while unannotated features are referred to as “unknown”. Data were finally exported as .msp file for MSMS spectral information and .csv files for metadata information (peak area, retention times, annotation results, etc.).

The MSP and metadata files generated after the MS-CleanR workflow were imported into MetGem (version 1.3.4) (Olivon *et al.* 2018). The data was filtered by removing all MS/MS peaks within ± 17 Da of the precursor m/z . MS/MS spectra were window filtered by using the top 6 peaks in the ± 50 Da window throughout the spectrum. MS/MS fragment ion tolerance was set to 0.2 Da. A mass spectral similarity network was created where edges were filtered to have a cosine score above 0.7 and at least six matched peaks. Further edges between two nodes were kept in the similarity network only if each of the nodes appeared on each other's top 10 most similar nodes. The resulting network was then imported into Cytoscape (version 3.8.2, Institute for Systems Biology, Seattle, WA, USA) to improve visualization (Shannon 2003). Nodes were colored according to their culture conditions and the node sizes indicate their normalized total peak area.

2.5.2 Statistical analysis

Statistical analysis were done by uploading the CSV file to the Metaboanalyst platform version 5.0 (<https://www.metaboanalyst.ca/>) (Chong *et al.* 2019). The data were pre-processed, first by normalization (by median) of the samples, then the variables were weighted by auto scale (mean-centered and divided by the square root of the standard deviation of each variable). A Principal Component Analysis (PCA) then a Partial Least Squares Discriminant Analysis (PLS-DA) were carried out. Finally, the m/z and the retention time of the top features associated to their normalized peak area were plotted on a heat map (hierarchical clustering) using ANOVA.

CHAPTER 3 TIME-VARIATIONS STUDY AND OSMAC APPROACH

3.1 Introduction

The OSMAC (One Strain - Many Compounds) approach is based on the potential of microorganisms to produce different compounds under specific conditions (Bode et al., 2002; Romano et al., 2018), focusing on the modification of the growth parameters for the induction of metabolites. This methodology has allowed the expansion of the chemical diversity of endophytic fungi, which are able to produce new metabolites with biological activity (Ariantari et al., 2019; Fan et al., 2019; Tran-Cong et al., 2019).

Among these growth parameters, the nutrient content, the time of incubation, the light conditions and other physicochemical parameters can be easily modified. As previously described as part of the strategies for metabolite induction (**Section 1.5.1**), a large proportion of the fungal genome can be regulated by light (Schumacher, 2017) including its different wavelengths (Yu and Fischer, 2019). The time for incubation is intimately related to the growth and development of each fungal strain, which is important to consider for the discovery of new metabolites and for monitoring specific compounds behavior (Peters et al., 2010). Taking into consideration the time-frame production for metabolite production has allowed the discovery of the potential novelty hidden in fungi for natural product discovery (Bertrand et al., 2014; Choi et al., 2010).

Under these concepts, this work has two main objectives: to study the metabolome changes of the endophytic fungus *C. mamane* during 28 days of culture and to evaluate its response when using two different culture media and light *versus* dark conditions at 2 different incubation times under the OSMAC approach. A special focus was given to the previously isolated thiodiketopiperazines (TDKPs), botrysosulfuranols A, B, C (Barakat et al., 2019) and D.

3.2 Results

3.2.1 Time-variations of the metabolome of *C. mamane*

General observations

During the first 24 and 48 hours of culture of *C. mamane* in 6-well plates containing MEA, the colonies started to grow as a white mycelium, as observed in **Figure 3.1**.

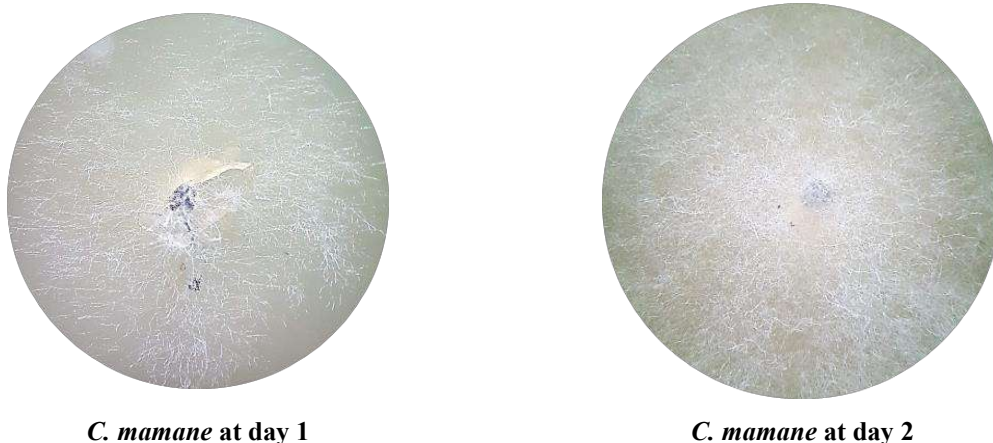


Figure 3.1. Closer view of the mycelium growth of *C. mamane* during the first 2 days of growing in 6-well plates containing MEA (upper view with stereoscope)

After 28 days of incubation in MEA, most of the colonies presented white mycelia with black spots, mainly observed from the bottom view (**Figure 3.2**), indicating a potential production of pigments.

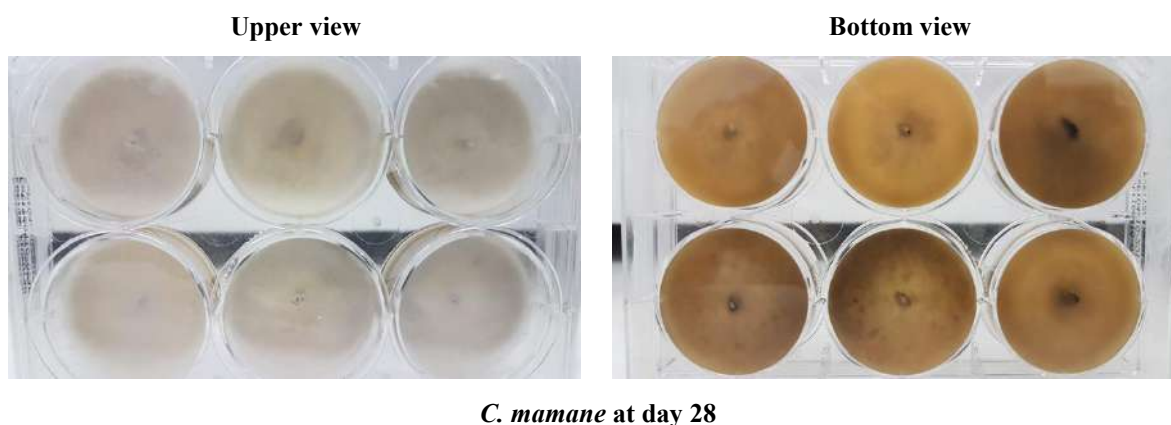


Figure 3.2. Growth of *C. mamane* after 28 days of incubation showing surface (upper view) and reverse appearance (bottom view) of the white mycelium with some black spots in MEA

Preliminary results of the general chemical profiling using quality controls (QC) samples (pool sample of replicates), allowed us to select some time-points (4, 8, 12, 16, 20, 24 and 28 days) for the metabolomics analysis. An overview of the chromatograms corresponding to the day 4 (D4), 8 (D8), 12 (D12), 16 (D16), 20 (D20), 24 (D24) and 28 (D28) days of culture indicate the general variations in the metabolome with major and minor peaks appearing at different culture times. For example, feature m/z 230.1383 at 4.58 min corresponds to a minor peak that appeared between D24 and D28; m/z 292.1536 at 5.12 min corresponds to a major peak that was detected during the whole culture while m/z 442.2441 at 6.85 min corresponds to a minor peak that appeared between D20 and D28 while the feature m/z 230.138 at 4.83 that appeared between D20 and D28 (**Figure 3.3**).

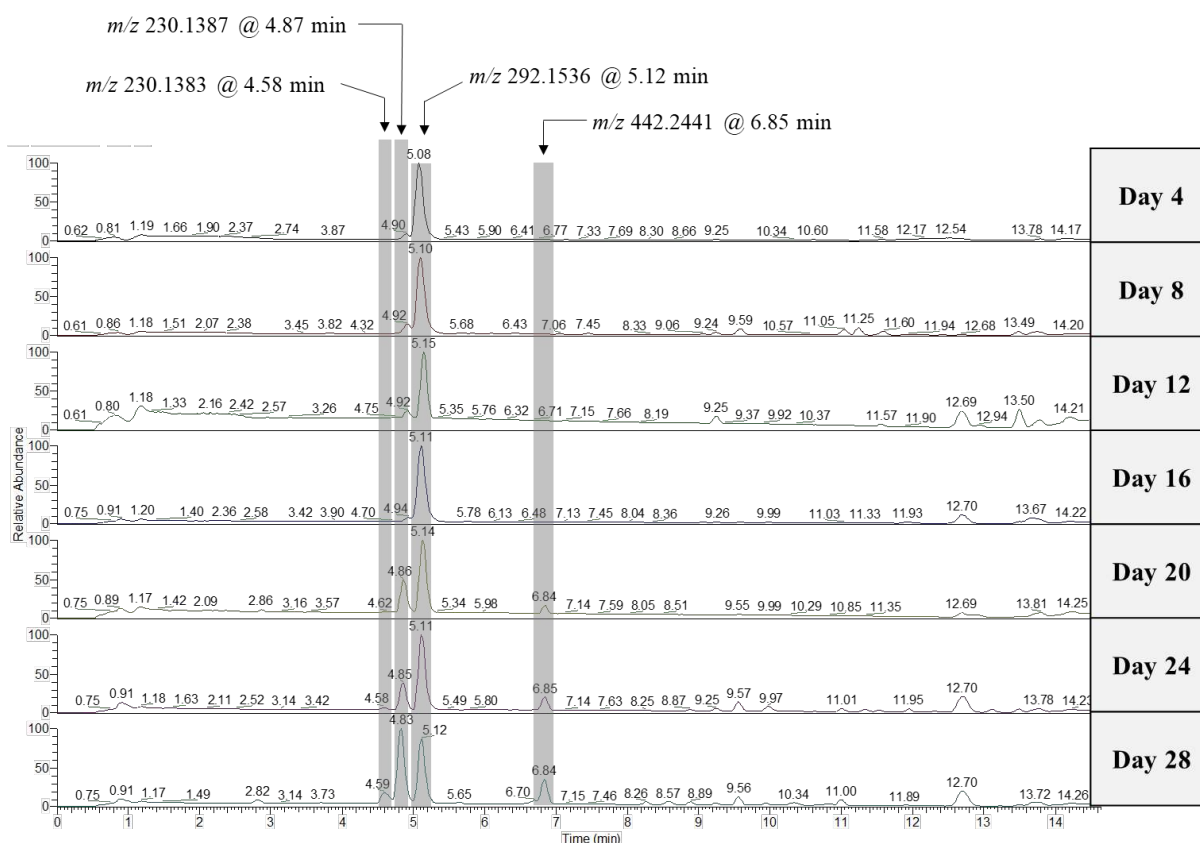


Figure 3.3. (+)ESI-HRMS base peak chromatograms from crude extracts of *C. mamane* cultivated at day 4, 8, 12, 16, 20, 24 and 28 in MEA

LC-MS profiles of all the 28 crude extracts from the time-series study afforded 303 features (m/z -RT pairs) in positive ionization (PI) mode, which were considered for the metabolomics and statistical analysis. Fifteen percent of these features (45 out of 303) were putatively annotated, corresponding to different chemical classes such as diketopiperazines (DKPs), cyclic peptides, fatty acids, phosphoethanolamines, phosphocholines (**Annexes 1**).

Multivariate classification (PLS-DA)

A supervised PLS-DA analysis was carried out on these data which indicated the evolution of metabolites of *C. mamane* through 28 days of culture (**Figure 3.4**). Separate clusters appear on the PLS-DA score plot (26 % of total variability). The component 1 (X-axis) explained 16.9 % of the variability, allowing the discrimination of the different clusters, mainly between D4-D8, D12-D16 and D20-D28. While in the component 2 (9.1%), we can observe a slight differentiation following a semicircle trajectory which represents the apparition and disappearance of the compounds found in crude extracts from D4 to D28.

The **Figure 3.4** presents the top 15 features that contributed to the differences between these clusters while some of them were putatively identified. Among them, we can find tiglylcarnitine, a type of acylcarnitine known to be involved in acyl-groups transport for mitochondrial normal functions and in lipid metabolism in mycorrhizal fungi (Laparre et al., 2014) which was detected during D28. A 1,3-dicarbonyl compound, previously isolated from a marine-derived strain of *Penicillium citrinum* (Pimenta et al., 2010) was also detected mainly during D28. The sugar alcohol derived from galactose, galacticol, found in different microorganisms including yeast and fungi (Jagtap et al., 2019), was mainly detected during D4 as well as a putatively identified cyclopentapeptide, previously isolated from an endophytic fungal strain of *Fusarium decemcellulare* (Li et al., 2016). These metabolites are detailed in

Table 3.1.

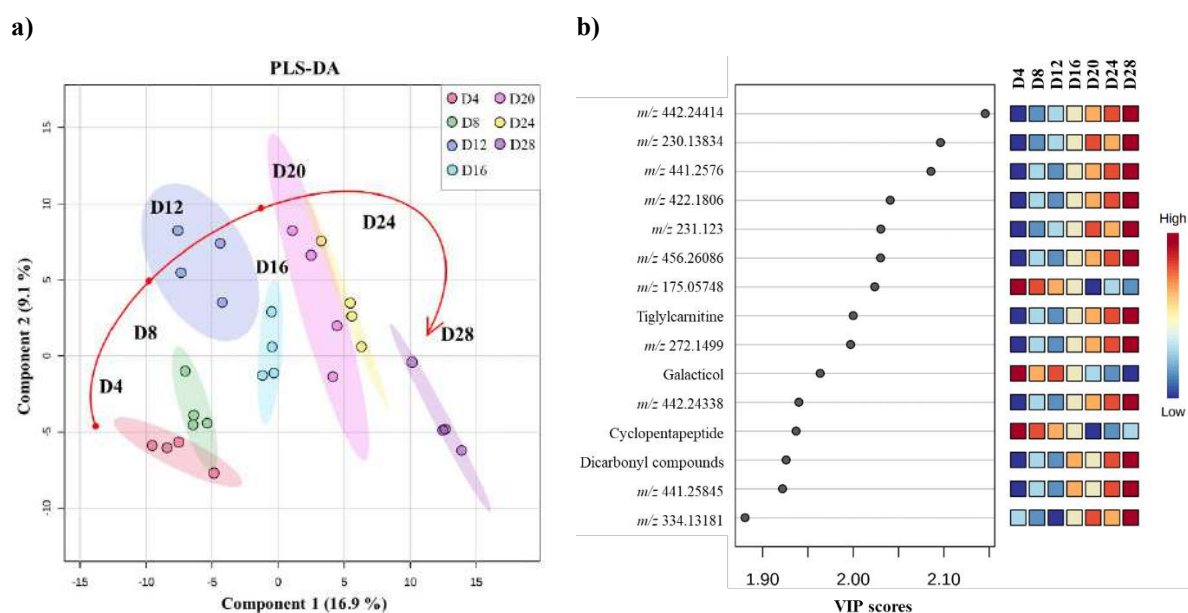


Figure 3.4. (a) Partial least squares discriminant analysis (PLS-DA) obtained from LC-(+)ESI-HRMS profiles corresponding to the extracts of *C. mamane* cultivated in MEA during 4, 8, 12, 16, 20, 24 and 28 days (4 replicates per day) based on features (m/z at Rt to its normalized peak area) detected in LC-MS chromatograms; (b) Variable importance in projection (VIP) score of the top 15 compounds from the PLS-DA analysis corresponding to each group (D4, D8, D12, D16, D20, D24, D28)

Table 3. 1. Top 15 features that significantly contribute to the difference in the PLS-DA analysis from LC-HRMS/MS data corresponding to the time-series study of *C. mamane*

| ID | Rt (min) | m/z | Adduct | Formula | Δ mass (ppm) | Putative identification | Ontology | VIP scores |
|-----|----------|----------|-----------------------------------|---|---------------------|---|--------------------------|------------|
| 109 | 6.84 | 442.2441 | [M+H] ⁺ | C ₂₃ H ₃₁ N ₅ O ₄ | 1.67 | Unknown | Unknown | 2.1457 |
| 240 | 4.58 | 230.1383 | [M+H] ⁺ | C ₁₁ H ₁₉ NO ₄ | 1.49 | Unknown | Unknown | 2.0965 |
| 105 | 8.26 | 441.258 | [M+NH ₄] ⁺ | C ₂₂ H ₃₃ N ₇ O ₇ | 4.37 | Unknown | Unknown | 2.0858 |
| 96 | 5.86 | 422.1806 | [M+H] ⁺ | C ₂₂ H ₂₃ N ₅ O ₄ | 3.98 | Unknown | Unknown | 2.0409 |
| 262 | 5.653 | 231.1230 | [M+H] ⁺ | C ₁₁ H ₁₈ O ₅ | 1.29 | Unknown | Unknown | 2.0305 |
| 116 | 7.32 | 456.2609 | [M+H] ⁺ | C ₂₄ H ₃₃ N ₅ O ₄ | 0.72 | Unknown | Unknown | 2.0302 |
| 160 | 0.88 | 175.0575 | [M+H] ⁺ | C ₃ H ₆ N ₆ O ₃ | 0.37 | Unknown | Unknown | 2.0238 |
| 252 | 5.42 | 244.1539 | [M+H] ⁺ | C ₁₂ H ₂₁ NO ₄ | 1.45 | Tiglylcarnitine | Fatty acyls | 2.0001 |
| 276 | 5.70 | 272.1499 | [M+H] ⁺ | C ₁₄ H ₁₇ N ₅ O | 2.52 | Unknown | Unknown | 1.9972 |
| 286 | 0.88 | 205.0683 | [M+Na] ⁺ | C ₆ H ₁₄ O ₆ | 0.39 | Galactitol | Sugar alcohols | 1.9636 |
| 107 | 6.70 | 442.2434 | [M+H] ⁺ | C ₂₂ H ₃₅ NO ₈ | 0.37 | Unknown | Unknown | 1.9401 |
| 174 | 9.37 | 583.4518 | [M+NH ₄] ⁺ | C ₃₀ H ₅₅ N ₅ O ₅ | 3.95 | Cyclo-(L-Ile-L-Leu-L-Leu-L-Leu-L-Leu)* | Cyclopeptides | 1.937 |
| 265 | 3.90 | 250.1800 | [M+H] ⁺ | C ₁₅ H ₂₃ NO ₂ | 0.45 | (E)-1-(2,3-dihydro-1H-pyrrrol-1-yl)-2-methyldec-8-ene-1,3-dione | 1,3-dicarbonyl compounds | 1.9261 |
| 106 | 8.05 | 441.2585 | [M+NH ₄] ⁺ | C ₂₂ H ₃₃ NO ₇ | 2.44 | Unknown | Unknown | 1.9221 |
| 39 | 5.26 | 334.1318 | [M+H] ⁺ | C ₁₄ H ₂₃ NO ₆ S | 0.22 | Unknown | Unknown | 1.8807 |

*unknown amino acids sequence

Hierarchical clustering analysis (Heat map)

Out of the 303 features, 156 showed a significant change over time (p-value < 0.05) using the analysis of variance (one-way ANOVA). The top 50 features with significant changes were plotted in the hierarchical clustering analysis (heat map) where three main clusters of metabolite behaviors are observed (**Figure 3.5**): a cluster of metabolites that appeared in the early days of culture, between D4 and D8; a cluster of metabolites that started to be produced during a middle stage, mainly between D8 and D12 and that were not observed after D16; and a cluster of metabolites that started to be produced at a late stage from D20 until D28. These results are consistent with PLS-DA analysis observed above.

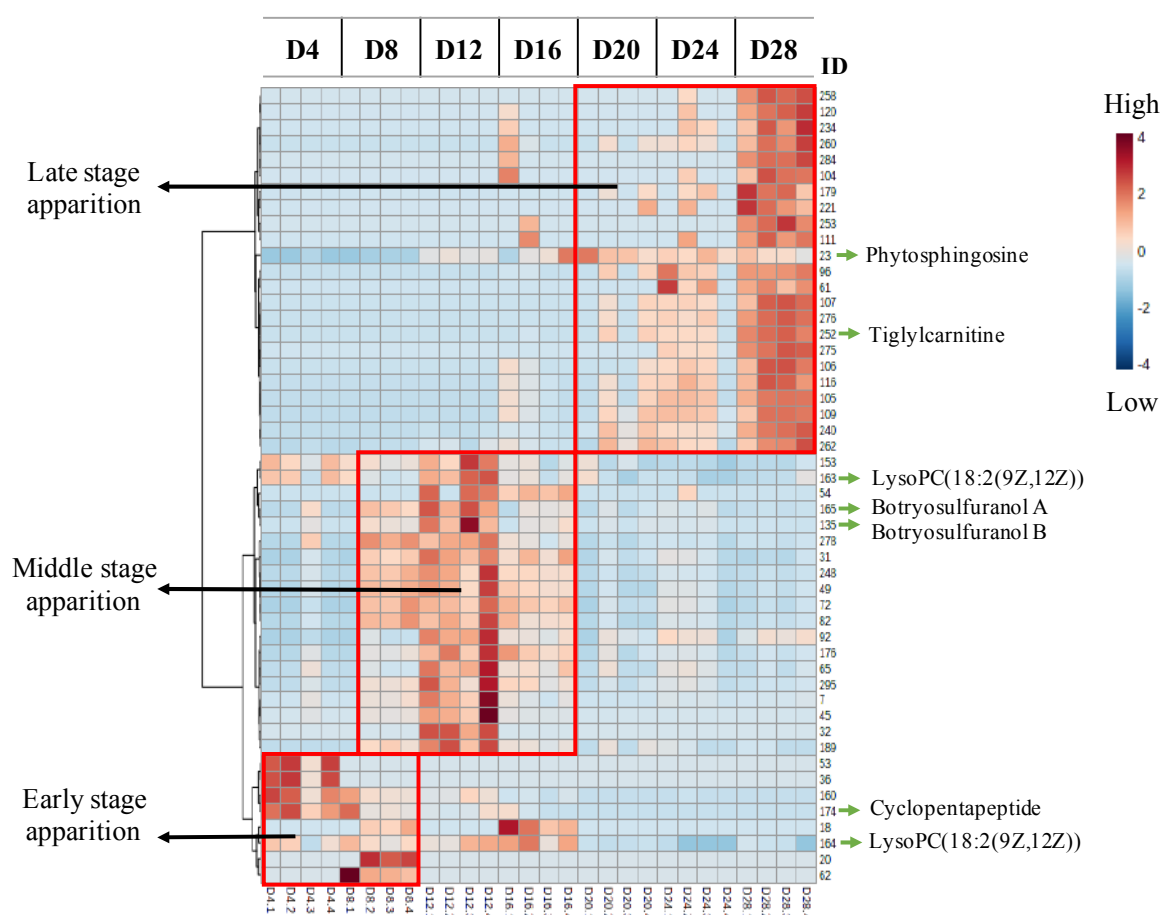


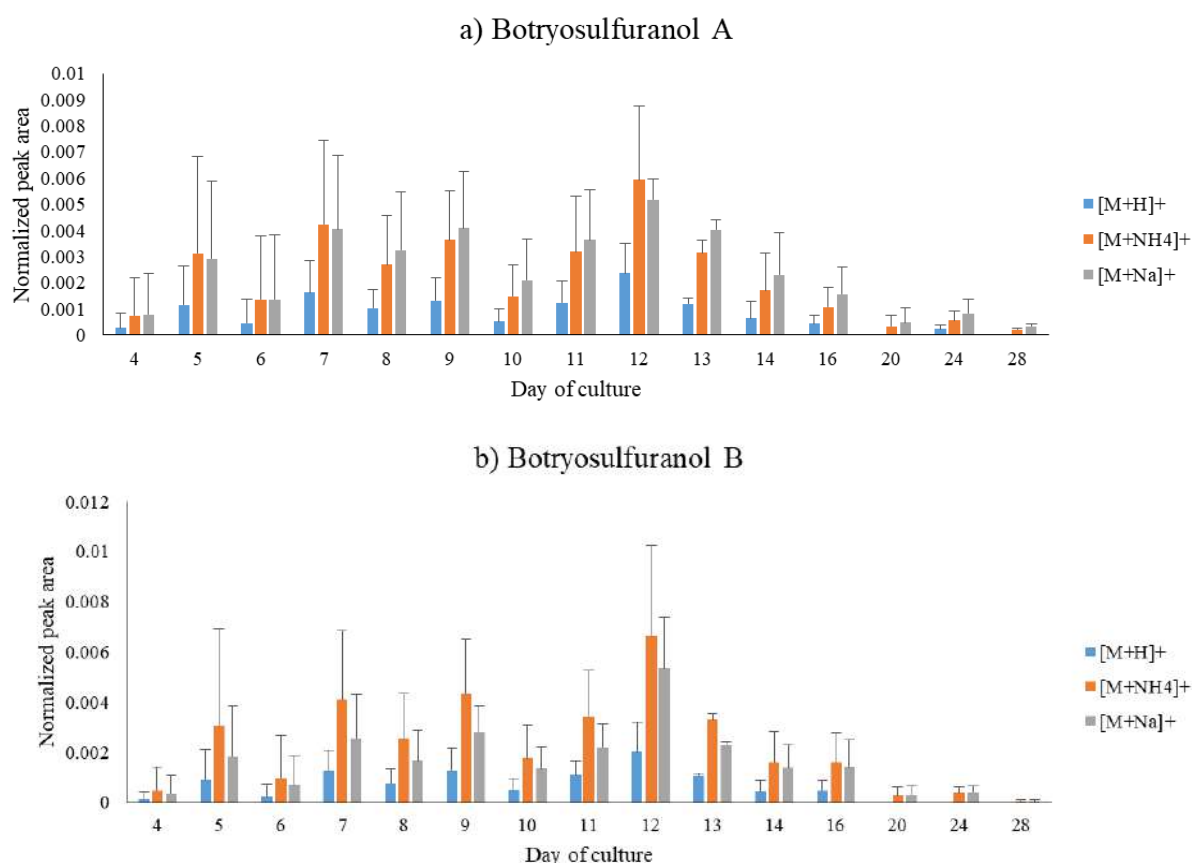
Figure 3.5. Hierarchical clustering of the top 50 features observed from day 4 (D4) to day 28 (D28) in *C. mamane*

Two metabolites putatively identified as glycerophosphocholines were produced during the early stage and middle stage while a cyclopentapeptide, mentioned above as part of top 15 features from the PLS-DA in **Figure 3.4**, was produced during the early stage. During the middle stage, we observed the production of the previously isolated TDKPs, botryosulfuranols A and B (Barakat et al., 2019) while a

tiglylcarnitine and a phytosphingosine, previously isolated from an unidentified fungus (Reindel et al., 1940), were produced during the late stage of *C. mamane* culture.

Thiodiketopiperazines evolution over time

Taking a closer look to the production of the TDKPs, botryosulfuranols A and B exhibited a significant change over time, being produced along the 28 days of culture with a maximum detection at D12 (**Figure 3.6**) while botryosulfuranol C presented a short time-frame production, being lowly detected between D6 and D9 (**Figure 3.6**). Different adducts were detected along the time-series study for the TDKPs, being $[M+NH_4]^+$ the major adduct, followed by $[M+Na]^+$ and $[M+H]^+$, except for botryosulfuranol C. Botryosulfuranol D was not detected in this study. The error bars also indicates us that these experiments need to be repeated in order to confirm our hypothesis, however this trend give us insights of their behavior over time.



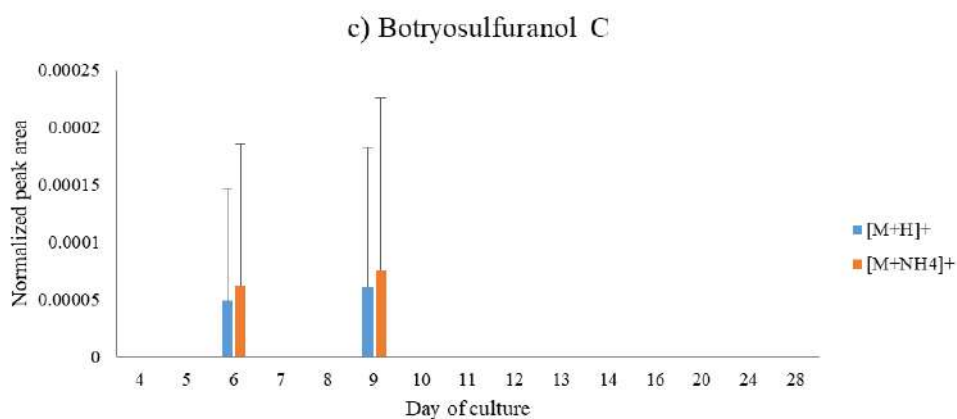


Figure 3.6. Normalized peak areas of the TDKPs (a) botryosulfuranols A, (b) B and (c) C under different incubation times observed for each detected adduct ($[M+H]^+$; $[M+NH_4]^+$ and $[M+Na]^+$)

Comparing and overlapping the production trends of the three TDKPs (**Figure 3.7**) considering the major adduct detected ($[M+NH_4]^+$), botryosulfuranols A and B present a similar pattern in contrast to botryosulfuranol C, which is almost not visible due to its lowly production and detection during the 28 days of culture. Moreover, their production tend to increase until D12 with a clear decrease from that day on.

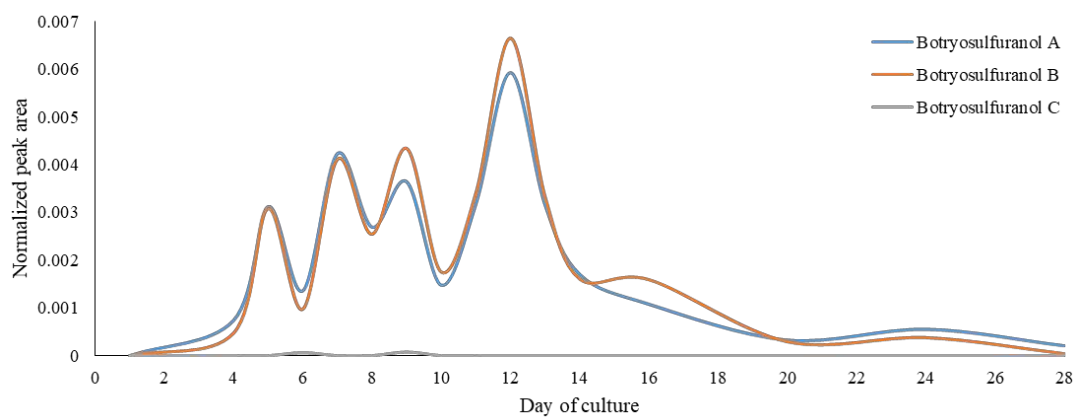


Figure 3.7. Production trend of TDKPs based on their normalized peak area

Molecular network analysis

To better visualize chemical relationships based on acquired MS-MS data, and to focus on the general chemical diversity based on annotated metabolites, a molecular networking was performed. Our analysis displayed a molecular network that contained 303 nodes and 29 spectral families with 161 connected

nodes while the rest of the nodes showed no link to others (self-loop). The size of the nodes represents VIP scores obtained for each feature (a bigger size, a higher VIP score) (**Figure 3.8**).

We observed the presence of chemical families such as the cluster containing long-chain fatty acids with the highest number of nodes possessing the highest VIP scores (bigger node sizes). Moreover, we can also observe the other clusters corresponding to phosphoethanolamines, phosphocholines, fatty amides, fatty acyls, steroid derivatives, amines, hybrid peptides, cyclopeptides and the cluster of TDKPs, botryosulfuranol A and B (**Figure 3.8**). The putatively identified metabolites found in the clusters within the molecular networking are detailed in **Table 3.2**.

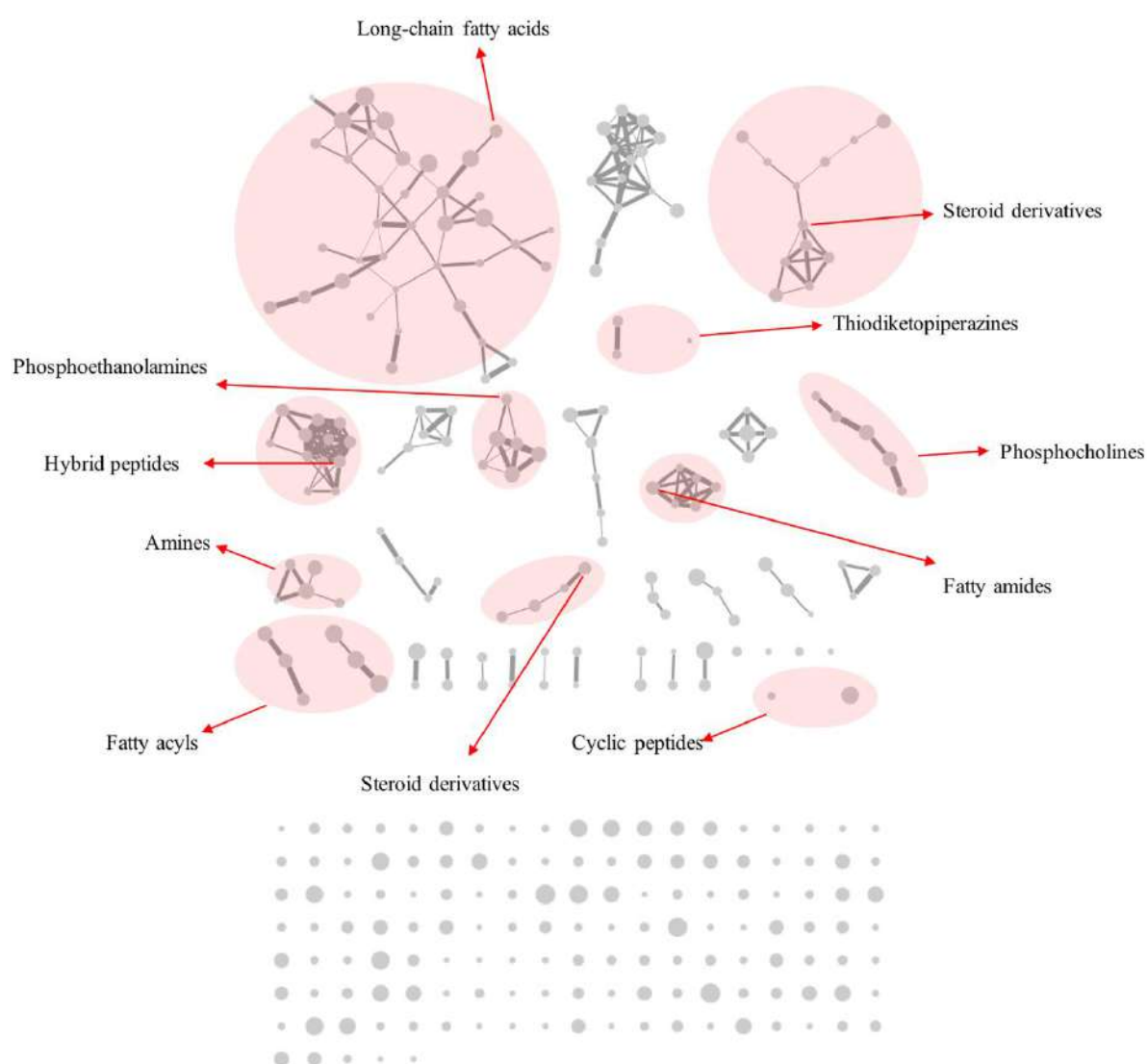


Figure 3.8. Molecular network showing the chemical families of the nodes corresponding to putatively annotated compounds clustered due to fragmentation pattern similarity

Table 3.2. Putative identification of metabolites detected during the time-series study of *C. mannii*

| ID | Rt (min) | m/z | Adduct | Formula | Δ mass (ppm) | Putative identification | Ontology |
|------------|-----------------|------------|-------------------------------------|--|---------------------|---|--------------------------|
| 23 | 8.39 | 318.2999 | [M+H] ⁺ | C ₁₈ H ₃₉ NO ₃ | 4.70 | Phytosphingosine | Sphingolipids |
| 265 | 3.90 | 250.1800 | [M+H] ⁺ | C ₁₅ H ₂₃ NO ₂ | 4.84 | (E)-1-(2,3-dihydro-1H-pyrrrol-1-yl)-2-methyldec-8-ene-1,3-dione | 1,3-dicarbonyl compounds |
| 252 | 5.42 | 244.1539 | [M+H] ⁺ | C ₁₂ H ₂₁ NO ₄ | 5.94 | Tiglylcarnitine | Fatty acyls |
| 163 | 9.25 | 542.3193 | [M+Na] ⁺ | C ₂₆ H ₅₀ NO ₇ P | 6.52 | LysoPC(18:2(9Z,12Z)) | Glycerophosphocholines |
| 164 | 9.06 | 520.3383 | [M+H] ⁺ | C ₂₆ H ₅₀ NO ₇ P | 5.00 | LysoPC(18:2(9Z,12Z)) | Glycerophosphocholines |
| 112 | 7.50 | 450.0783 | [M+NH ₄] ⁺ | C ₁₉ H ₁₆ N ₅ O ₆ S ₂ | 3.46 | Botryosulfuranol C | Thiodiketopiperazines |
| 165 | 7.05 | 485.0816 | [M+Na] ⁺ | C ₂₁ H ₂₂ N ₅ O ₆ S ₂ | 1.39 | Botryosulfuranol A | Thiodiketopiperazines |
| 135 | 7.32 | 487.0961 | [M+Na] ⁺ | C ₂₁ H ₂₄ N ₅ O ₆ S ₂ | 3.68 | Botryosulfuranol B | Thiodiketopiperazines |
| 172 | 9.03 | 552.4119 | [M+H] ⁺ | C ₂₉ H ₅₃ N ₅ O ₅ | 2.12 | Cyclo-(L-Leu-L-Leu-D-Leu-L-Leu-L-Leu-L-Val)* | Cyclic peptides |
| 174 | 9.37 | 583.4518 | [M+NH ₄] ⁺ | C ₃₀ H ₅₅ N ₅ O ₅ | 5.83 | Cyclo-(L-Ile-L-Leu-L-Leu-L-Leu-L-Leu)* | Cyclic peptides |
| 58 | 11.93 | 339.3256 | [M+H-H ₂ O] ⁺ | C ₂₂ H ₄₄ O ₃ | 3.72 | 22-Hydroxydocosanoic acid | Long-chain fatty acids |
| 127 | 9.03 | 478.2936 | [M+H] ⁺ | C ₂₃ H ₄₄ NO ₇ P | 0.69 | LysoPE(18:2(9Z,12Z)/0:0) | Phosphoethanolamines |
| 202 | 11.19 | 799.5190 | [M+H] ⁺ | C ₄₃ H ₇₀ N ₆ O ₆ S | 3.64 | Symplostatin 1 | Hybrid peptides |
| 291 | 12.43 | 284.2949 | [M+H] ⁺ | C ₁₈ H ₃₇ NO | 3.61 | Octadecanamide | Fatty amides |

*unknown amino acids sequence

3.2.2 *C. mamane* culture under the OSMAC approach

General observations

The endophytic fungus *C. mamane* was cultivated in 6-well plates containing Czapek (CZA) and ISP2 (see **Chapter 2** for methodology) during 6 and 10 days at 27 °C. To observe the influence of light on the metabolism, *C. mamane* was cultured under natural light (L) and total darkness (D) (**Figure 3.9**). At first sight, colonies presented white mycelium with bigger black spot areas than those observed in the colonies grown on MEA under the time-series study (shown above in **Figure 3.2**), which might indicate the production of pigments except when *C. mamane* was cultured in ISP2 during 6 days at dark conditions, suggesting a probable influence of light for mycelium coloration under this culture medium.

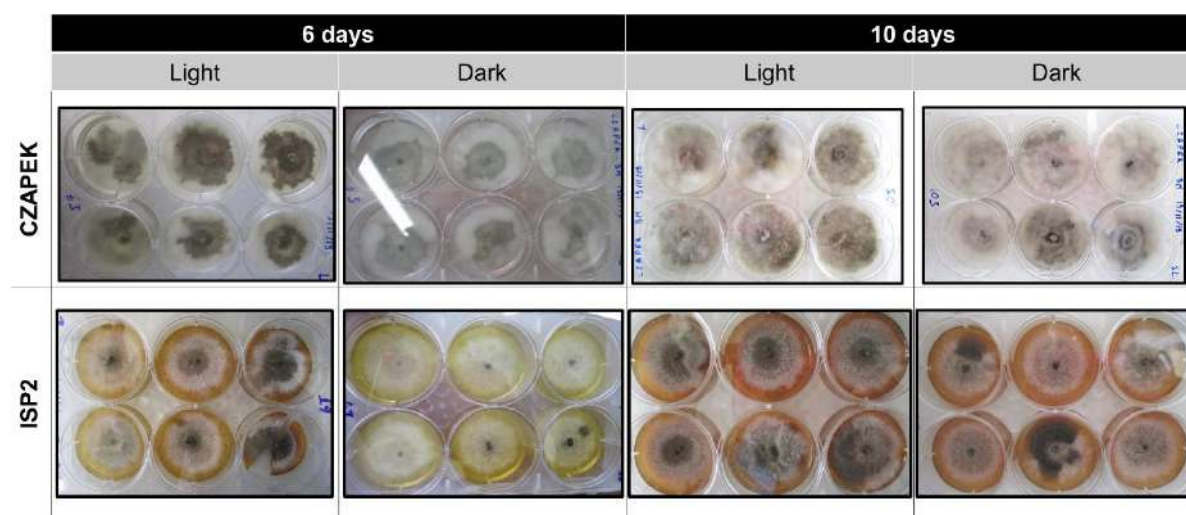


Figure 3.9. Morphological changes of *C. mamane* (upper view) cultivated in CZA and ISP2 media during 6 and 10 days under natural light and dark conditions

LC-(+)ESI-HRMS profiles of all the 48 extracts from the OSMAC experiment afforded 456 features (m/z -RT pairs) in PI mode, which were considered for the metabolomics and statistical analysis. A total of 16% of these features were putatively annotated (73 out of 456), corresponding to different chemical classes such as DKPs, cyclic peptides, fatty acids, fatty amides, flavonoids (**Annexes 2**).

Multivariate classification (PLS-DA)

A supervised PLS-DA analysis was carried out on these data indicating the different responses of *C. mamane* when cultured in ISP2 or CZA during 6 and 10 days under light or total darkness (**Figure 3.10**).

Separate clusters appear on the PLS-DA score plot (31 % of total variability). The component 1 (X-axis) explained 22.1 % of the variability, allowing the discrimination of the clusters corresponding to CZA and ISP2. While in the component 2 (9.9%), we can observe the influence of 6 and 10 days of culture and a slight differentiation between the clusters of light (L) and total darkness (D).

The **Figure 3.10** presents the top 15 features that contributed to the differences between these clusters. Among them, a metabolite mainly detected in ISP2, was putatively identified as 3-formylindole, a compound previously isolated from the endophytic fungus *Nigrospora* sp. (Kjer, 2009). Another compounds, mainly detected in CZA, was identified as botryosulfuranol C. The top 15 metabolites are detailed in **Table 3.3**.

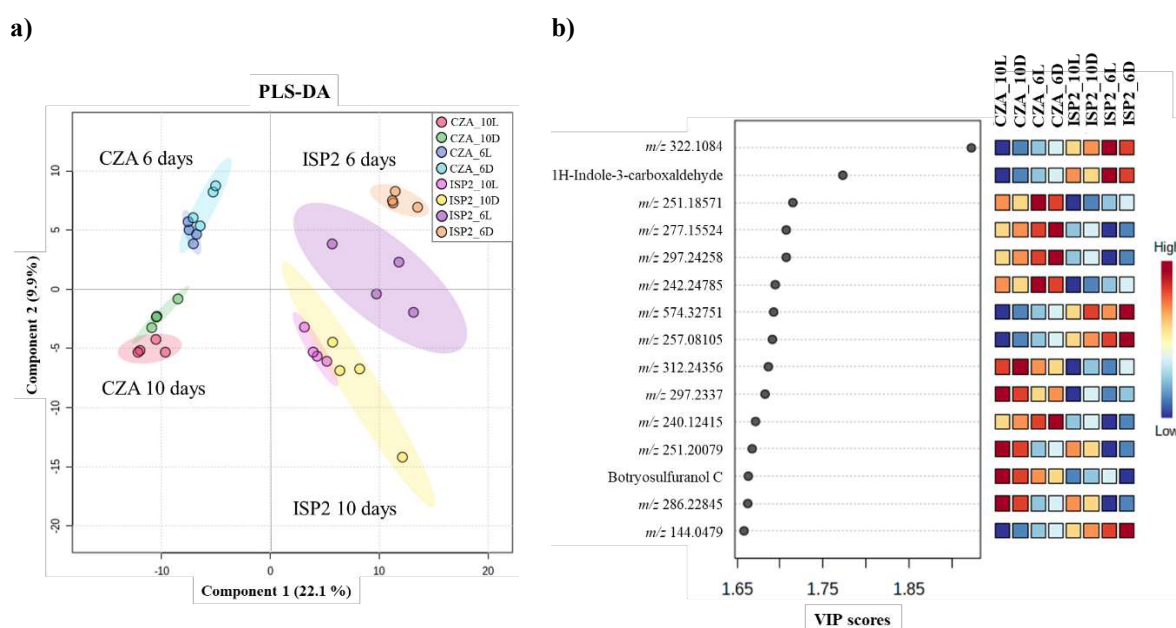


Figure 3.10. (a) Partial least squares discriminant analysis (PLS-DA) obtained from LC-MS profiles corresponding to the extracts of *C. mamane* cultivated in Czapek (CZA) and ISP2 during 6 and 10 days based on features (m/z at R_t to its normalized peak area), (b) Variable importance in projection (VIP) score of the top 15 compounds from the PLS-DA analysis corresponding to each condition of culture

Table 3.3. Top compounds that significantly contribute to the difference in the PLS-DA analysis from LC-HRMS/MS data corresponding to the OSMA_C approach of *C. mamane* culture

| ID | Rt (min) | m/z | Adduct | Formula | Δ mass (ppm) | Putative identification | Ontology | VIP scores |
|-----|----------|----------|-------------------------------------|--|--------------|-------------------------|-----------------------|------------|
| 182 | 3.69 | 322.1084 | [M+H] ⁺ | C ₁₁ H ₁₆ N ₃ O ₆ S | 3.15 | Unknown | Unknown | 1.9221 |
| 208 | 3.34 | 146.0604 | [M+H] ⁺ | C ₉ H ₇ NO | 2.46 | 3-formylindole | Indoles | 1.7728 |
| 75 | 5.70 | 251.1857 | [M+H] ⁺ | C ₁₂ H ₂₆ O ₅ | 1.59 | Unknown | Unknown | 1.7148 |
| 88 | 4.25 | 277.1552 | [M+H] ⁺ | C ₁₅ H ₂₀ N ₂ O ₃ | 1.91 | Unknown | Unknown | 1.7072 |
| 131 | 10.10 | 297.2425 | [M+NH ₄] ⁺ | C ₁₁ H ₃₇ NS ₃ | 0.46 | Unknown | Unknown | 1.7072 |
| 43 | 10.97 | 242.2478 | [M+H] ⁺ | C ₁₅ H ₃₁ NO | 0.16 | Unknown | Unknown | 1.6944 |
| 344 | 9.43 | 574.3275 | [M+H-H ₂ O] ⁺ | C ₃₄ H ₄₅ N ₅ O ₆ | 0.08 | Unknown | Unknown | 1.6925 |
| 61 | 5.64 | 257.0810 | [M+H-H ₂ O] ⁺ | C ₁₅ H ₁₄ O ₅ | 0.64 | Unknown | Unknown | 1.6911 |
| 163 | 9.16 | 312.2435 | [M+H] ⁺ | C ₂₀ H ₂₉ N ₃ | 0.24 | Unknown | Unknown | 1.6864 |
| 128 | 7.29 | 297.2337 | [M+H] ⁺ | C ₂₀ H ₂₈ N ₂ | 3.9 | Unknown | Unknown | 1.6826 |
| 38 | 5.36 | 240.1241 | [M+H] ⁺ | C ₁₂ H ₁₇ NO ₄ | 4.40 | Unknown | Unknown | 1.6715 |
| 132 | 8.47 | 251.2007 | [M+H] ⁺ | C ₁₆ H ₂₆ O ₂ | 0.56 | Unknown | Unknown | 1.6676 |
| 287 | 7.67 | 455.0363 | [M+Na] ⁺ | C ₁₉ H ₁₆ N ₂ O ₆ S ₂ | 4.60 | Botryosulfuranol C | Thiodiketopiperazines | 1.6631 |
| 102 | 8.48 | 286.2284 | [M+NH ₄] ⁺ | C ₁₈ H ₂₄ N ₂ | 2.18 | Unknown | Unknown | 1.6625 |
| 196 | 1.80 | 144.0479 | [M+H] ⁺ | C ₆ H ₉ NOS | 0.96 | Unknown | Unknown | 1.6582 |

Hierarchical clustering analysis (Heat map)

Out of the 456 features, 253 showed a significant change over time (p -value < 0.05) using the analysis of variance (one-way ANOVA) and Fisher test (F-test). The top 50 features with significant changes were plotted in the hierarchical clustering analysis (heat map) where four main clusters of metabolite behaviors are observed (**Figure 3.11**): **(a)** a cluster of metabolites induced in CZA regardless of the culture time or light condition; **(b)** a cluster of metabolites induced in CZA at day 10 regardless the light condition, **(c)** a cluster of metabolites induced in ISP2 at day 6 under dark conditions, **(d)** a cluster of metabolites induced in ISP2 at day 6, **(e)** a cluster of metabolites induced in ISP2 at day 10 under light conditions and **(f)** a cluster of metabolites induced in ISP2 at day 10.

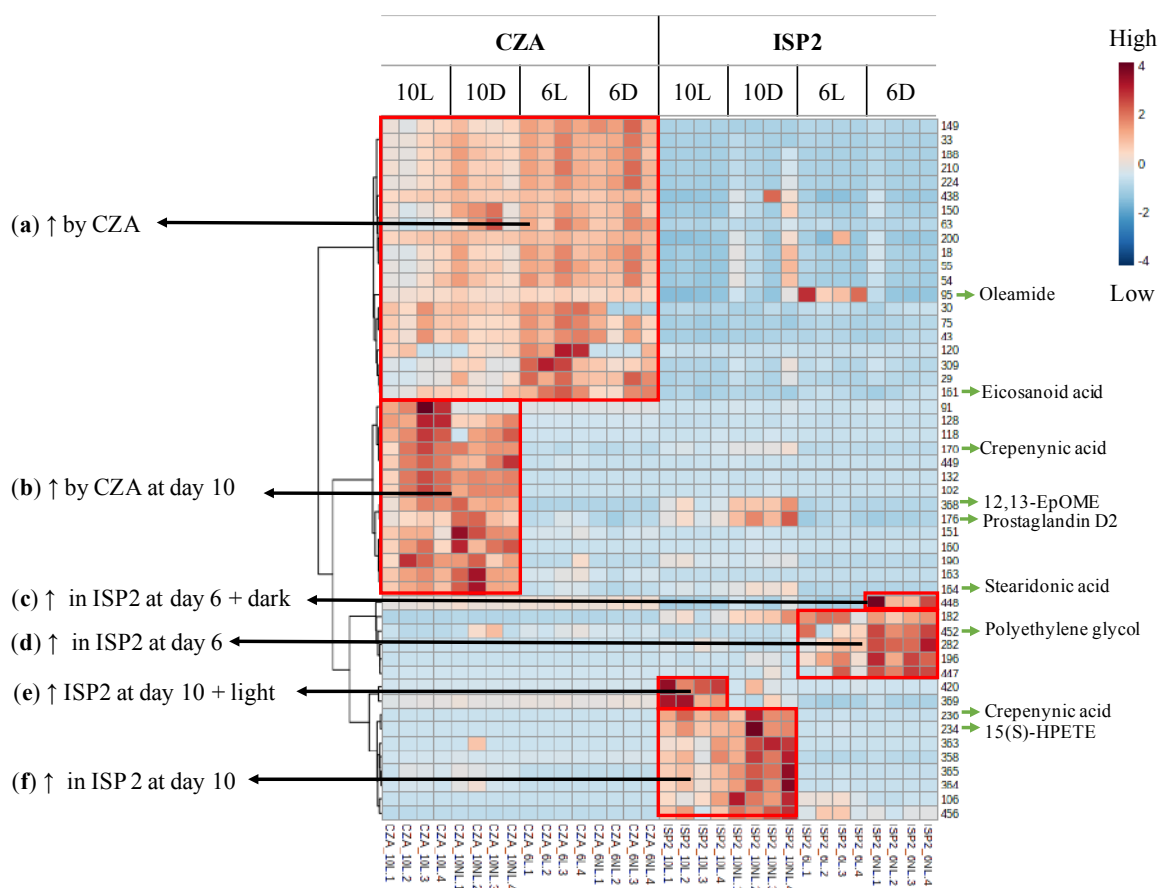


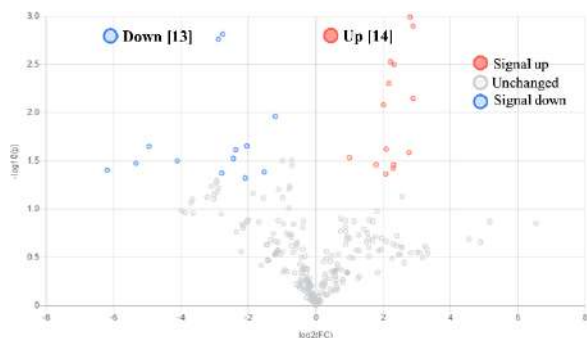
Figure 3.11. Hierarchical clustering of the top 50 features observed in *C. mamane* cultured under the OSMAC approach

Most of the putatively identified metabolites from the top 50 correspond to fatty acids, fatty acyl, fatty amides and prostaglandins such as oleamide, eicosanoid acid, 12,13-EpOME, prostaglandin D2.

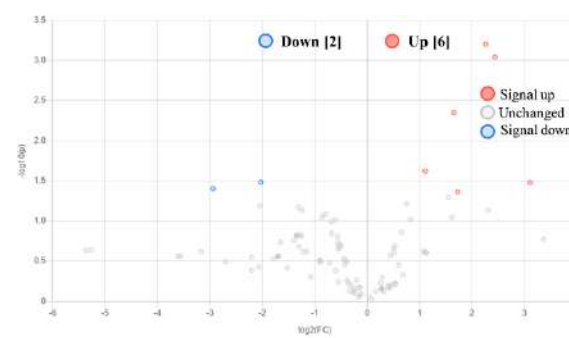
Differential production of metabolites (Volcano plot analysis)

Regarding the influence of light more in details as shown in the Volcano plot representations (**Figure 3.12**), a total of 14, 38, 6 and 10 features were up-regulated when *C. mamane* was cultivated under light conditions in ISP2 during 6 days (**Figure 3.12a**) and 10 days (**Figure 3.12c**) and in CZA during 6 days (**Figure 3.12b**) and 10 days (**Figure 3.12d**), respectively. Notably, culturing *C. mamane* in ISP2 medium during 10 days was the condition that induced the highest number of metabolites. Moreover, some metabolites were down-regulated when *C. mamane* was cultivated under light conditions, meaning that total darkness was a suitable condition for the production of these metabolites. However, due to the low annotation rate, metabolites with the most important significant changes could not be putatively annotated.

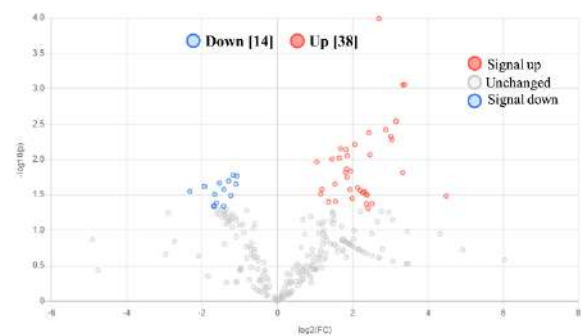
a) CM grown on ISP2 during 6 days



b) CM grown on Czapek during 6 days



c) CM grown on ISP2 during 10 days



d) CM grown on Czapek during 10 days

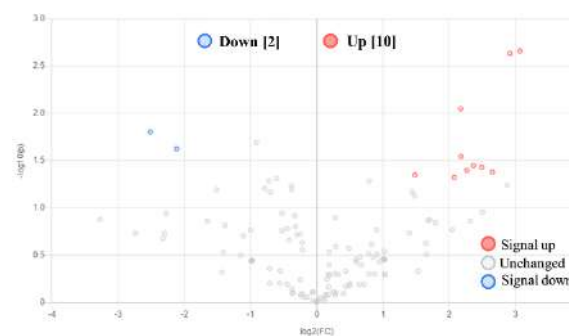


Figure 3.12. Volcano plot representations of *C. mamane* response to light conditions in ISP2 (a, c) and CZA (b, d) during 6 (a, b) and 10 days of incubation (c, d). Each dot represents a feature (m/z at R_t), the red and blue colors indicate a significant change (p -value < 0.05 and fold-change ratio > 2) of the features under the light influence in up-regulating (red) or down-regulating (blue) their production while the numbers in brackets indicate the number of significant features observed.

Focusing on the TDKPs, we can observe that no TDKP was detected when *C. mamane* was growth on ISP2 during 6 days under dark conditions (ISP2_6D), indicating that under these conditions, the presence of light was crucial for their production or that their production was delayed as it is observed during 10 days under dark conditions. Besides this, the production of botryosulfuranol C was

significantly different (p -value < 0.05) between CZA and ISP2 media, between 6 and 10 days of culture. Botryosulfuranol D was not detected in this study. Despite the high variability observed (error bars), this indicates that TDKPs production by *C. mamane* tend to be higher in CZA than in ISP2, specifically during 10 days under light conditions (**Figure 3.13**).

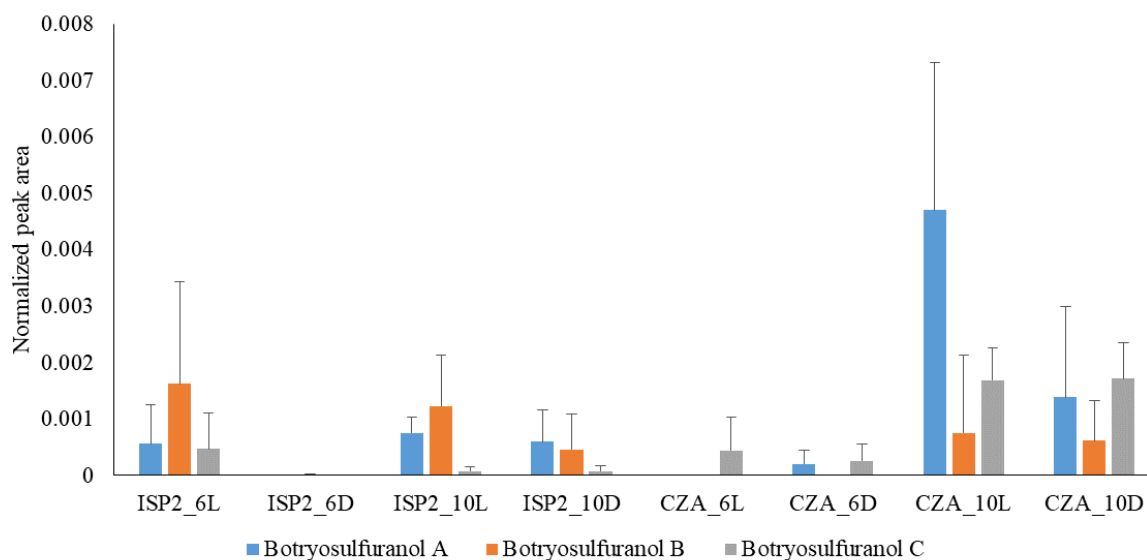


Figure 3.13. Normalized peak areas of TDKPs botryosulfuranols A, B and C when culturing *C. mamane* in ISP2 and CZA media during 6 and 10 days under light (L) and dark (D) conditions

Molecular network analysis

To better visualize chemical relationships based on acquired MS-MS data, and to focus on potential derivatives of annotated metabolites, a molecular networking was performed. Our analysis displayed a molecular network that contained 456 nodes and 38 spectral families with 252 connected nodes while the rest of the nodes showed no link to others (self-loop). The size of the nodes represents the total average of the detected peak areas for each compound in all the conditions while the colors depend on their detection in CZA and/or ISP2 medium (**Figure 3.14**). A cluster containing metabolites mostly produced in ISP2 was observed with no putatively annotated features.

The putatively annotated DKP, brevianamide F, was previously isolated from *Penicillium brevicompactum* (Birch and Russell, 1972), also from an endophytic fungal strain of *Aspergillus fumigatus* cultured in three different culture media under the OSMAC approach (Zhang et al., 2013) and more recently, from an endophytic fungal strain of *Colletotrichum gloeosporioides* (Yang et al., 2019). This DKP was found to be linked to two unknown metabolites with m/z 288.0716 at 3.8 min ($C_7H_{14}N_2O_5S_2$) and with m/z 300.1708 at 5.4 min ($C_{17}H_{21}N_3O_2$). Regarding the nodes corresponding to the TDKPs,

botryosulfuranols A, B and C were not linked to each other, contrary to what expected and observed in the time-series study.

Another DKP, cordycedipeptide A, previously isolated from the fungus *Cordyceps sinensis* (Jia et al., 2005) was found to be linked to one unknown metabolite with m/z 228.1341 at 2.95 min ($C_{10}H_{14}N_2O_3$). Moreover, the molecular network highlighted the presence of a cluster corresponding to cyclic peptides, containing putatively annotated metabolites previously isolated from an endophytic fungal strain of *Fusarium decemcellulare* with m/z 552.4145 at 9.176 min ($C_{29}H_{53}N_5O_5$), m/z 588.4099 at 9.504 min ($C_{30}H_{55}N_5O_5$) (Li et al., 2016) and m/z 600.4117 at 9.491 min ($C_{33}H_{53}N_5O_5$) from a non-identified endophytic fungal strain (Li et al., 2004). However, the order of the amino acids within the structure are not known and cannot be determined by this methodology.

Four metabolites were annotated as long-chain fatty acids such as the eicosanoid OPC-8:0, previously isolated from the fungus *Fusarium oxysporum* (Miersch et al., 1999), one eicosanoid acid; one sphingolipid phytosphingosine, previously isolated from an unknown fungus (Reindel et al., 1940), a prostaglandine D2, fatty acids crepenynic acid and 12-13-EpOME. These metabolites are detailed in **Table 3.4**.

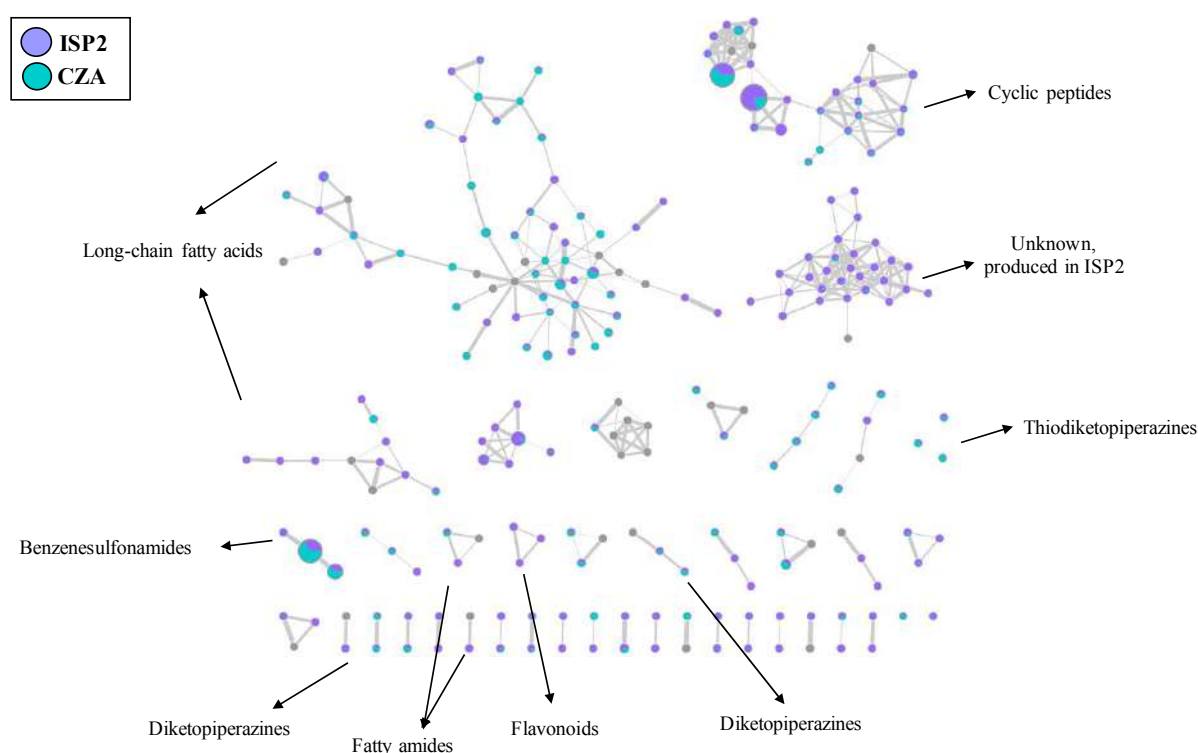


Figure 3.14. Molecular networking showing the chemical families of putatively annotated compounds detected under ISP2 and/or CZA

Table 3.4. Putative identified compounds detected in the OSMAC approach

| ID | Rt (min) | m/z | Adduct | Formula | Δ mass (ppm) | Putative identification | Ontology |
|-----|----------|----------|--------------------------------------|--|---------------------|--|---|
| 95 | 11.55 | 282.2793 | [M+H] ⁺ | C ₁₈ H ₃₅ NO | 0.56 | Oleamide | Fatty amides |
| 161 | 11.20 | 311.2945 | [M+H] ⁺ | C ₂₀ H ₃₈ O ₂ | 0.13 | Eicosenoic acid | Eicosanoids |
| 170 | 9.64 | 279.2323 | [M+H] ⁺ | C ₁₈ H ₃₀ O ₂ | 1.58 | Crepenymic acid | Long-chain fatty acids |
| 368 | 9.64 | 319.2244 | [M+Na] ⁺ | C ₁₈ H ₃₂ O ₃ | 0.11 | 12,13-EpOME | Long-chain fatty acids |
| 176 | 9.06 | 317.2098 | [M+H-2H ₂ O] ⁺ | C ₂₀ H ₃₂ O ₅ | 4.16 | Prostaglandin D2 | Prostaglandins and related compounds |
| 164 | 9.06 | 277.2174 | [M+H] ⁺ | C ₁₈ H ₂₈ O ₂ | 4.30 | Stearidonic acid | Unsaturated fatty acids and derivatives |
| 452 | 4.39 | 188.1288 | [M+H] ⁺ | C ₉ H ₁₇ NO ₃ | 3.61 | Polyethylene glycol | Medium-chain fatty acids |
| 236 | 10.73 | 279.2322 | [M+H] ⁺ | C ₁₈ H ₃₀ O ₂ | 1.22 | Crepenymic acid | Long-chain fatty acids |
| 234 | 10.21 | 359.2195 | [M+Na] ⁺ | C ₂₀ H ₃₂ O ₄ | 0.60 | 15(S)-HPETE | Fatty acyls |
| 208 | 3.34 | 146.0604 | [M+H] ⁺ | C ₉ H ₇ NO | 2.46 | 1H-Indole-3-carboxaldehyde | Indoles |
| 301 | 7.19 | 463.0989 | [M+H] ⁺ | C ₂₁ H ₂₂ N ₂ O ₆ S ₂ | 0.50 | Botrysulfuranol A | Thiodiketopiperazines |
| 305 | 7.44 | 487.0989 | [M+Na] ⁺ | C ₂₁ H ₂₄ N ₂ O ₆ S ₂ | 4.33 | Botrysulfuranol B | Thiodiketopiperazines |
| 287 | 7.67 | 455.0363 | [M+Na] ⁺ | C ₁₉ H ₁₆ N ₂ O ₆ S ₂ | 4.60 | Botrysulfuranol C | Thiodiketopiperazines |
| 98 | 4.82 | 284.1395 | [M+H] ⁺ | C ₁₆ H ₁₇ N ₅ O ₂ | 0.62 | Brevianamide F | Diketopiperazines |
| 166 | 9.76 | 295.2267 | [M+H] ⁺ | C ₁₈ H ₃₀ O ₃ | 0.24 | 3-Oxo-2-(2-entenyl)cyclopentaneoctanoic acid;OPC-8:0 | Eicosanoids |
| 351 | 9.50 | 588.4099 | [M+Na] ⁺ | C ₃₀ H ₅₁ N ₅ O ₅ | 0.73 | Cyclo-(L-Ile-L-Leu-L-Leu-L-Leu-L-Leu)* | Cyclic peptides |
| 332 | 9.17 | 552.4144 | [M+H] ⁺ | C ₂₉ H ₅₃ N ₅ O ₅ | 4.60 | Cyclo-(L-Leu-L-Leu-D-Leu-L-Leu-L-Leu-L-Val)* | Cyclic peptides |
| 360 | 9.49 | 600.4118 | [M+H] ⁺ | C ₃₃ H ₅₃ N ₅ O ₅ | 0.13 | Cyclo-(L-Phe-L-Leu-L-Leu2-L-Leu3-L-Ile)* | Cyclic peptides |
| 28 | 3.17 | 228.1345 | [M+H] ⁺ | C ₁₀ H ₁₇ N ₃ O ₃ | 1.23 | Cordycepidipeptide A;(-)-Cordycepidipeptide A | Diketopiperazines |
| 178 | 9.98 | 318.3002 | [M+H] ⁺ | C ₁₈ H ₃₉ NO ₃ | 0.22 | Phytosphingosine | Sphingolipids |

*unknown amino acids sequence

3.3 Discussion

3.3.1 General morphological observations

Morphological changes were observed under both methodologies, mainly the darkening of mycelium pigmentation. Over 28 days of culture in MEA, most of the colonies maintained a white pigmentation while only some of them showed dark spots on their mycelium (**Figure 3.2**). In a previous study, *C. mamane* colonies showed a dark pigmentation of their mycelium when it was cultured in MEA during 10 days (Triastuti et al., 2021), probably indicating the aging of the strain, a different inoculation method or different light conditions in comparison to our study. Also, the first morphological descriptions of this species indicate a rapid change of mycelium color from medium to dark gray when the culture was exposed to natural light (Gardner, 1997). Similarly, another member of the Botryosphaeriaceae family, *Botryosphaeria dothidea*, showed initially colorless colonies that changed to dark with age (Phillips et al., 2005). Under the OSMAC approach, most of the colonies grown on ISP2 and CZA showed a darkening of their mycelium at day 6 and 10 of culture, except in ISP2 at day 6 under dark conditions (**Figure 3.9**).

Light can influence visible changes such as the mycelium growth (Kim et al., 2005) as observed with *C. mamane* when white colonies were produced on ISP2 during 6 days under dark conditions. Also, the correlation between mycelium color changes and metabolite production has been previously reported in, for example, the fungus *Menisporopsis theobromae* (Madla et al., 2006) but no pigments could be identified in this study. In general, fungi produce a wide array of pigments corresponding to different chemical classes such as melanins, carotenoids and quinones (Kalra et al., 2020) which possess many pharmacological activities that help the fungi to protect against biotic and abiotic stress (Berthelot et al., 2017; Dufossé et al., 2014).

3.3.2 Time-series study

Variations in the metabolome of *C. mamane* across the 28 days of culture in MEA allowed us to identify three main metabolite behaviors: early, middle and late stage (**Figure 3.5**). In agreement with our findings, similar patterns were observed in the metabolome of the fungus *Colletotrichum sublineolum* corresponding to 3 time periods: early adaptation, transition and stationery. It is postulated that in the transition period, the fungus can respond to environmental signals for a better adaptation to decreasing nutrient conditions (Tugizimana et al., 2019). Similarly, a time-variations study of the metabolome of two marine-derived fungal strains, *Penicillium canescens* and *Penicillium* sp., indicates three phases according the morphological changes and six groups of compounds with different behaviors according to their apparition or disappearance during the whole culture time (Roullier et al., 2016).

Among the metabolites that contributed to the cluster differentiation in the multivariate analysis (PLS-DA), we found a cyclic pentapeptide, which exhibited an early-stage apparition between D4 and D8 and a rapid decrease, being almost undetected after this period. Cyclic peptides produced by fungi have been reviewed elsewhere, indicating their important biological activities and potential applications (Wang et al., 2017b), however, their ecological roles have been very less discussed. Their relevance in interspecies interactions as crosstalk communication molecules has been proposed as for example for the hexocyclopeptides produced by the endophytic fungus *Fusarium solani* due to their accumulation under the co-culture with an endophytic bacterium (Wang et al., 2015). Also, cyclic pentapeptides produced by the endophytic fungus *Fusarium decemcellulare* exhibited a weak antimicrobial activity against another fungal endophyte in co-culture (Li et al., 2016), showing their role in microbial neighbor communication and in proving certain benefits in colonization. This might explain the rapid decrease in the production of the cyclic pentapeptide along the 28 days of culture of *C. mamane*, considering also that secondary metabolites stop being produced over time or after many subculture generations under axenic conditions (Kusari et al., 2014) in the absence of signals from their natural environment.

Other metabolites also showed a change in their production over time, including two glycerophosphocholines that were produced during the early stage and middle stage, which is consistent with a lipidomic study of *Trichoderma brevicompactum*, where the glycerophospholipids were found to be significantly altered at the early phase of a 15-day culture (Bai et al., 2019). Also, a phytosphingosine was produced during a late stage. Fungal sphingolipids have shown to possess antifungal activity, playing important functions in cell differentiation and pathogen recognition and virulence (Mota Fernandes and Del Poeta, 2020) such as two new sphingolipids with antifungal activity, previously isolated from the marine alga endophytic fungus *Aspergillus niger* (Zhang et al., 2007). Besides the reports showing their potent and novel antimicrobial activities (Barthélemy et al., 2019), it is known that lipids possess important functions in fungi, especially in the pathogen recognition by the host and signaling between cells during infection or during nutrient exchange (Siebers et al., 2016). In general, lipids are known to be important metabolites as signaling molecules during development and pathogenicity in fungi (Shea and Del Poeta, 2006) and in this case, their induction in CZA or ISP2 appeared to be significantly different. Moreover, molecular network analysis allowed us to observe the presence of many other fatty acids contained in one of the clusters from our molecular network analysis, as well as other chemical families based on the putatively annotated metabolites.

3.3.3 OSMAC approach

Among culture media used to cultivate *C. mamane* according to literature, ISP2 and CZA were not previously reported (Barakat et al., 2019; Gardner, 1997; Pongcharoen et al., 2007). CZA medium contains 3 salts as source of ions, being sodium nitrate the unique source of inorganic nitrogen (St-

Germain and Summerbell, 2003). Metal ions have demonstrated to have a physiological influence on microorganisms (Thorneley, 1990), triggering the synthesis of new compounds (Jiang et al., 2014). CZA medium could be considered as an oligotrophic medium, which could be favorable for the activation of some gene clusters, which may remain silent when fungi are cultured in eutrophic media such as MEA or ISP2. This might explain the cluster of metabolites induced when *C. mamane* was cultured in CZA but not by ISP2 (**Figure 3.11**). Although ISP2 medium does not contain any source of ions or supplements, it has been useful for the discovery of new metabolites and the induction of other known compounds in fungi (Wakefield et al., 2017) as also observed with the cluster of metabolites induced when *C. mamane* was cultured in ISP2 but in CZA (**Figure 3.11**).

On the other side, the influence of light seemed to be more important when culturing *C. mamane* in ISP2 during 10 days given the number of metabolites induced under natural light conditions (**Figure 3.12**) in comparison to the other conditions. In general, fungal endophytes have a prominent dark habitat in the internal host plant tissues and this condition has shown a great impact in, for example, taxol production by *Paraconiothyrium* spp. as this endophytic fungus is mainly found in the deep vascular system of the host plant (Soliman and Raizada, 2018). By contrast, *C. mamane* was isolated from the leaves of *Bixa orellana*, a plant that grows in tropical regions (Vilar et al., 2014) with natural light conditions varying from full sunlight to partial shade, since light quality is also important for the secondary metabolism of the host plant (Faria et al., 2019).

We were not able to determine the chemical classes of the metabolites that might be specifically induced by each culture media or the different light conditions. However, molecular networking showed us a cluster of metabolites that was mostly produced by *C. mamane* in ISP2 (**Figure 3.14**) but no metabolite belonging to this cluster could be putatively annotated either. Similar chemical families observed in the time-series study were observed under the OSMAC approach, including the fatty acids, fatty amides, cyclic peptides and TDKPs. Moreover, the presence of two DKPs, putatively annotated as brevianamide F and cordycedipeptide A linked with other metabolites in different clusters could indicate the presence of their derivatives or similar compounds that will need further future investigations.

3.3.4 Special focus on the TDKPs

We observed the production of botryosulfuranols A and B (**Figure 3.6**) during the whole culture period of 28 days with a significant higher production during the middle stage at D12 (**Figure 3.7**) while botryosulfuranol D was undetected in both studies, indicating its production is less constant or in undetected quantities. In contrast, botryosulfuranol C was only detected between D6 and D9, highlighting the short time frame for its detection. This TDKP possesses a di-sulfur bridge, which plays an important role in biological activity (Barakat et al., 2019; Jiang and Guo, 2011). In addition to this,

di-sulfur bridge might represent certain toxicity to the producer organism, which activates defense mechanisms involving the opening of the di-sulfur bridge as it was observed for the biosynthesis of the DKP gliotoxin produced by *Aspergillus fumigatus* (Scharf et al., 2014). This situation might explain the short time-frame production of botryosulfuranol C by *C. mamane*.

In this study, we also observed that the presence of light was important to avoid the production delay of the TDKPs when growing *C. mamane* in ISP2 during 6 days. It is known that for the biosynthesis of DKPs, two main enzymes are needed, nonribosomal peptide synthetases (NRPSs) and cyclodipeptide synthases (CDPSs) (Borgman et al., 2019) whose coding gene clusters have shown to respond to environmental signals such as light and nutrient sources (Brakhage, 2013), which can explain our observations of the influence of light over the TDKPs production. Moreover, culturing *C. mamane* in CZA during 10 days under light conditions allowed a higher production of the TDKPs, specifically a significant induction of botryosulfuranol C, in comparison to the other conditions used under the OSMAC approach. Similarly, the endophytic fungus *Penicillium brocae* produced four new TDKPs when cultured in CZA (Meng et al., 2017). Also, the fungus *Aspergillus sydowii* allowed the isolation of four bioactive DKPs when cultured in three different culture media under the OSMAC approach (da Silva Lima et al., 2018).

Our interest in understanding the production trend over time of the TDKPs and their production patterns under different culture conditions lies on their biological activity as cytotoxic (Barakat et al., 2019) and as antileishmanial metabolites (unpublished data) as well as in the different biological activities reported in the literature for these type of metabolites (Song et al., 2021; Wang et al., 2017a). Their ecological roles in fungi are still not fully understood, however DKPs might act as quorum sensing (QS) molecules, a mechanism that is observed in yeast and filamentous fungi through the production of tyrosol, farnesol (Mondal and Majumdar, 2019) and mycotoxins (Venkatesh and Keller, 2019). This role has been recently reported for DKP-producing bacteria (Bofinger et al., 2017; Zhu et al., 2019) and in *Aspergillus fumigatus*, where the production of DKPs emestrins A and B were induced in presence of bacterial QS molecules, probably acting as QS inhibitors to modulate or attenuate bacterial populations (Rateb et al., 2013).

3.4 Conclusions

During the time variations study, we observed that a cyclopentapeptide appeared significantly produced during the early stage of *C. mamane*. This metabolite is probably involved in QS mechanisms as reported for other fungi and the absence of other signals might have cause the decline of its production in later stages. Moreover, the production of the TDKPs, botryosulfuranols A and B showed a significant increase during the middle stage at day 12 while botryolsulfuranol C exhibited a short time frame

production, probably due to the toxicity that the di-sulfur bridge might generate in *C. mamane* as reported for other DKPs such as the gliotoxin.

The OSMAC approach allowed us to observe the different cluster of metabolites that were induced under CZA and ISP2, the latter being the culture medium that induced most of the metabolites under light conditions. Interestingly, TDKPs were not detected in ISP2 during 6 days of culture under dark conditions, indicating the importance of light for an earlier production. The culture of *C. mamane* in CZA during 10 days under natural light conditions allowed a higher production (6 times higher) of the TDKPs in comparison to the other conditions. Different light wavelengths will be contemplated for future studies involving the influence of light, as it has been shown that light influence the secondary metabolism in fungi. In order to better understand the biological activities and the ecological roles of botryosulfuranols A, B and C, optimal culture conditions for their production are needed where different physicochemical conditions have to be followed up. This will allow us to obtain significant quantities of TDKPs for future experiments under different approaches and to discover new bioactive metabolites that this endophyte might produce.

Molecular network analysis allowed us to identify different chemical classes produced under the time variations study and OSMAC approach. Despite the low percentage of putative annotation in this study and in other untargeted metabolomics studies using mass spectrometry databases, some of the putatively annotated metabolites gave us insights about the chemical diversity produced by *C. mamane* under different conditions.

CHAPTER 4 ADDITION OF EPIGENETIC MODIFIERS AND AMINO ACIDS

This Chapter has been prepared from the submitted original research article

Chemical modulation of the metabolism of an endophytic fungal strain of *Cophinforma mamane* using epigenetic modifiers and amino-acids

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Abstract

Endophytic fungi are capable of producing a great diversity bioactive metabolites. However, the presence of silent and lowly expressed genes represents a main challenge for the discovery of novel secondary metabolites with different potential uses. Epigenetic modifiers have shown to perturb the production of fungal metabolites through the induction of silent biosynthetic pathways leading to an enhanced chemical diversity. Moreover, the addition of bioprecursors to the culture medium has been described as a useful strategy to induce specific biosynthetic pathways. The aim of this study was to assess the effects of different chemical modulators on the metabolic profiles of an endophytic fungal strain of *Cophinforma mamane* (Botryosphaeriaceae), known to produce 3 bioactive thiodiketopiperazine (TDKPs) alkaloids (botryosulfuranols A-D). Four epigenetic modifiers, 5-azacytidine (AZA), sodium butyrate (SB), nicotinamide (NIC), homoserin lactone (HSL) and 2 amino acids, L-phenylalanine and L-tryptophan, as bioprecursors of diketopiperazines, were used. The metabolic profiles were analysed by UHPLC-HRMS/MS under an untargeted metabolomics approach. Our results show that the addition of the two amino acids in *C. mamane* culture and the treatment with AZA significantly reduced the production of botryosulfuranols A, B and C. Interestingly, the treatment with HSL significantly induced the production of different classes of DKPs. The treatment with AZA resulted as the most effective epigenetic modifier for the alteration of the secondary metabolite profile of *C. mamane* by promoting the expression of cryptic genes.

Keywords: endophytic fungi, *Cophinforma mamane*, epigenetic modifiers, metabolomics, diketopiperazines

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4.1 Introduction

Microorganisms such as bacteria, filamentous actinomycetes and fungi, have produced most of the known antibiotics due to the large chemical diversity and biological activities of their metabolites (Boufridi & Quinn 2018; Abdel-Razek *et al.* 2020). Among them, endophytic fungi are considered as a goldmine of secondary metabolites (Strobel & Daisy 2003), represented by little explored and characterized microorganisms capable of produce compounds with potential for pharmaceutical and commercial uses (Strobel 2018; Deshmukh *et al.* 2018; White *et al.* 2019). However, the conditions for gene expression in fungi are not fully understood and the presence of silent or lowly-expressed genes represent one of the main challenges for the isolation of novel secondary metabolites (Brakhage & Schroeckh 2011). Biosynthetic gene clusters in fungi are often found in the distal telomeric regions of the chromosome (Shwab *et al.* 2007; Williams *et al.* 2008), which are transcriptionally controlled by epigenetic regulation, specifically histone acetylation and methylation (Brakhage 2013; Pfannenstiel & Keller 2019). When fungal DNA is organized onto histone proteins and is densely packed into heterochromatin, it becomes transcriptionally silent (Okada & Seyedsayamdost 2017). In this regard, highly acetylated histones are generally loosely packed into euchromatin and therefore genes are considered as transcriptionally active (Cichewicz 2010) while methylation of histones often results in gene silencing (Suzuki & Bird 2008).

Recently, a very well-reviewed strategy to induce cryptic genes has consisted in the treatment of fungal endophytes with epigenetic modifiers such as histone deacetylase inhibitors (HDACi) and DNA methyltransferase inhibitors (DNMTi) (Toghueo *et al.* 2020; Gupta *et al.* 2020). For instance, the addition of 5-azacytidine, a DNMTi, enhanced the production of the alkaloid camptothecine in *Botryosphaeria rhodina* (Botryosphaeriaceae), a finding that could give insights of the signaling mechanisms between the host plant and its endophytic fungus (Vasanthakumari *et al.* 2015). Similarly, metabolite production in *Diaporthe* sp. (Diaporthaceae) was enhanced when it was subjected to epigenetic treatment with the HDACi suberohydroxamic acid and valproic acid (Jasim *et al.* 2019). The addition of the HDACi nicotinamide to the culture of the endophytic fungus *Graphiopsis chlorocephala* (Cladosporiaceae), led to a notable enrichment in secondary metabolite production and also allowed the isolation of new benzophenones and cephalanones (Asai *et al.* 2013). A clear advantage of using epigenetic-based methods is that they do not require genetic manipulation and they can be applied to any fungal strain, potentially allowing the annotation of new secondary metabolites and the enhancement of the synthesis of low-yield metabolites.

Based on these promising results, we decided to use epigenetic modifiers for the induction of cryptic genes of an endophytic fungal strain of *Cophinforma mamane* (D.E. Gardner) A. J. L. Phillips & A. Alves (Botryosphaeriaceae) isolated from the leaves of *Bixa orellana* (Bixaceae) (Barakat *et al.* 2019). The first attempt to induce the metabolite production of *C. mamane* using epigenetic modifiers allowed

the detection of 8 metabolites produced *de novo* (Triastuti *et al.* 2019). Additionally, to date, 10 compounds have been isolated from *C. mamane*, including 3 new thiodiketopiperazines alkaloids, botryosulfuranols A, B and C isolated by our team, exhibiting cytotoxic activity against different cancer cell lines (Barakat *et al.* 2019). This class of diketopiperazines (DKPs) are derived from two phenylalanines residues and present an unprecedented skeleton with two spirocyclic centers which are currently under biological investigation to go further in elucidation of their mechanism of action. Moreover, as described in Welch and Williams (2014) (Welch & Williams 2014), thiodiketopiperazines are biogenetically derived from at least one aromatic amino acid (phenylalanine, tyrosine and/or tryptophan), therefore the addition of these type of bioprecursors into the culture media might induce their biosynthetic pathway. The amino-acid directed strategy has shown to induce cryptic biosynthetic pathways, allowing the isolation of many novel fungal bioactive compounds in fungi, specifically alkaloids (Huang *et al.* 2017; Yuan *et al.* 2019; Qiu *et al.* 2020; Guo *et al.* 2020). Other molecules previously isolated from *C. mamane* include a new dihydrobenzofuran derivative, botryomaman and a known molecule, primin (Pongcharoen *et al.* 2007).

In the present study, the effects on secondary metabolism of *C. mamane* through the addition of four different epigenetic modifiers to the culture medium were investigated. Two HDACi, sodium butyrate (SB) and nicotinamide (NIC), one DNMTi, 5-azacytidine (AZA) and one bacterial *quorum-sensing* (QS) molecule, N-Butyryl-DL-homoserine lactone (HSL), were used for this purpose. In a separated experiment, culture medium was supplemented with 2 amino acids, L-phenylalanine and L-tryptophan, precursors of nitrogenous compounds such as DKPs, to evaluate their influence on the metabolism of this strain, especially on the production of the three known phenylalanine-derived DKPs previously isolated. This work presents two final objectives: to assess the effects of chemical elicitors on the secondary metabolites profile of *C. mamane* by promoting the expression of cryptic genes, and 2) to obtain a better yield of botryosulfuranols A, B and C needed for further biological investigations in future research.

4.2 Results and Discussion

4.2.1 General observations

The general overview of the chromatograms of the experiment carried out with the epigenetic modifiers show five main peaks produced in all the conditions, including the control (untreated *C. mamane*) corresponding to the three TDKPs, botryosulfuranol A, botryosulfuranol B, botryosulfuranol C and two cyclopentapeptides (**Figure 4.1**). Endophytic fungi have shown a great potential to produce diverse

DKPs and cyclopeptides under standard culture conditions, which possess interesting biological activities reported in the literature (Wang *et al.* 2017a,b).

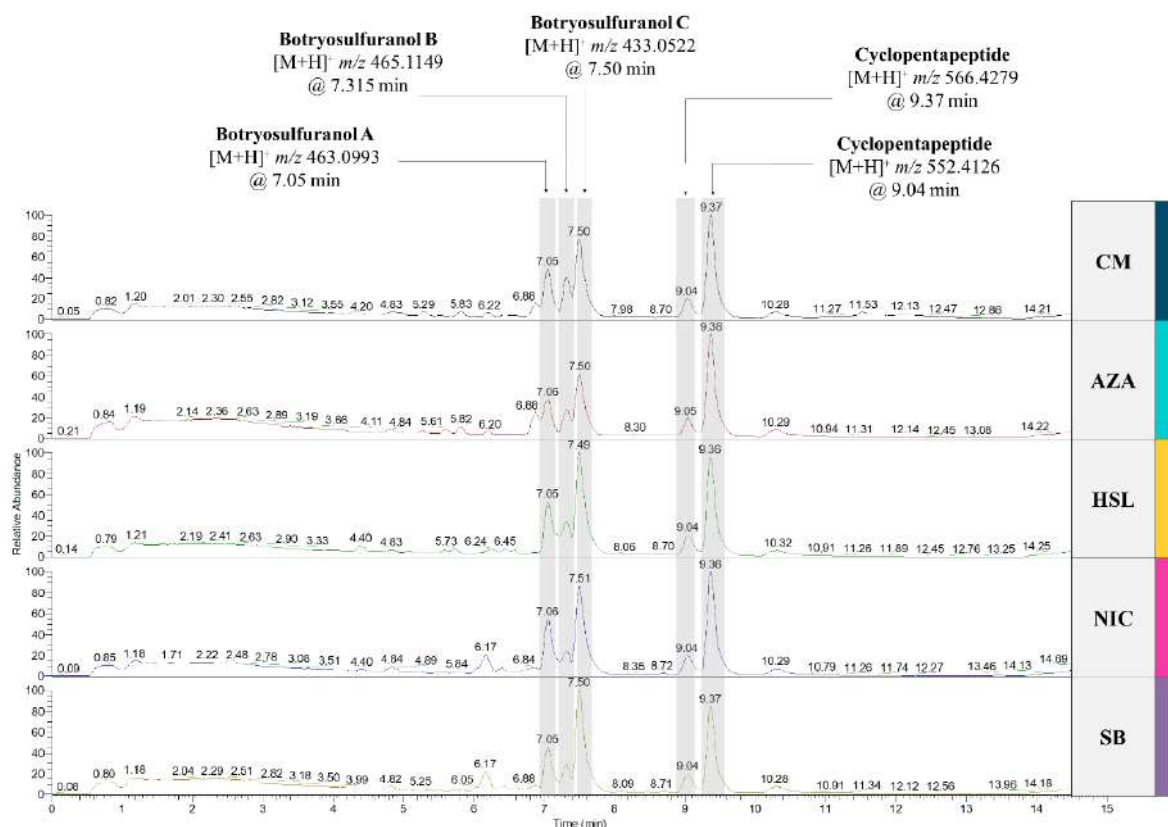


Figure 4.1. UHPLC-ESI-HRMS (total ion chromatogram) from crude extracts of *C. mamane* (CM) cultivated in PDB and treated with 5-azacytidine (AZA), homoserine-lactone (HSL), nicotinamide (NIC) and sodium butyrate (SB).

A metabolomic profile of 20 extracts used in the epigenetic modifier treatment obtained by UHPLC-ESI-HRMS afforded 294 features (m/z -RT pairs), which were considered for the metabolomics and statistical analysis. Twenty percent of these features were putatively annotated, corresponding to metabolites belonging to different chemical classes such as diketopiperazines, cyclic peptides, fatty acyls, phosphoethanolamines, and ubiquinones (**Annexes 3**).

4.2.2 Multivariate classification (PLS-DA)

As a first step, a PCA was applied as an unsupervised overview of the general LC-MS profile (data not shown). This analysis clustered all independent biological replicates from the same conditions. Then, a supervised PLS-DA analysis was carried out on these same data which indicated the different responses of *C. mamane* (CM) when treated with the HDACi (NIC and SB), the DNMTi (AZA) and the QS

molecule (HSL) in comparison to the untreated culture of *C. mamane* (**Figure 4.2**). Separate clusters appear on the PLS-DA score plot (65.3% of total variability). The component 1 (X-axis) explained 56.2% of the variability, allowing the discrimination of the AZA cluster. While in the component 2 (9.1%), we can observe two clusters: NIC/SB and CM/HSL, however a slight differentiation can be observed between CM and HSL.

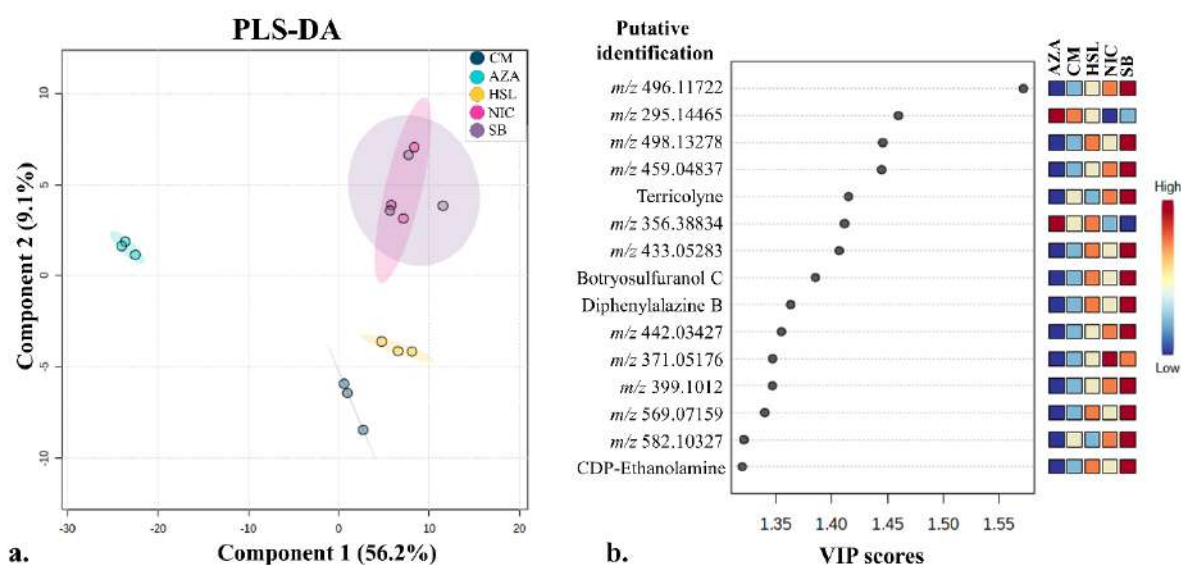


Figure 4.2. a) PLS-DA corresponding to the extracts of *C. mamane* cultivated in PDB (CM) and treated with nicotinamide (NIC), sodium butyrate (SB), 5-azacytidine (AZA) and homoserin lactone (HSL), based on features (*m/z* at Rt to its normalized peak area) detected in LC-MS chromatograms; (b) PLS-DA-VIP projection for the top 15 features corresponding to each group (AZA, CM, HSL, NIC, SB).

The **Figure 4.2** presents the top 15 features that contributed to the differences between the treatments while some of them were putatively identified. Among them, a metabolite was annotated as fatty acyl terricolyne, a metabolite that was previously isolated from an endophytic fungal strain of *Neurospora terricola* (Sordariaceae) isolated from a lichen, therefore categorized as an endolichenic fungus (Zhang *et al.* 2009). Another annotated metabolite was the phospholipid cytidine diphosphate ethanolamine (CDP-ethanolamine) which has previously shown to be critical for mycelial growth and whose biosynthesis is involved in the mycotoxin production and virulence in *Fusarium graminearum* (Nectriaceae) (Wang *et al.* 2019). The annotated DKP diphenylalazine B was previously isolated from *Epicoccum nigrum* (Didymellaceae) which colonized *Cordyceps sinensis* (Cordycipitaceae) (Guo *et al.* 2009) and the TDKP, botryosulfuranol C, previously isolated from the strain of *C. mamane* (Barakat *et al.* 2019) used in this study. The aforementioned metabolites are detailed in **Table 4.1**

Table 4.1. Top 15 features that significantly contribute to the difference in the PLS-DA analysis from LC-MS data under the treatment with epigenetic modifiers in *C. mamane* culture

| ID | Rt (min) | m/z | Adduct | Formula | Δ mass (ppm) | Putative identification | Ontology | VIP scores |
|-----|----------|----------|-----------------------------------|---|---------------------|--|-----------------------|------------|
| 7 | 5.49 | 193.0857 | [M+H] ⁺ | C ₁₁ H ₁₂ O ₃ | 0.73 | Terricolyne | Fatty acyls | 1.415 |
| 99 | 5.81 | 295.1446 | [M+H] ⁺ | C ₁₈ H ₁₈ N ₂ O ₂ | 1.84 | Unknown | Unknown | 1.46 |
| 125 | 6.90 | 323.1393 | [M+H] ⁺ | C ₁₉ H ₁₈ N ₂ O ₃ | 1.08 | Diphenylalazine B; (+)-Diphenylalazine B | Diketopiperazines | 1.3631 |
| 148 | 10.80 | 356.3883 | [M+H] ⁺ | C ₂₃ H ₄₉ NO | 0.98 | Unknown | Unknown | 1.4116 |
| 155 | 7.44 | 371.0517 | [M+H] ⁺ | C ₁₈ H ₁₄ N ₂ O ₃ S ₂ | 0.26 | Unknown | Unknown | 1.3469 |
| 168 | 7.83 | 399.1012 | [M+H] ⁺ | C ₂₀ H ₁₈ N ₂ O ₅ S | 0.7 | Unknown | Unknown | 1.3467 |
| 181 | 6.62 | 433.0528 | [M+H] ⁺ | C ₂₃ H ₁₂ O ₉ | 5.95 | Unknown | Unknown | 1.4068 |
| 189 | 4.79 | 442.0342 | [M+H] ⁺ | C ₁₄ H ₁₆ N ₇ O ₂ PS ₃ | 1.06 | Unknown | Unknown | 1.355 |
| 191 | 7.07 | 447.0678 | [M+H] ⁺ | C ₁₁ H ₂₀ N ₄ O ₁₁ P ₂ | 0.34 | CDP-Ethanolamine | Phosphoethanolamine | 1.3198 |
| 198 | 6.43 | 459.0483 | [M+H] ⁺ | C ₁₅ H ₁₉ N ₆ O ₃ PS ₃ | 1.62 | Unknown | Unknown | 1.4449 |
| 222 | 5.76 | 496.1172 | [M+H] ⁺ | C ₂₁ H ₂₅ N ₃ O ₇ S ₂ | 6.94 | Unknown | Unknown | 1.5721 |
| 224 | 6.06 | 498.1327 | [M+H] ⁺ | C ₂₄ H ₂₃ N ₃ O ₇ S | 0.33 | Unknown | Unknown | 1.4459 |
| 255 | 6.01 | 569.0715 | [M+H] ⁺ | C ₃₀ H ₁₆ O ₁₂ | 0.24 | Unknown | Unknown | 1.3399 |
| 261 | 6.96 | 582.1032 | [M+NH ₄] ⁺ | C ₂₇ H ₂₀ N ₂ O ₈ S ₂ | 5.7 | Unknown | Unknown | 1.3212 |
| 278 | 7.49 | 433.0521 | [M+H] ⁺ | C ₁₉ H ₁₆ N ₂ O ₆ S ₂ | 0.24 | Botryosulfuranol C | Thiodiketopiperazines | 1.3854 |

4.2.3 Hierarchical clustering analysis (Heat-map)

Out of the 294 features, 221 showed significant changes under the treatment with epigenetic modifiers (p -value < 0.05) using the analysis of variance (one way ANOVA) and Fisher test (F-test). The top 150 features with significant changes were plotted in the hierarchical clustering analysis (heat map) where five main clusters of metabolite behaviors are detected (**Figure 4.3**): a cluster corresponding to the up-regulation with AZA treatment, a cluster corresponding to the up-regulation with NIC and SB treatment, a cluster corresponding to the up-regulation with HSL treatment and a cluster corresponding to the down-regulation with the 4 epigenetic modifiers treatment. These results are consistent with PLS-DA analysis observed above.

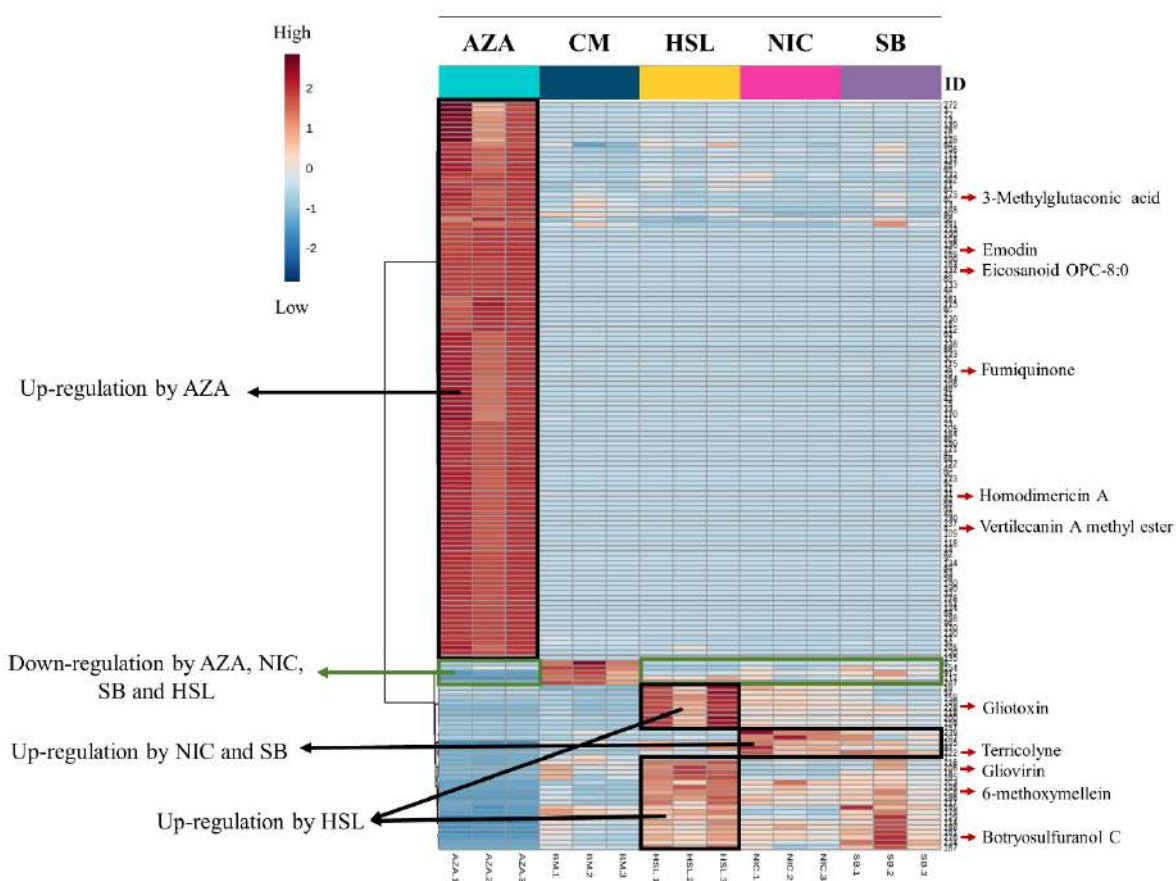


Figure 4.3. Hierarchical clustering of the main features observed in *C. mamane* in the positive mode after the treatment with the epigenetic modifiers

AZA significantly induced 113 metabolites among the top 150, initially lowly produced by *C. mamane*, highlighting the great capacity of this epigenetic modifier to alter the gene expression through the induction of DNA demethylation in the endophytic fungus. An analysis of the differential expression of endophytic fungi under the treatment with 7 different HDACi and DNMTi (including AZA, NIC and

SB) published in 2016 showed that AZA was the epigenetic modifier that induced most of the changes in the secondary metabolite profile of the ascomycete *Dothiora* sp. (Dothioraceae) (González-Menéndez *et al.* 2016), which is consistent with our results. Metabolites annotated as 3-methylglutanonic acid, eicoisanoid, emodin, fumiquinone A, homodimericin A and vertilecanin A methyl ester were also induced under the treatment with AZA as observed in **Figure 4.3**.

Interestingly, the metabolites identified as DKPs gliotoxin, gliovirin and botryosulfuranol C were up-regulated under the treatment with HSL, indicating that the recognition of a bacterial QS molecule by *C. mamane* triggered a signal that induced the production of these type of metabolites. Similarly, the metabolite annotated as isocoumarin 6-methoxymellein was also up-regulated by HSL, first isolated from the fungus *Preussia bipartita* (Sporormiaceae) while different isocoumarin derivatives have been also isolated from Botryosphaeriaceae family members, including *Botryosphaeria mamane*, *B. obtusa* and *B. rhodina* (Reveglia *et al.* 2020). Moreover, the fatty acyl terricolone was up-regulated by NIC and SB but not by AZA or HSL (**Figure 4.3**), suggesting that NIC and SB might trigger similar effects as they both are HDACi.

4.2.4 Differential production of metabolites (Volcano plot analysis)

Regarding the influence of the epigenetic modifiers more in details as shown in the Volcano plot representations (**Figure 4.4**), a total of 113, 46, 39 and 18 metabolites were up-regulated when *C. mamane* culture was treated with AZA (**Figure 4.4a**), HSL (**Figure 4.4b**), NIC (**Figure 4.4c**) and SB (**Figure 4.4d**), respectively. Notably, as observed above in the heat map, AZA was the epigenetic modifier that induced and down-regulated the highest number of metabolites. On the contrary, SB is the one that alters the metabolism the least, inducing and down-regulating the smallest number of metabolites.

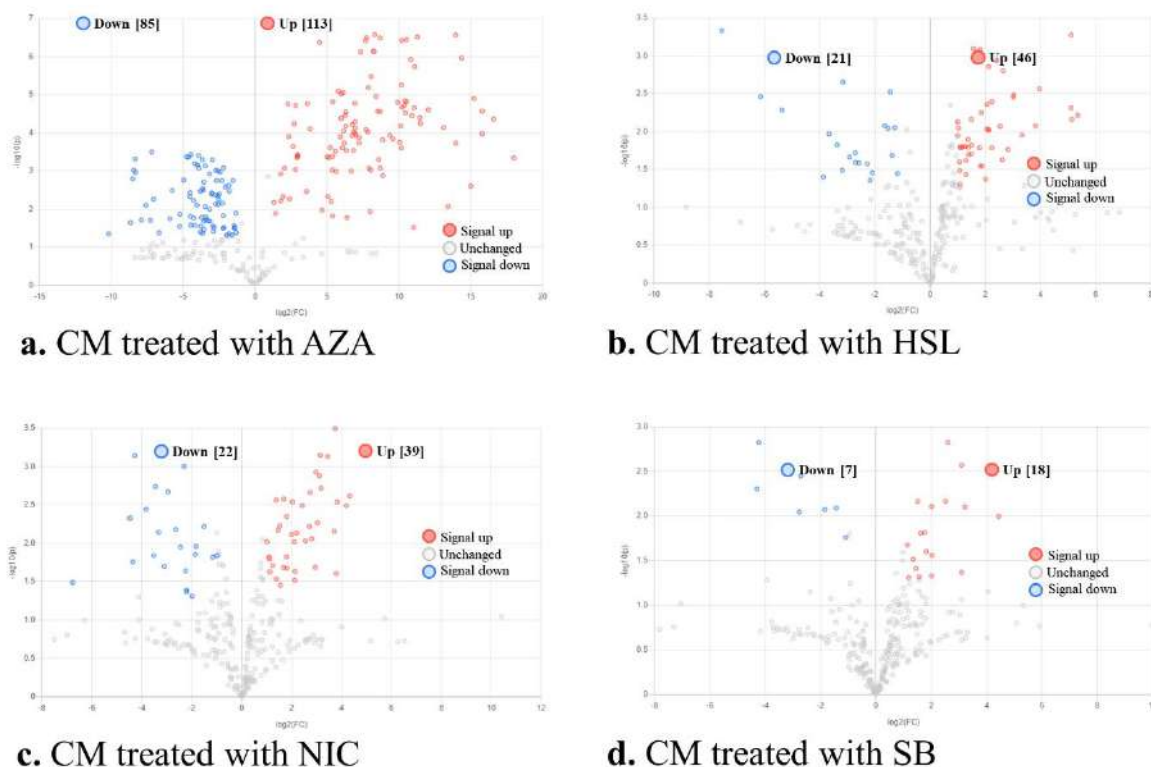


Figure 4.4. Volcano plot representations of *C. mamane* response to the treatment with epigenetic modifiers (a) azacytidine (AZA), (b) homoserin lactone (HSL), (c) nicotinamide (NIC) and (d) sodium butyrate (SB). Each dot represents a feature (m/z at R_t), the red and blue colors indicate a significant change (p -value < 0.05 and fold-change ratio > 2) of the features under treatment in up-regulating (red) or down-regulating (blue) their production in comparison with the untreated culture of CM, while the numbers in brackets indicate the number of significant features observed.

Among the metabolites with the highest significant changes (p -value < 0.05 and fold-change ratio > 2) shown in

Table 4.2, the metabolite annotated as vertilecanin A methyl ester was significantly up-regulated by the addition of AZA but also by NIC. This phenopicolinic acid derivative was previously isolated from the entomopathogenic fungus *Verticillium lecanii* (Soman *et al.* 2001), a species that is currently classified within the genus *Lecanicillium* (Nicoletti & Becchimanzi 2020).

The metabolite annotated as fumiquinone A, previously isolated from *Aspergillus fumigatus* (Hayashi *et al.* 2007) was significantly up-regulated by AZA. Another ubiquinone, fumiquinone B, was isolated from the endophytic fungus *Neopestalotiopsis* sp. (Grigoletto *et al.* 2019). AZA, NIC and HSL significantly up-regulated the metabolite annotated as hydroxyanthraquinone emodin, a compound known to be produced by different fungal species as a pigmented metabolite (Wells *et al.* 1975). Emodin has also been described in different endophytic fungal species including *Aspergillus versicolor* (Hawas *et al.* 2012), *Alternaria* sp., *Epicoccum nigrum* (Vigneshwari *et al.* 2019) and an endophytic strain of *Penicillium citrinum* isolated from a medicinal plant (Luo *et al.* 2019).

The metabolite annotated as homodimericin, significantly up-regulated by AZA and SB, has been described as a complex fungal polyketide isolated from *Trichoderma harzianum*, whose biosynthesis occurs in a similar manner to an epicolactone produced by an endophytic fungal strain of *Epicoccum* sp. ((Mevers *et al.* 2016; Long *et al.* 2018).

Although both NIC and SB inhibit the deacetylation of histones (HDACi), they differently induce the production of compounds in *C. mamane*, probably because each of these HDACi interacts in different manner with different histone deacetylases. As described in the literature, SB inhibits HDAC class I and II, while NIC inhibits HDAC class III (Harrison *et al.* 2019). In agreement with these observations, El-Hawary *et al.*, (2018) observed that when the marine-derived fungus *Penicillium brevicompactum* is treated with NIC and SB, different compounds were induced, obtaining more phenolic metabolites with NIC than with SB (El-Hawary *et al.* 2018).

While the majority of compounds produced by *C. mamane* were up-regulated with AZA treatment, many other compounds were down-regulated. Similar results were reported in the literature. As an example, when the endophytic fungus *Diaporthe* sp. is treated with AZA, Deepika *et al.*, (2020) demonstrated a significant increase in most metabolites, specifically in colchicine production, while a negative regulation in the production of other metabolites in the presence of demethylating agents was observed (Deepika *et al.* 2020).

Table 4.2. Most significant metabolites up-regulated by the treatment with epigenetic modifiers in *C. mamane* culture based on volcano plot analysis. Up-regulation is shown with a significant (*) or not significant (N.S.) fold-change (FC) value. Significant values with *p*-value < 0.05 and fold-change ratio > 2

| ID | Rt (min) | m/z | Adduct | Formula | Δ mass (ppm) | Putative identification | Ontology | <i>C. mamane</i> | | | |
|-----|----------|----------|---------------------|---|--------------|------------------------------------|---------------------------------|----------------------------------|----------------------------------|---------------------------------|----------------------------------|
| | | | | | | | | and AZA Log ₂ (FC) | and NIC Log ₂ (FC) | and SB Log ₂ (FC) | and HSL Log ₂ (FC) |
| 45 | 6.57 | 244.0967 | [M+H] ⁺ | C ₁₄ H ₁₃ N ₃ O ₃ | 0.49 | Vertilecanin A methyl ester | Phenopicolinic acid derivatives | 16.58* | 4.18* | N.S. | 4.41 |
| 60 | 5.30 | 255.0864 | [M+H] ⁺ | C ₁₂ H ₁₄ O ₆ | 0.45 | Fumiquinone A | Ubiquinones | 10.43* | N.S. | N.S. | N.S. |
| 76 | 6.11 | 271.060 | [M+H] ⁺ | C ₁₅ H ₁₀ O ₅ | 0.36 | Emodin | Hydroxyanthraquinones | 13.95* | 3.8* | N.S. | 3.82* |
| 107 | 6.41 | 300.1439 | [M+H] ⁺ | C ₁₄ H ₂₁ N ₃ O ₆ | 0.87 | Unknown | Unknown | 6.62* | 6.54 | N.S. | 5.35* |
| 124 | 9.12 | 320.2221 | [M+H] ⁺ | C ₁₉ H ₂₉ N ₃ O ₃ | 0.24 | Unknown | Unknown | 15.22* | N.S. | 1.18 | 2.52 |
| 148 | 10.81 | 356.3883 | [M+H] ⁺ | C ₂₃ H ₄₉ NO | 1.09 | Unknown | Unknown | 2.26* | N.S. | 2.02* | N.S. |
| 149 | 4.39 | 357.0904 | [M+H] ⁺ | C ₁₅ H ₂₀ N ₂ O ₄ S ₂ | 9.2 | Bisdethiobis (methylthio)gliotoxin | Epipolythiodiketopiperazine | N.S. | 3.16* | 1.4 | 3.97* |
| 156 | 4.13 | 375.0678 | [M+H] ⁺ | C ₁₅ H ₁₄ N ₆ O ₂ S ₂ | 3.84 | Unknown | Unknown | N.S. | 1.18 | 3.22* | N.S. |
| 157 | 5.59 | 375.1009 | [M+H] ⁺ | C ₁₈ H ₁₈ N ₂ O ₅ S | 0.05 | Unknown | Unknown | N.S. | 3.83* | 2.52 | 5.13* |
| 158 | 4.09 | 375.1012 | [M+H] ⁺ | C ₁₈ H ₁₈ N ₂ O ₅ S | 0.79 | Unknown | Unknown | N.S. | 3.75* | 3.1 | 5.11* |
| 159 | 5.73 | 375.1014 | [M+H] ⁺ | C ₁₈ H ₁₈ N ₂ O ₅ S | 1.28 | Unknown | Unknown | N.S. | 3.72* | N.S. | 5.11* |
| 161 | 5.77 | 378.1189 | [M+H] ⁺ | C ₁₈ H ₁₉ NO ₈ | 1.47 | Unknown | Unknown | 15.78* | 3.18 | N.S. | 4.26 |
| 175 | 4.06 | 417.1186 | [2M+H] ⁺ | C ₁₁ H ₁₂ O ₂ S | 0.66 | Unknown | Unknown | 17.99* | N.S. | 1.24 | N.S. |
| 190 | 4.39 | 446.1968 | [M+H] ⁺ | C ₂₀ H ₂₇ N ₇ O ₃ S | 0.19 | Unknown | Unknown | 15.78* | 2.54 | N.S. | N.S. |
| 221 | 9.76 | 491.1675 | [M+H] ⁺ | C ₂₈ H ₂₆ O ₈ | 5.17 | Homodimericin A | Diterpenoids | 6.95* | N.S. | 2.61* | N.S. |
| 227 | 9.88 | 498.3788 | [M+H] ⁺ | C ₂₈ H ₁₅ NO ₆ | 0.23 | Unknown | Unknown | N.S. | N.S. | 2* | N.S. |
| 242 | 5.73 | 530.1049 | [M+H] ⁺ | C ₁₈ H ₂₄ N ⁺ O ₆ PS ₂ | 1.72 | Unknown | Unknown | N.S. | 4.32* | N.S. | N.S. |
| 271 | 9.52 | 681.4417 | [M+H] ⁺ | C ₃₄ H ₄₄ O ₁₃ | 0.39 | Unknown | Unknown | N.S. | N.S. | 3.09* | N.S. |

Focusing on the three novel DKPs previously isolated from *C. mamane* (Barakat *et al.* 2019), botryosulfuranols A, B and C were significantly down-regulated by AZA (**Figure 4.5a,b**), while only botryosulfuranol C showed a significant up-regulation by HSL (**Figure 4.5c**). The HDACi SB had no influence on their production, contrary to the results of a previous study, where the 3 botryosulfuranols were found to be induced when *C. mamane* culture was treated with the HDACi sodium valproate (Triastuti *et al.* 2019). This could have been explained by an interaction of the HDACi with different histone deacetylases, however, sodium valproate and SB belong to the same class of HDACi (Shukla & Tekwani 2020) and they were expected to present the same effect on *C. mamane* metabolism.

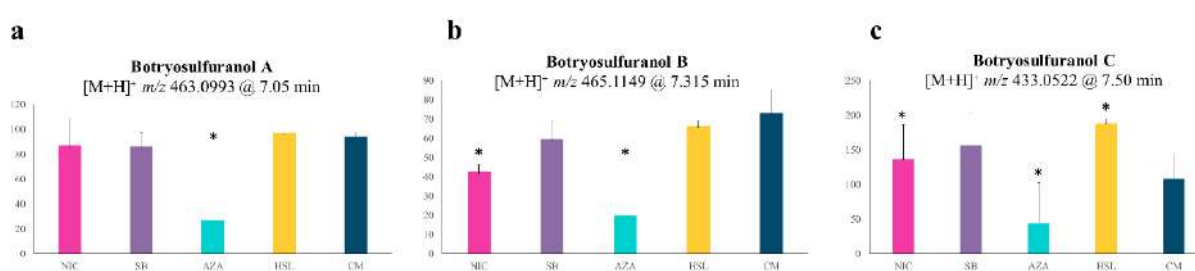


Figure 4.5. Normalized peak area of the TDKPs (a) botryosulfuranol A, (b) botryosulfuranol B and (c) botryosulfuranol C detected under the treatment with the epigenetic modifiers nicotinamide (NIC), sodium butyrate (SB), 5-azacytidine (AZA) and homoserin lactone (HSL) in comparison to the untreated culture of *C. mamane* (CM).

Homoserin lactones are well-known molecules involved in QS in bacterial communities and their role in gram-negative bacteria has already been reported, indicating their modulatory activity in regulatory proteins for gene expression (Fuqua & Greenberg 2002). The roles of DKPs in QS have also been reported during bacterial interactions (Bofinger *et al.* 2017), suggesting a similar role of botryosulfuranols in *C. mamane* when treated with HSL. Moreover, mechanisms of QS are also observed in yeast and filamentous fungi through the production of tyrosol, farnesol (Mondal & Majumdar 2019) and mycotoxins (Venkatesh & Keller 2019). As observed with the up-regulation of botryosulfuranol C by HSL, QS are molecules known to be capable of inducing the production of cyclic DKPs, such as emestrin A and emestrin B, produced by *Aspergillus fumigatus* in presence of HSL. In spite of their lack of *in vitro* activity against bacteria, it was hypothesized that these DKPs could act as QS inhibitors to modulate or attenuate bacterial populations (Rateb *et al.* 2013).

In our study, the metabolite annotated as DKP diphenylalazine B was also significantly up-regulated by HSL. A similar compound, diphenylalazine C, has been isolated from the fungus *Schizophyllum commune* (Chunyu *et al.* 2017), recently described as an endophytic fungus isolated from the medicinal plant *Alchornea glandulosa* (Euphorbiaceae), being a source of many other DKPs (Rabal Biasetto *et al.* 2019). Furthermore, the metabolite annotated as DKP bisdethiobis (methylthio)-gliotoxin was

significantly up-regulated by HSL and NIC. In a previous work, this metabolite was isolated from an endophytic fungal strain of *Aspergillus fumigatus* isolated from a Chinese medicinal plant (Zhang *et al.* 2018).

4.2.5 Molecular network analysis

To better visualize chemical relationships based on acquired MS-MS data, and to focus on derivatives of annotated metabolites, a molecular networking was performed. Our analysis displayed a molecular network that contained 294 nodes and 26 spectral families with 109 connected nodes while the rest of the nodes showed no link to others (self-loop). The size of the nodes represents the total average of the detected peak areas for each compound in all the conditions while the colors depend on their detection under the treatment with each epigenetic modifier (**Figure 4.6**).

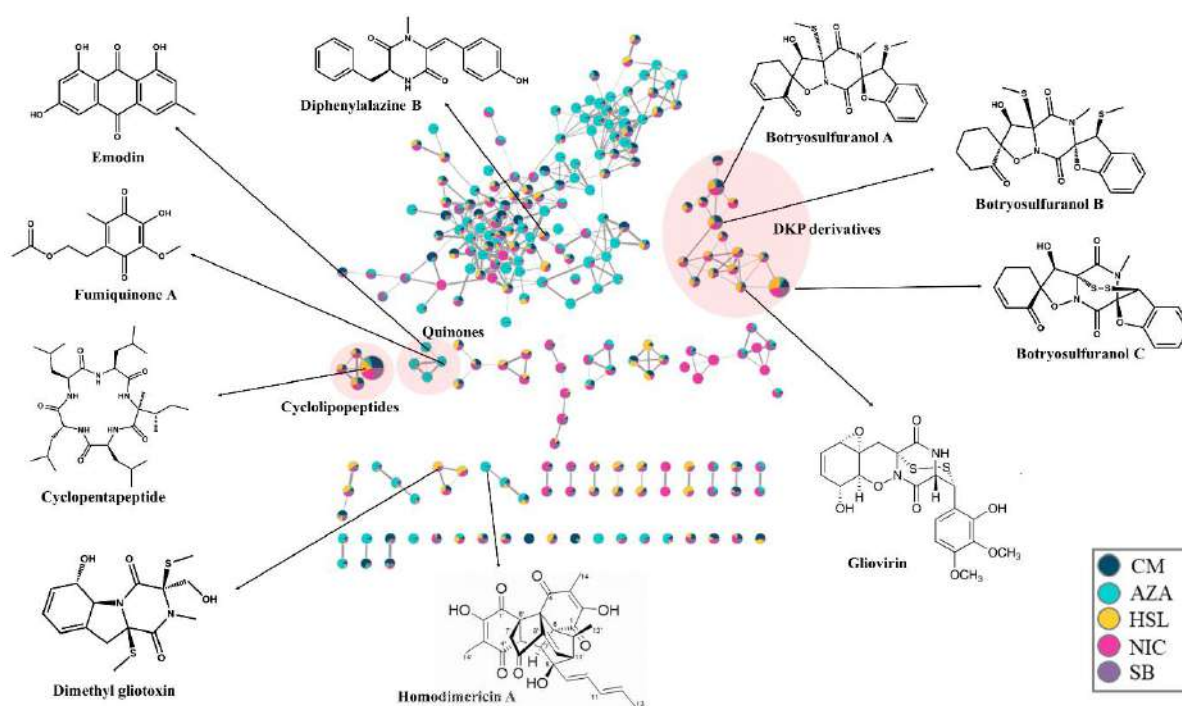


Figure 4.6. Molecular networking corresponding to the extracts of *C. mamane* treated with 4 epigenetic modifiers based on features (m/z at R_t to its normalized peak area) detected in LC-MS chromatograms.

The metabolite annotated as gliovirin, an epidithiodiketopiperazine obtained from the marine-derived fungus *Trichoderma* sp. isolated from a red alga (Yamazaki *et al.* 2015), was up-regulated by SB and appeared within the cluster containing the 3 TDKPs, botryosulfuranols A, B and C. Additionally, in the same cluster of DKP derivatives, a feature with m/z 463.0991 at 6.272 min was found to possess the

same molecular formula than botryosulfuranol A, linked with a cosine value of 0.89 (data not shown), possibly indicating an isomer of this metabolite. The metabolite annotated as bisdethiobis (methylthio)-gliotoxin was found in a different cluster linked to two unknown metabolites (**Figure 4.6**). Moreover, the molecular network highlighted the presence of another cyclopentapeptide linked to the two main cyclopentapeptides observed above in the LC-MS chromatograms (**Figure 4.1**). In the cluster of quinones, we observed two metabolites annotated as fumiquinone A and emodin linked to two other metabolites: one annotated as chrysophanol, previously isolated from marine fungus *Aspergillus* sp. (Qian *et al.* 2011) and a metabolite with m/z 285.0763 at 5.56 min ($C_{16}H_{12}O_5$) that did not show any putative annotation. The metabolite annotated as homodimericin A was also linked to two unidentified metabolites with m/z 505.1832 at 10.04 min ($C_{24}H_{28}N_2O_{10}$) and m/z 519.1989 at 10.01 min ($C_{30}H_{30}O_8$).

The putatively annotated metabolites mentioned above are listed in **Table 4.3**.

4.2.6 Addition of amino acids for DKPs induction

Regarding the experiment with amino acids, our hypothesis consisted of an increase in the production of botryosulfuranols and other DKPs after the addition of the bioprecursors L-phe and L-trp to the culture medium. Some studies have shown that the addition of different amino acids in the culture of fungi led to the induction of different metabolites, including new compounds such as bioactive alkaloids, in, for example, the fungus *Pseudallescheria boydii* (Microascaceae) (Huang *et al.* 2017) and in the marine fungus *Aspergillus* sp. (Qiu *et al.* 2020). However, contrary to what was expected, botryosulfuranol A, B and C showed a significant down-regulation under the addition of L-phe and L-trp (p-value < 0.05 and fold-change ratio >2) in comparison to the untreated culture of *C. mamane* (**Figure 4.7**). Other metabolites, including alkaloids, might have been induced but not detected (data not shown). Different concentrations of other amino acids as well as different culture media and incubation times will be contemplated for future experiments.

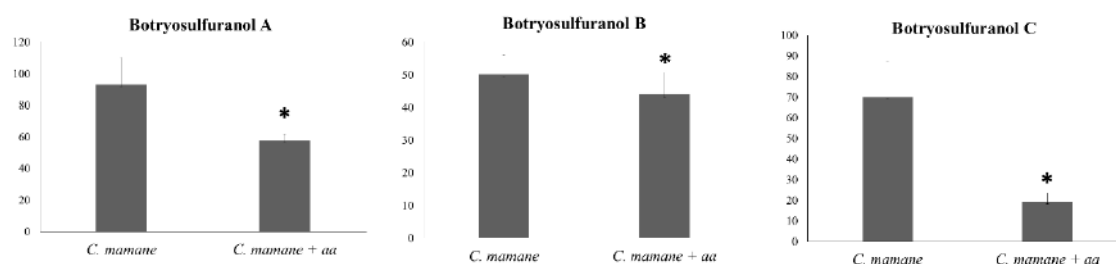


Figure 4.7. Normalized peak area of botryosulfuranols A, B and C detected under the addition of amino acids (*C. mamane* + aa) in comparison to the untreated culture of *C. mamane*.

Table 4.3. List of putative identified compounds detected under the treatment with epigenetic modifiers when culturing *C. mamane*

| ID | Rt (min) | m/z | Adduct | Formula | A mass (ppm) | Putative identification | Ontology |
|-----|----------|----------|--------------------|---|--------------|--|---------------------------------|
| 262 | 9.36 | 566.4279 | [M+H] ⁺ | C ₃₀ H ₅₃ N ₅ O ₅ | 0.67 | Cyclo-(L-Ile-L-Leu-L-Leu-L-Leu-L-Leu)* | Cyclopentapeptides |
| 257 | 9.03 | 552.4126 | [M+H] ⁺ | C ₂₉ H ₅₃ N ₅ O ₅ | 1.18 | Cyclo-(L-Leu-L-Leu-D-Leu-L-Leu-L-Val)* | Cyclopentapeptides |
| 263 | 9.25 | 600.4108 | [M+H] ⁺ | C ₃₃ H ₅₃ N ₅ O ₅ | 1.75 | Cyclo-(L-Phe-L-Leu-L-Leu-L-Leu-L-Ile)* | Cyclopentapeptides |
| 283 | 7.31 | 465.1149 | [M+H] ⁺ | C ₂₁ H ₂₄ N ₂ O ₆ S ₂ | 0.22 | Botryosulfuranol B | Thiodiketopiperazines |
| 215 | 7.04 | 463.0992 | [M+H] ⁺ | C ₂₁ H ₂₂ N ₂ O ₆ S ₂ | 0.14 | Botryosulfuranol A | Thiodiketopiperazines |
| 278 | 7.49 | 433.0521 | [M+H] ⁺ | C ₁₉ H ₁₆ N ₂ O ₆ S ₂ | 0.24 | Botryosulfuranol C | Thiodiketopiperazines |
| 216 | 6.51 | 481.0734 | [M+H] ⁺ | C ₂₀ H ₂₀ N ₂ O ₈ S ₂ | 0.22 | Gliovirin | Epithiodiketopiperazines |
| 201 | 6.27 | 463.0990 | [M+H] ⁺ | C ₂₁ H ₂₂ N ₂ O ₆ S ₂ | 0.24 | Botryosulfuranol A derivative | Thiodiketopiperazines |
| 125 | 6.90 | 323.1393 | [M+H] ⁺ | C ₁₉ H ₁₈ N ₂ O ₃ | 1.08 | Diphenylalazine B;(+)Diphenylalazine B | Diketopiperazines |
| 149 | 4.39 | 357.0904 | [M+H] ⁺ | C ₁₅ H ₂₀ N ₂ O ₄ S ₂ | 9.3 | Bisdethiobis (methylthio)-gliotoxin | Epipolythiodiketopiperazines |
| 45 | 6.57 | 244.0967 | [M+H] ⁺ | C ₁₄ H ₁₃ NO ₃ | 0.49 | Vertilecanin A methyl ester | Phenopicolinic acid derivatives |
| 60 | 5.30 | 255.0864 | [M+H] ⁺ | C ₁₂ H ₁₄ O ₆ | 0.45 | Fumiquinone A | Ubiquinones |
| 76 | 6.11 | 271.0600 | [M+H] ⁺ | C ₁₅ H ₁₀ O ₅ | 0.36 | Emodin | Hydroxyanthraquinones |
| 221 | 9.76 | 491.1675 | [M+H] ⁺ | C ₂₈ H ₂₆ O ₈ | 5.17 | Homodimericin A | Diterpenoids |
| 191 | 7.07 | 447.0678 | [M+H] ⁺ | C ₁₁ H ₂₀ N ₄ O ₁₁ P ₂ | 0.34 | CDP-Ethanolamine | Phosphoethanolamines |
| 7 | 5.49 | 193.0857 | [M+H] ⁺ | C ₁₁ H ₁₂ O ₃ | 0.73 | Terricolyne | Fatty acyls |
| 18 | 6.92 | 209.0809 | [M+H] ⁺ | C ₁₁ H ₁₂ O ₄ | 0.41 | 6-Methoxyxymellein | 2-benzopyrans |
| 173 | 1.76 | 145.0493 | [M+H] ⁺ | C ₆ H ₈ O ₄ | 1.21 | 3-Methylglutaconic acid | Methyl-branched fatty acids |
| 100 | 9.81 | 295.2268 | [M+H] ⁺ | C ₁₈ H ₃₀ O ₃ | 0.23 | 3-Oxo-2-(2-entenyl)cyclopentaneoctanoic acid;OPC-8:0 | Eicosanoids |

*unknown amino acids sequence

4.3 Additional information

The following results have not been included in the draft article and they correspond to the addition of L-Trp and L-Phe into the culture of *C. mamane* in order to evaluate additional results besides the observed for the TDKPs botryosulfuranols A, B and C.

4.3.1 General observations

The general overview of the chromatograms (**Figure 4.8**) shows five main metabolites produced in all conditions: botryosulfuranol A, botryosulfuranol B, botryosulfuranol C and two others putatively identified as cyclopeptides (m/z 566.4279 and m/z 552.4126). In this chromatogram, the peak corresponding to botryosulfuranol C appears to be less intense in the presence of amino acids.

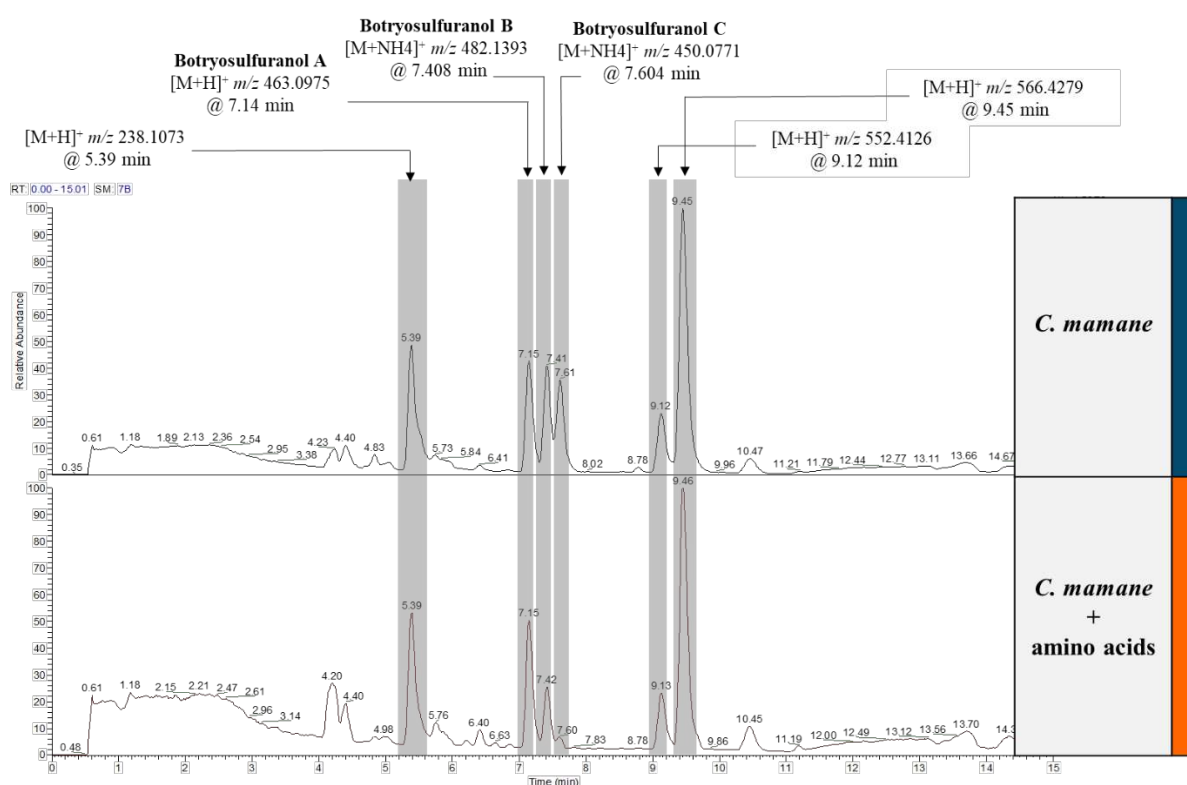


Figure 4.8. UHPLC-(+)ESI-HRMS base peak chromatograms from crude extracts of *C. mamane* cultivated in MEA with the addition of L-phenylalanine and L-tryptophan

A metabolic profile of the 7 extracts obtained by UHPLC-(+)ESI-HRMS afforded 132 features (m/z -Rt pairs), which were considered for the metabolomics and statistical analysis. 20% of these features (23 out of 132) were putatively annotated (**Annexes 4**).

4.3.1 Multivariate classification (PLS-DA)

A supervised PLS-DA analysis was carried out on these data which indicated the different responses of *C. mamane* when cultivated in a medium supplemented with two amino acids (CM + aa) in comparison to the untreated culture of *C. mamane* (CM) (Figure 4.9). Separate clusters appear on the PLS-DA score plot (80.5% of total variability). The component 1 (X-axis) explained 60.9% of the variability, allowing the discrimination of the CM and CM+aa cluster.

The Figure 4.9 presents the top 15 features (Table 4.4) that contributed to the differences between the treatments. Among them, the features m/z 566.4270 at 9.448 min and m/z 574.3937 at 9.125 min were putatively identified as cyclic pentapeptides while similar metabolites were previously isolated from an endophytic fungal strain of *Fusarium decemcellulare* (Li *et al.* 2016). Feature m/z 299.1385 at 4.637 min was putatively identified as a prenylated tryptophan derivative called luteoride A, first isolated from an entomopathogenic fungus *Torrubiella luteorostrata* (Asai *et al.* 2013) while another derivative, luteoride E was isolated from a marine-derived fungus *Aspergillus terreus* (Liu *et al.* 2018). Moreover, feature m/z 176.0704 at 1.852 min was putatively identified as 7-amino-4-methylcoumarin, first isolated from endophytic fungus *Xylaria* sp. (Liu *et al.* 2008).

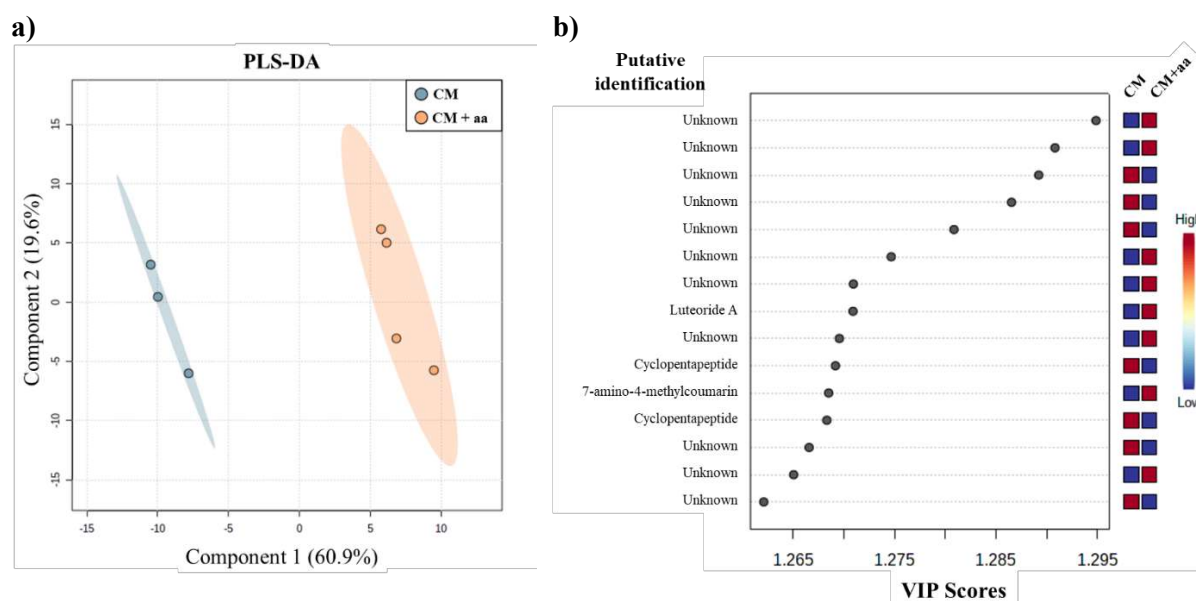


Figure 4.9. a) Partial least squares discriminant analysis (PLS-DA) obtained from LC-(+)ESI-HRMS profiles corresponding to the extracts of *C. mamane* cultivated in MEB (CM) and under the addition of L-phe and L-trp (CM+aa); based on features (m/z at Rt to its normalized peak area) detected in LC-MS chromatograms; (b) Variable importance in projection (VIP) score of the top 15 compounds from the PLS-DA analysis corresponding to each condition

Table 4.4. Top 15 features that significantly contribute to the difference in the PL-S-DA analysis from LC-HRMS/MS data under the addition of amino acids in *C. mannine* culture

| ID | Rt (min) | m/z | Adduct | Formula | Δ mass (ppm) | Putative identification | Ontology |
|-----|----------|----------|-------------------------------------|---|---------------------|--|--|
| 118 | 4.80 | 285.1228 | [M+H] ⁺ | C ₁₆ H ₁₆ N ₂ O ₃ | 1.99 | Unknown | Unknown |
| 127 | 4.17 | 301.1176 | [M+H] ⁺ | C ₁₆ H ₁₆ N ₂ O ₄ | 2.26 | Unknown | Unknown |
| 44 | 5.06 | 501.0752 | [M+Na] ⁺ | C ₁₂ H ₂₄ N ₄ O ₁₂ P ₂ | 1.22 | Unknown | Unknown |
| 62 | 7.60 | 903.0509 | [M+H] ⁺ | C ₃₈ H ₅₆ N ₆ O ₁₃ S ₄ | 0.49 | Unknown | Unknown |
| 75 | 2.57 | 220.1180 | [M+H] ⁺ | C ₉ H ₁₇ NO ₅ | 0.23 | Unknown | Unknown |
| 100 | 10.14 | 253.1336 | [M+H] ⁺ | C ₁₆ H ₁₆ N ₂ O | 0.23 | Unknown | Unknown |
| 117 | 4.69 | 285.1228 | [M+H] ⁺ | C ₁₆ H ₁₆ N ₂ O ₃ | 1.99 | Unknown | Unknown |
| 125 | 4.63 | 299.1385 | [M+H] ⁺ | C ₁₇ H ₁₈ N ₂ O ₃ | 1.73 | Luteinide A | Indolyl carboxylic acids and derivatives |
| 18 | 1.85 | 176.0704 | [M+H] ⁺ | C ₁₀ H ₆ NO ₂ | 1.16 | 7-Amino-4-methylcoumarin | Coumarins and derivatives |
| 35 | 7.14 | 480.0511 | [M+H] ⁺ | C ₂₂ H ₁₃ N ₃ O ₈ S | 3.09 | Unknown | Unknown |
| 51 | 9.44 | 566.4270 | [M+H] ⁺ | C ₃₀ H ₅₅ N ₅ O ₅ | 1.05 | Cyclo-(L-Ile-L-Leu-L-Leu-L-Leu-L-Leu)* | Oligopeptides |
| 56 | 9.12 | 574.3938 | [M+Na] ⁺ | C ₂₉ H ₅₃ N ₅ O ₅ | 0.15 | Cyclo-(L-Leu-L-Leu-D-Leu-L-Leu-L-Val)* | Oligopeptides |
| 110 | 6.63 | 269.0918 | [M+H-H ₂ O] ⁺ | C ₁₅ H ₁₄ N ₂ O ₄ | 0.99 | Unknown | Unknown |
| 116 | 5.83 | 284.1180 | [M+H] ⁺ | C ₁₉ H ₁₃ N ₃ | 0.78 | Unknown | Unknown |

*amino acids sequence not known

4.3.1 Differential production of metabolites (Volcano plot analysis)

Out of the 132 features, 81 showed significant changes under the treatment with amino acids (p -value < 0.05) using the analysis of variance (one way ANOVA) and Fisher test (F-test). Regarding the influence of the amino acids more in details as shown in the Volcano plot representations (**Figure 4.10**), a total of 41 metabolites were down-regulated and 23 were up-regulated.

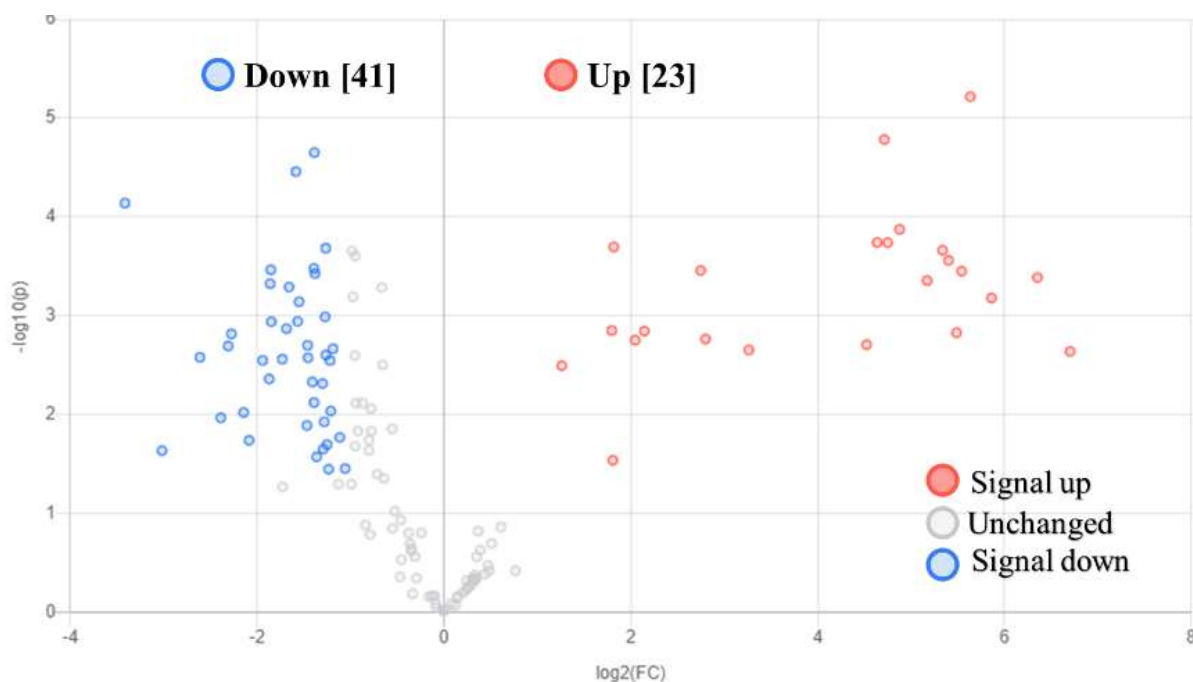


Figure 4.10. Volcano plot representations of *C. mamane* response to the addition of amino acids L-phenylalanine and L-tryptophan. Each dot represents a feature (m/z at Rt), the red and blue colors indicate a significant change (p -value < 0.05 and fold-change ratio > 2) of the features under treatment in up-regulating (red) or down-regulating (blue) their production in comparison with the untreated culture of CM, while the numbers in brackets indicate the number of significant features observed.

Among the metabolites with the highest significant changes (**Table 4.5**) (p -value < 0.05 and fold-change ratio > 2), considering their up-regulation under the addition of the amino acids based on the Volcano plot analysis, feature m/z 178.0863 at 4.315 min, putatively identified as (-)-3a-hydroxyfuroindoline, was first isolated from an endophytic fungal strain of *Fusarium incarnatum* (Li *et al.* 2008) while feature m/z 210.0759 at 3.837 min, putatively identified as penidilamine, was isolated from *Penicillium* sp. (Kimura *et al.* 2000). Feature m/z 241.0966 at 6.175 was also significantly up-regulated under the presence of the amino acids and it was putatively identified as 1,2,3,11b-tetrahydroquinolactacide, which was first isolated from an endophytic fungal strain of *Penicillium citrinum* (El-Neketi *et al.* 2013).

Table 4.5. Most important compounds up-regulated by the treatment with epigenetic modifiers in *C. mamane* culture. Up-regulation is shown with a significant (*) or not significant (N.S.) fold-change (FC) value. Significant values with *p*-value < 0.05 and fold-change ratio > 2

| ID | Rt (min) | <i>m/z</i> | Adduct | Formula | Δ mass (ppm) | Putative identification | Ontology | Log2 (FC) |
|-----|----------|------------|---------------------|---|---------------------|------------------------------------|-----------------------------------|-----------|
| 2 | 6.25 | 313.0812 | [M+H] ⁺ | C ₁₆ H ₂₅ N ₂ O ₅ | 2.22 | Unknown | Unknown | 5.86* |
| 14 | 1.38 | 174.0546 | [M+H] ⁺ | C ₁₀ H ₇ NO ₂ | 2.03 | Unknown | Unknown | 2.8* |
| 17 | 3.07 | 176.0704 | [M+H] ⁺ | C ₁₀ H ₆ N ₂ O ₂ | 1.16 | Unknown | Unknown | 3.26* |
| 20 | 4.31 | 178.0863 | [M+H] ⁺ | C ₁₀ H ₁₁ NO ₂ | 0.25 | (-)-3a-Hydroxyfuroindoline | Indolines | 4.52* |
| 28 | 6.75 | 440.2170 | [M+H] ⁺ | C ₂₄ H ₂₉ N ₃ O ₅ | 2.26 | Unknown | Unknown | 5.17* |
| 68 | 3.83 | 210.0759 | [M+H] ⁺ | C ₁₀ H ₁₁ NO ₄ | 0.87 | Penidilamine(+)-Penidilamine | Alpha amino acids and derivatives | 1.26* |
| 76 | 5.05 | 221.0706 | [M+H] ⁺ | C ₁₄ H ₈ N ₂ O | 1.53 | Unknown | Unknown | 6.35* |
| 79 | 5.34 | 227.0808 | [M+H] ⁺ | C ₁₃ H ₁₀ N ₂ O ₂ | 3.1 | Unknown | Unknown | 2.14* |
| 89 | 6.17 | 241.0966 | [M+H] ⁺ | C ₁₄ H ₁₂ N ₂ O ₂ | 2.29 | 1,2,3,11b-tetrahydroquinolactamide | Hydroquinolones | 5.54* |
| 98 | 9.64 | 253.1334 | [M+H] ⁺ | C ₁₆ H ₁₆ N ₂ O | 0.55 | Unknown | Unknown | 5.48* |
| 105 | 5.63 | 259.1227 | [M+H] ⁺ | C ₁₈ H ₁₄ N ₂ | 1.06 | Unknown | Unknown | 1.8* |
| 124 | 8.38 | 297.1225 | [M+H] ⁺ | C ₁₇ H ₁₆ N ₂ O ₃ | 2.92 | Unknown | Unknown | 1.79* |
| 128 | 4.73 | 301.1185 | [M+Na] ⁺ | C ₁₁ H ₂₂ N ₂ O ₅ S | 2.48 | Unknown | Unknown | 2.74* |
| 130 | 5.20 | 307.1286 | [M+H] ⁺ | C ₁₅ H ₁₈ N ₂ O ₅ | 0.8 | Unknown | Unknown | 6.7* |

4.3.1 Molecular network analysis

To better visualize chemical relationships based on acquired MS-MS data, and to focus on derivatives of annotated metabolites, a molecular networking was performed. Our analysis displayed a molecular network that contained 132 nodes and 10 spectral families with 86 connected nodes while the rest of the nodes showed no link to others (self-loop). The size of the nodes represents the total average of the detected peak areas for each compound in all the conditions while the colors depend on their detection under the treatment with each epigenetic modifier (**Figure 4.11**).

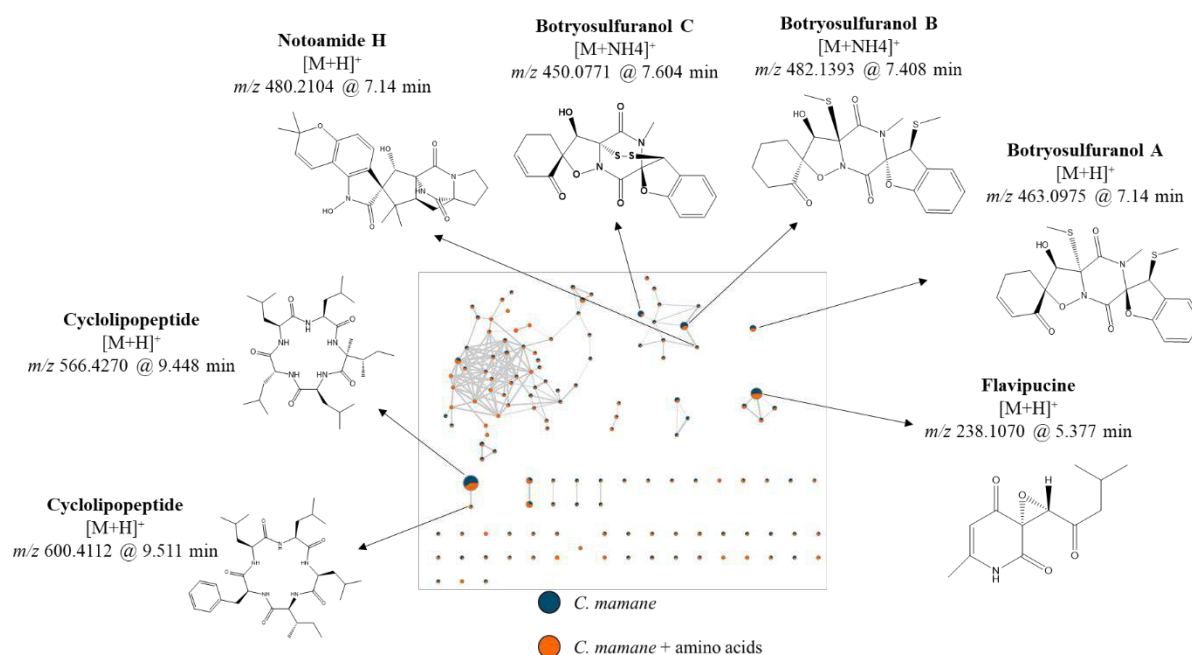


Figure 4.11. Molecular networking corresponding to the extracts of *C. mamane* treated with amino acids based on features (m/z at R_t to its normalized peak area) detected in LC-MS chromatograms.

Focusing on the cluster containing the thiodiketopiperazines botryosulfuranols B and C, feature m/z 480.21042 at 7.14 min was putatively identified as a diketopiperazine alkaloid analogue, notoamide H, isolated from the marine-derived endophytic fungus *Aspergillus* sp. (Yang *et al.* 2021) linked to the node corresponding to botryosulfuranol B with a cosine value of 0.97. Moreover, 4 other features linked in the same cluster were putatively identified as flavipucine (or isomers), a compound isolated from an endophytic fungal strain of *Phoma* sp. (Loesgen *et al.* 2011) (**Figure 4.11**). Feature m/z 600.4112 at 9.51 min, putatively identified as a cyclic pentapeptide previously isolated from a non-identified endophytic fungal strain (Li *et al.* 2004) was found to be linked to cyclic pentapeptide (m/z 566.4270 at 9.448 min). The list of putatively identified compounds for this experiment is detailed in **Table 4.6**.

Table 4.6. List of putative identified compounds detected under the addition of amino acids in the culture of *C. mannii*

| ID | Rt (min) | m/z | Adduct | Formula | Δ mass (ppm) | Putative identification | Ontology |
|-----|----------|----------|-----------------------------------|--|---------------------|--|--|
| 60 | 9.51 | 600.4112 | [M+H] ⁺ | C ₃₃ H ₅₃ N ₅ O ₅ | 1.25 | Cyclo-(L-Phe-L-Leu1-L-Leu2-L-Leu3-L-Ile)* | Cyclopentapeptides |
| 51 | 9.44 | 566.4270 | [M+H] ⁺ | C ₃₀ H ₅₅ N ₅ O ₅ | 1.05 | Cyclo-(L-Ile-L-Leu-L-Leu-L-Leu-L-Leu-L-Leu)* | Cyclopentapeptides |
| 56 | 9.12 | 574.3938 | [M+Na] ⁺ | C ₂₉ H ₅₃ N ₅ O ₅ | 0.15 | Cyclo-(L-Leu-L-Leu-D-Leu-L-Leu-L-Leu-L-Val)* | Cyclopentapeptides |
| 103 | 5.38 | 238.1071 | [M+H] ⁺ | C ₁₂ H ₁₅ N ₄ O ₄ | 1.06 | (±)-Flavipucine** | Tetrahydropyridines |
| 109 | 5.75 | 238.1069 | [M+H] ⁺ | C ₁₂ H ₁₅ N ₄ O ₄ | 1.94 | (±)-Flavipucine** | Tetrahydropyridines |
| 86 | 6.41 | 238.1069 | [M+H] ⁺ | C ₁₂ H ₁₅ N ₄ O ₄ | 1.9 | (±)-Flavipucine** | Tetrahydropyridines |
| 87 | 5.96 | 238.1071 | [M+H] ⁺ | C ₁₂ H ₁₅ N ₄ O ₄ | 1.31 | (±)-Flavipucine** | Tetrahydropyridines |
| 36 | 7.14 | 463.0975 | [M+H] ⁺ | C ₂₁ H ₂₂ N ₂ O ₆ S ₂ | 3.61 | Botrysulfuranol A | Thiodiketopiperazines |
| 39 | 7.41 | 482.1393 | [M+NH ₄] ⁺ | C ₂₁ H ₂₄ N ₂ O ₆ S ₂ | 4.27 | Botrysulfuranol B | Thiodiketopiperazines |
| 30 | 7.60 | 450.0771 | [M+NH ₄] ⁺ | C ₁₉ H ₁₆ N ₂ O ₆ S ₂ | 3.8 | Botrysulfuranol C | Thiodiketopiperazines |
| 37 | 7.14 | 480.2104 | [M+H] ⁺ | C ₂₆ H ₂₉ N ₃ O ₆ | 5.18 | Notoamide H(-)-Notoamide H | Diketopiperazines |
| 125 | 4.64 | 299.1385 | [M+H] ⁺ | C ₁₇ H ₁₈ N ₂ O ₃ | 1.73 | Luteoride A | Indolyl carboxylic acids and derivatives |
| 68 | 3.84 | 210.0759 | [M+H] ⁺ | C ₁₀ H ₁₁ N ₄ O ₄ | 0.87 | Pendilamine(+)-Pendilamine | Alpha amino acids and derivatives |
| 18 | 1.85 | 176.0704 | [M+H] ⁺ | C ₁₀ H ₉ N ₂ O ₂ | 1.16 | 7-Amino-4-methylcoumarin | Coumarins and derivatives |
| 20 | 4.32 | 178.0863 | [M+H] ⁺ | C ₁₀ H ₁₁ N ₂ O ₂ | 0.25 | (-)-3a-Hydroxyfuroindoline | Indolines |
| 89 | 6.17 | 241.0966 | [M+H] ⁺ | C ₁₄ H ₁₂ N ₂ O ₂ | 2.29 | 1,2,3,11b-tetrahydroquinolactamide | Hydroquinolones |

*Amino acid sequence not known

*Isomers of this metabolite could also correspond to this putatively annotation

4.3.1 Discussions

According to the literature, amino acid – directed strategy has shown to promote nitrogen-containing compounds, such as alkaloid production in *Dichotomomyces cejpai* when the culture medium was supplemented with L-tryptophan and L-phenylalanine (2 g/L), obtaining new aliphatic amides, polyketides and (Chen *et al.* 2017; Wu *et al.* 2018) or the 22 diverse alkaloids, including 14 new compounds of which 12 are fumiquinazoline analogues produced by the fungus *Scedosporium apiospermum* when the culture medium was supplemented with L-tryptophan, L-phenylalanine, L-threonine and D-L-methionine (2 g/L) (Huang *et al.* 2017).

Surprisingly, this experiment did not lead to an increase in the production of the botryosulfuranols, nor to the detection and identification of new diketopiperazines or more generally other alkaloids.

One possible explanation for our unexpected results is the low rate in compound annotation, due to a lack of data in the databases used, or a non-detection due to a low level of production, or their absence in the molecular network because of a lack of fragmentation due the methodology used.

Another explanation is the probable elevated and toxic intracellular levels of specific amino acids previously observed in prokaryotic and eukaryotic cells. For instance, *Saccharomyces cerevisiae* is capable to synthesize all amino acids de novo or to obtain them from the environment using plasma membrane transporters, however, the addition of high levels of amino acids (0.5-5 mM) in culture media induce growth inhibition and growth defects (Ruiz *et al.* 2020).

4.4 Conclusions

Our results show that treatment with HDACi and DNMTi effectively activated biosynthetic pathway due to the induction of lowly produced metabolites when *C. mamane* was grown without any treatment. This indicates that methylation and deacetylation are mechanisms that regulates expression in the endophytic fungus, specifically the up-regulation obtained with the treatment with the DNMTi AZA. Meanwhile, deacetylation or methylation of histones were necessary for those compounds that were down-regulated in presence of the epigenetic modifiers. The induction different classes of DKPs by the treatment with the QS molecule HSL represents a promising result for future experiments involving a different induction approach such as the co-culture of this endophytic fungus with bacteria. This also suggests a possible role of DKPs as QS molecules in *C. mamane* as this potential role is suggested for other DKPs in other fungi and bacteria. Possible analogues or derivatives of putatively identified metabolites were indeed uncovered thanks to the molecular networking which can be improved by a better adapted LC-MS methodology for a larger diversity of metabolites, including the DKPs.

This work consisted in a preliminary step to modulate the culture of *C. mamane* in order to find better conditions to induce the production of botryosulfuranols A, B and C and new metabolites. In view of these results, further experiments will consist in performing large-scale cultures of *C. mamane* with AZA, in order to isolate and to structurally characterize compounds induced by this epigenetic modifier.

CHAPTER 5 - ADDITION OF HEAT-KILLED PARASITES AND CO-CULTURE WITH BACTERIA

5.1 Introduction

Fungal endophytes represent an important reservoir of metabolites presenting antibacterial (Deshmukh *et al.* 2014), antifungal (Deshmukh *et al.* 2018) and anti-parasitic activity (Hzounda Fokou *et al.* 2021), offering a great opportunity to search for novel compounds with bioactivity against diverse microorganisms. Neglected tropical diseases, including leishmaniasis caused by *Leishmania* spp., collectively affect approximately 2 billion people located mainly in tropical and subtropical regions (Engels & Zhou 2020). Moreover, multi-drug resistance in bacteria is a global health and development threat, specifically the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) which are the leading cause of nosocomial infections throughout the world and one of the greatest challenges in clinical practice (Santajit & Indrawattana 2016). Currently, the absence of effective vaccines and the development of resistance to the current drugs make these infections a major problem in endemic or developing countries and highlight the need for novel molecules acting on new targets.

Besides all the new bioactive compounds isolated from endophytes under standard culture conditions, new methodologies have been applied for the induction of novel metabolites such as co-culturing fungi with other fungi or bacteria, which has shown to naturally enhance the production of cryptic compounds that were not detected in axenic culture or that were lowly produced (Kamdem *et al.* 2018). This increased production might be the result of competition for nutrients or antagonism in their development between microorganisms sharing the same environment (Deshmukh *et al.* 2018).

A study on the fungal diversity associated to medicinal plants conducted in our laboratory led to the isolation of more than 400 endophytic fungal strains from twenty South American plants used in traditional medicine for their anti-parasitic activities (unpublished data). Among these strains, the ethyl acetate extract of an endophytic fungal strain of *Cophinforma mamane* (Botryosphaeriaceae), isolated from the leaves of the medicinal plant *Bixa orellana* (Bixaceae) collected in Peruvian amazon, exhibited antileishmanial activity against the axenic amastigotes of *Leishmania infantum* (Triastuti *et al.* 2016). Three thiodiketopiperazines (TDKPs), botryosulfuranols A, B and C presenting cytotoxic activity against four cancer lines were isolated from *C. mamane* (Barakat *et al.* 2019), along with a fourth TDKP, botryosulfuranol D (unpublished data). All the TDKPs were tested *in vitro* against axenic amastigotes of *Leishmania infantum*. Botryosulfuranol D exhibited the strongest activity with an IC₅₀ value of 0.03 μM and a selective index (SI) of 190 followed by botryosulfuranol C (IC₅₀ = 0.44 μM); botryosulfuranol A (IC₅₀ = 0.69 μM) and botryosulfuranol B (IC₅₀ = 3.87 μM) (unpublished data).

As *C. mamane* produces antileishmanial metabolites, we hypothesized that a co-culture between these two microorganisms could enhance the production of these bioactive compounds. However, *Leishmania* spp. and filamentous micromycetes need very different culture media and conditions to grow, therefore a way to allow biochemical interactions, involving the recognition of cell surface components with no biological interactions is using heat-killed cells (thermal inactivation) (Liang *et al.* 2019). In this case, we cultivated *C. mamane* in presence of heat-killed *Leishmania* parasites. To the best of our knowledge, the use of heat-killed inducers to reveal cryptic pathways has been described in the literature only for bacteria and fungi (Fourati-Ben Fguira *et al.* 2008; Liang *et al.* 2019, 2020) but not for parasites.

Furthermore, in a previous study performed in our team, *C. mamane* was confronted with *Fusarium solani* allowing the detection of 5 *de novo* compounds produced only under co-culture but not detected in mono-culture (Triastuti *et al.* 2021). This result highlighted that *C. mamane* is sensitive to the presence of another microorganism in its own culture medium, and can modulate its metabolome in response to this presence. Additionally, the experiment using epigenetic modifiers (see **Chapter 4**) has shown that the production of botryosulfuranol C was up-regulated when *C. mamane* was cultivated in presence of N-butyryl-DL-homoserine lactone (HSL), a main *quorum*-sensing molecule in gram-negative bacteria.

In the present study, two strategies to induce the production of potential antimicrobial metabolites were followed: the co-culture of *C. mamane* with three pathogenic bacterial strains (two gram-negative bacteria, *Escherichia coli* ATCC® 25922™ and *Pseudomonas aeruginosa* ATCC® 27853™ and a gram-positive bacterium, *Staphylococcus aureus* ATCC® 25923™) and the culture of *C. mamane* with thermally inactivated parasites of *Leishmania infantum* as a heat-killed inducer. The global chemical response of *C. mamane* was evaluated, with a focus on the production of TDKPs.

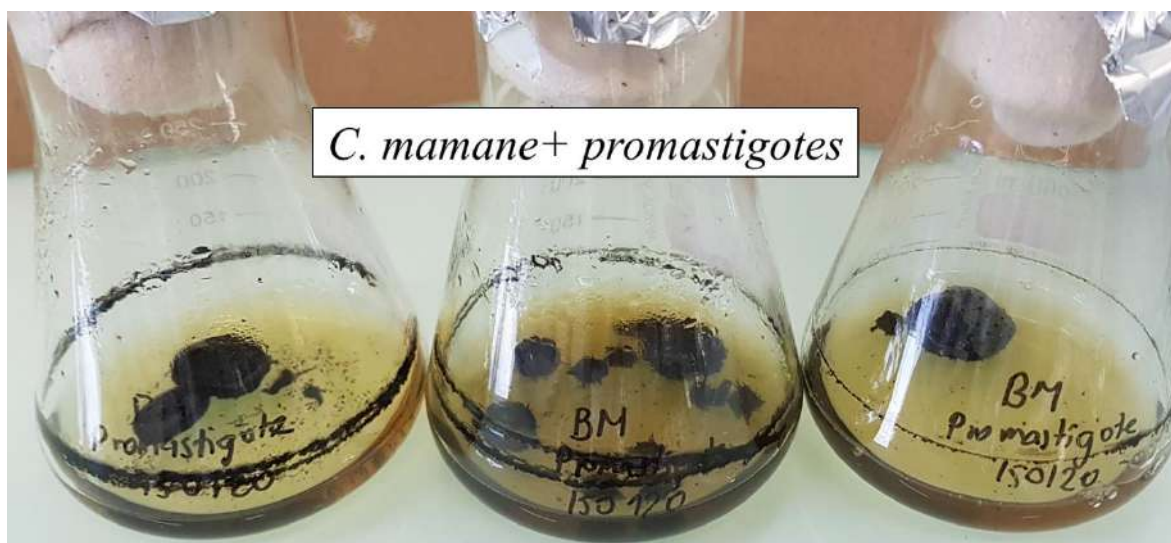
5.2 Results

5.2.1 Addition of heat-killed leishmanial parasites

Preliminary results

In a preliminary analysis, in order to compare the global chemical profile alteration and to observe if the production of the TDKPs responded differently to different parasitic forms, two developmental stages of *L. infantum* were used, promastigotes and amastigotes (**Figure 5.1**), at an unknown concentration of parasites/mL of culture medium. The intracellular *Leishmania* amastigote replicates within macrophages of the host while the extracellular promastigote develops within the insect vector.

a. Addition of heat-killed promastigotes of *L. infantum* into *C. mamane* culture



b. Addition of heat-killed amastigotes of *L. infantum* into *C. mamane* culture

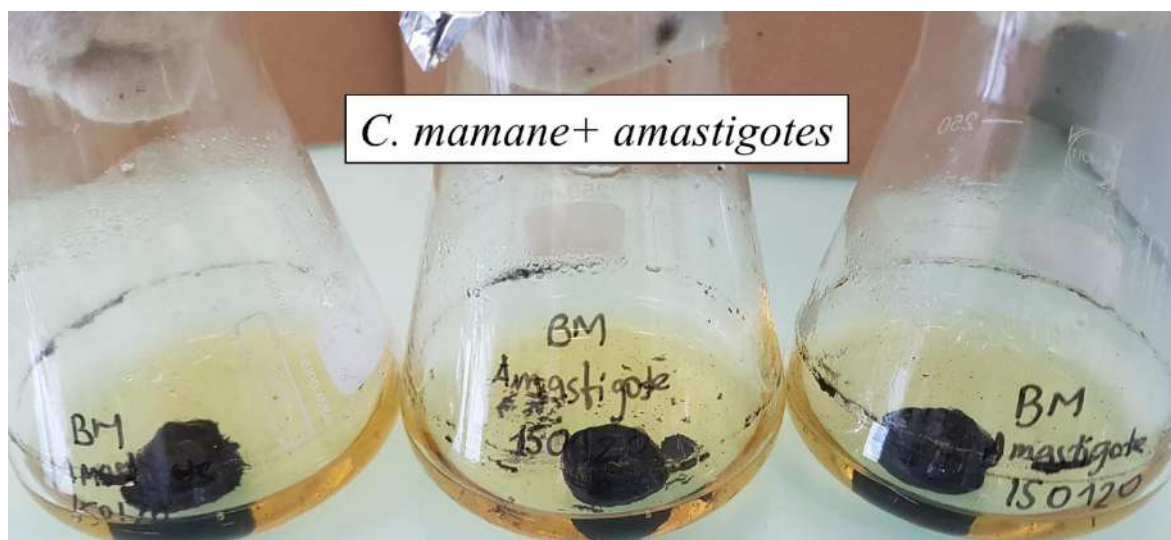


Figure 5.1. Culture of *C. mamane* in presence of heat-killed promastigotes and amastigotes of *L. infantum*

UHPLC-(+)ESI-HRMS chromatograms of extracts from the 3 groups (control, *C. mamane* + promastigotes and *C. mamane* + amastigotes) were compared. At a first look, we can observe that untreated *C. mamane* and *C. mamane* + amastigotes present a qualitatively quite similar global chemical profile, with 5 major peaks corresponding to the TDKPs, botryosulfuranols A, B and C and to two cyclopentapeptides (m/z 552.4164 at 8.69 min and m/z 566.4324 at 9.35 min) previously annotated. On the contrary, the extract of *C. mamane* cultured with promastigotes of *Leishmania infantum* present a different chemical profile, with only 2 major peaks corresponding to the cyclopentapeptides while botryosulfuranols are almost undetectable in the chromatogram under this culture condition (**Figure 5.2**).

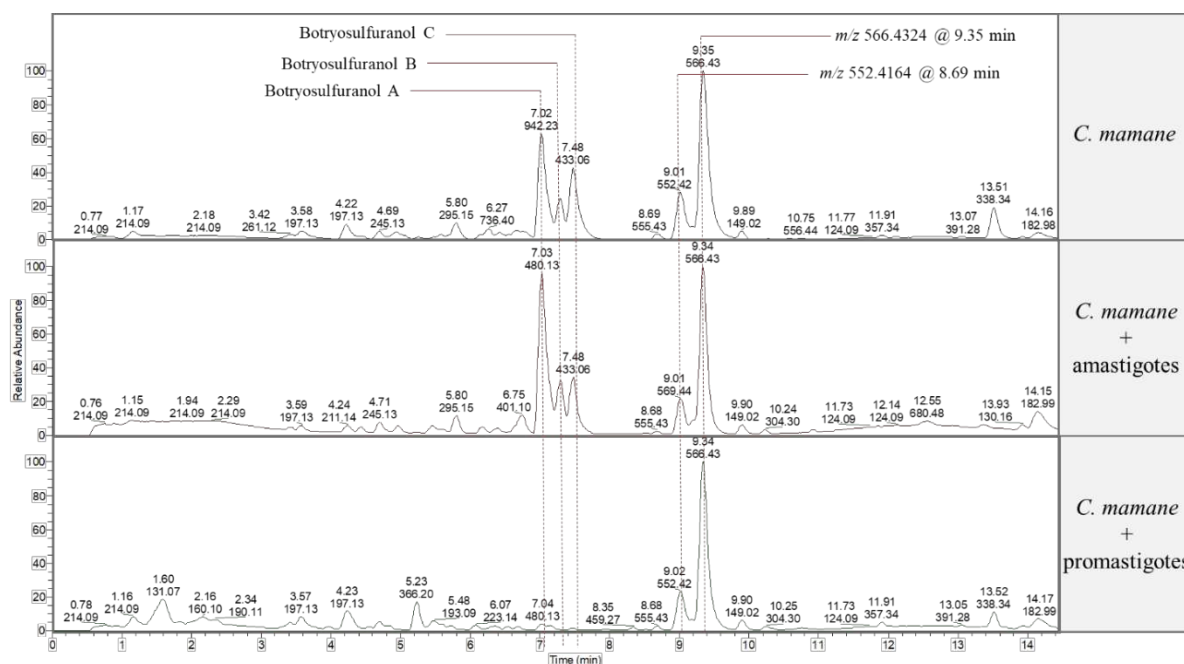


Figure 5.2. UHPLC-(+)ESI-HRMS base peak chromatograms of crude extracts of *C. mamane* treated with heat-killed promastigotes and amastigotes of *Leishmania infantum* in comparison to the control of *C. mamane*

Therefore, to repeat the experiment with more replicates and known concentrations of heat-killed parasites to be able to do statistical analyses, we decided to work only with amastigotes of *Leishmania infantum*. We also expected that the higher the concentration of parasites, the greater the induction of TDKPs will be.

General observations: Culture of C. mamane with known concentrations of heat-killed amastigotes of Leishmania infantum

Three different concentrations of *L. infantum* amastigotes were evaluated: C1=5.2 x 10⁵, C2=1.06 x 10⁶ and C4=2.12 x 10⁶ parasites/mL (C1 < C2 < C4) to observe additional alterations of the metabolome. The general overview of the chromatograms is presented in **Figure 5.3**, slightly different from the previous chromatogram (**Figure 5.2**) probably due to the different concentrations used. At a first look, we can observe peaks corresponding to the TDKPs, botryosulfuranols A and B in all conditions, while botryosulfuranol C seems to be less present under the presence of heat-killed parasites. The feature *m/z* 255.1318 at 5.192 min (not detected in any control or blank sample) appears to be induced under the presence of heat-killed parasites in comparison to the untreated culture of *C. mamane* (**Figure 5.3**).

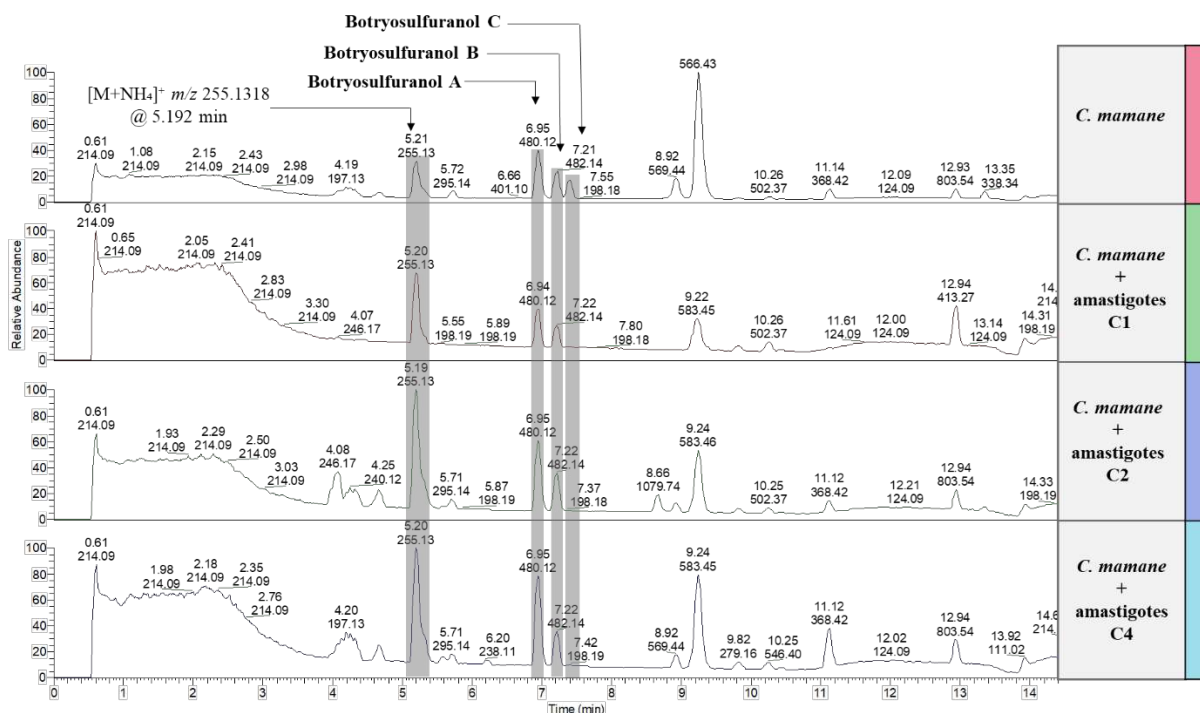


Figure 5.3. UHPLC-(+)ESI-HRMS base peak chromatograms from crude extracts of *C. mamane* treated with heat-killed amastigotes of *Leishmania infantum* at 3 different concentrations: C1, C2 and C4

UHPLC-(+)ESI-HRMS profiles of all the 12 crude extracts from this study afforded 93 features (m/z-RT pairs), which were considered for the metabolomics and statistical analysis. Thirty-three percent of these features (27 out of 93) were putatively annotated, corresponding to diketopiperazines (DKPs), cyclic peptides, tetrahydropyridines, eicosanoids etc (**Annexes 5**).

Multivariate classification (PLS-DA)

A supervised PLS-DA analysis was carried out on these data which indicated the response of *C. mamane* to the different concentrations of heat-killed amastigotes of *L. infantum* (**Figure 5.4**). Separate clusters appear on the PLS-DA score plot (37.3 % of total variability). The component 1 (X-axis) explained 18.5 % of the variability, allowing a slight the discrimination of the different clusters, mainly between the untreated *C. mamane* (CM) culture, CM+C1 - CM+C2 culture and CM+C4 culture. Component 2 (Y-axis) explained 18.8% of the variability with overlapping clusters, reflecting the few changes of the general metabolome of *C. mamane* when cultured in presence of heat-killed parasites.

The **Figure 5.4** also presents the top 15 compounds that contribute to the differences between these clusters while some of them were putatively identified. For instance, TDKPs botryosulfuranols A and C, which seems to have a lower detection under the addition of the parasites compared to the control. On the contrary, (\pm)flavipucine or its isomers ((\pm)isoflavipucine, fruit rot toxin A, sapinopyridion),

compounds isolated from an endophytic fungal strain of *Phoma* sp. (Loesgen *et al.* 2011), appear to have a higher detection under the addition of the parasites (CM+C4), along with other 5 other unidentified features (**Figure 5.4**).

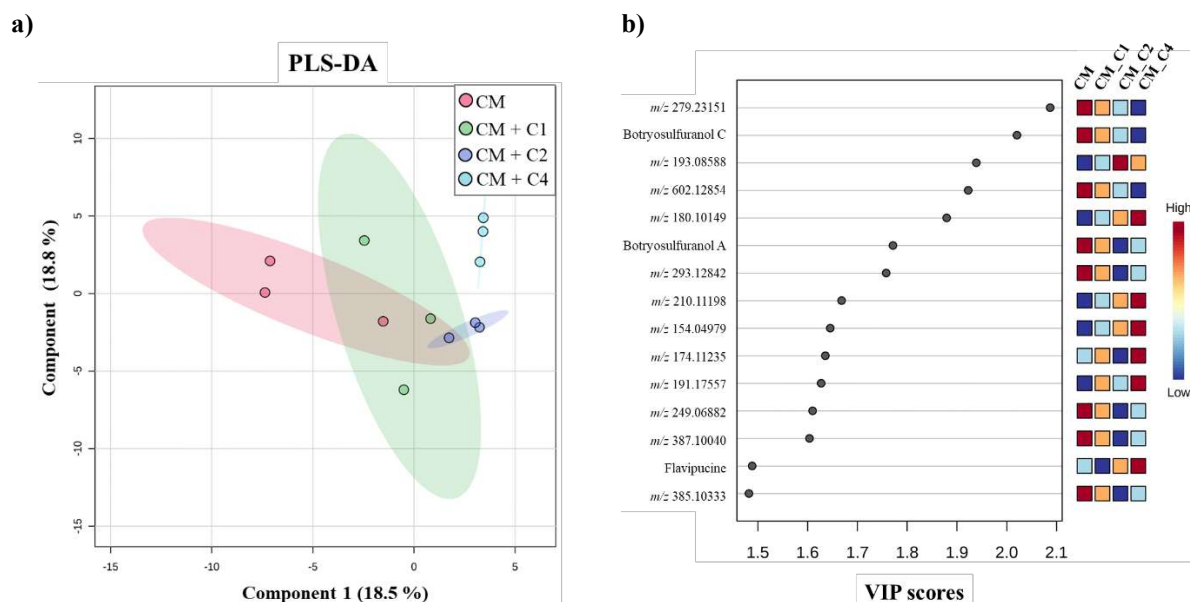


Figure 5.4. a) Partial least squares discriminant analysis (PLS-DA) obtained from UHPLC-(+)ESI-HRMS profiles corresponding to the extracts of *C. mamane* (CM) cultivated under the addition of heat-killed *Leishmania* parasites at 3 different concentrations (C1, C2 and C4); based on features (m/z at R_t to its normalized peak area) detected in LC-MS chromatograms; (b) Variable importance in projection (VIP) score of the top 15 compounds from the PLS-DA analysis corresponding to each condition

Table 5.1. Top 15 features that significantly contribute to the difference in the PLS-DA analysis from LC-HRMS/MS data under the addition of heat-killed parasites in *C. mamane* culture

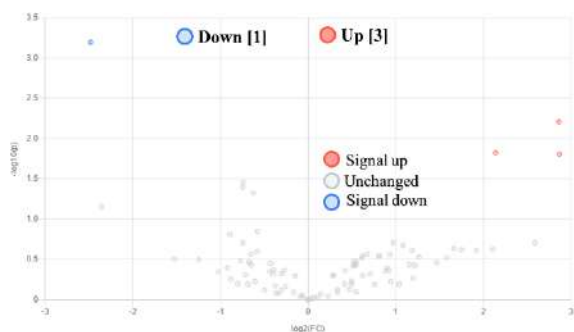
| ID | Rt (min) | Observed m/z | Adduct | Formula | Δ mass (ppm) | Putative identification | Ontology | VIP scores |
|----|----------|----------------|-----------------------------------|--|---------------------|-------------------------|-----------------------|------------|
| 69 | 9.37 | 279.2315 | [M+H] ⁺ | C ₁₈ H ₃₀ O ₂ | 1.24 | Unknown | Unknown | 2.09 |
| 16 | 7.39 | 455.0332 | [M+Na] ⁺ | C ₁₉ H ₁₆ N ₂ O ₆ S ₂ | 2.12 | Botryosulfuranol C | Thiodiketopiperazines | 2.02 |
| 38 | 5.40 | 193.0858 | [M+H] ⁺ | C ₁₁ H ₁₂ O ₃ | 0.21 | Unknown | Unknown | 1.94 |
| 31 | 6.59 | 602.1285 | [M+NH ₄] ⁺ | C ₃₁ H ₂₀ O ₁₂ | 1.26 | Unknown | Unknown | 1.92 |
| 12 | 3.90 | 180.1014 | [M+H] ⁺ | C ₁₀ H ₁₃ NO ₂ | 2.3 | Unknown | Unknown | 1.88 |
| 21 | 6.94 | 480.1244 | [M+NH ₄] ⁺ | C ₂₁ H ₂₂ N ₂ O ₆ S ₂ | 2.65 | Botryosulfuranol A | Thiodiketopiperazines | 1.77 |
| 76 | 6.11 | 293.1284 | [M+H] ⁺ | C ₁₈ H ₁₆ N ₂ O ₂ | 0.11 | Unknown | Unknown | 1.76 |
| 45 | 6.81 | 210.1119 | [M+H] ⁺ | C ₁₁ H ₁₅ NO ₃ | 2.33 | Unknown | Unknown | 1.67 |
| 80 | 3.68 | 154.0497 | [M+Na] ⁺ | C ₅ H ₉ NO ₃ | 7.97 | Unknown | Unknown | 1.65 |
| 8 | 3.72 | 174.1123 | [M+H] ⁺ | C ₈ H ₁₅ NO ₃ | 0.68 | Unknown | Unknown | 1.64 |
| 33 | 2.14 | 191.1755 | [M+H] ⁺ | C ₉ H ₂₂ N ₂ O ₂ | 0.86 | Unknown | Unknown | 1.63 |
| 53 | 6.43 | 249.0688 | [M+H] ⁺ | C ₁₂ H ₁₂ N ₂ O ₂ S | 1.62 | Unknown | Unknown | 1.61 |
| 3 | 6.94 | 387.1004 | [M+H] ⁺ | C ₁₉ H ₁₈ N ₂ O ₅ S | 1.34 | Unknown | Unknown | 1.60 |
| 49 | 6.22 | 238.1069 | [M+H] ⁺ | C ₁₂ H ₁₅ NO ₆ | 1.9 | (±)-Flavipucine | Tetrahydropyridines | 1.49 |
| 2 | 7.42 | 385.1033 | [M+H] ⁺ | C ₁₉ H ₁₆ N ₂ O ₇ | 0.78 | Unknown | Unknown | 1.48 |

Differential production of metabolites (Volcano plot analysis)

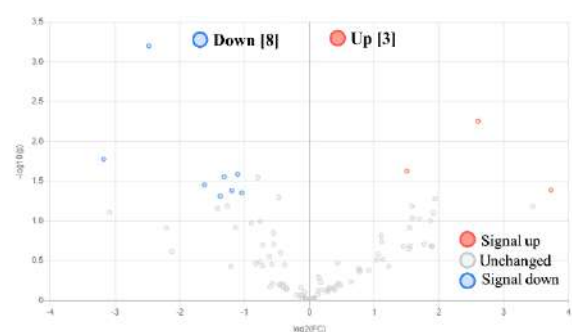
Regarding the influence of heat-killed amastigotes more in details as shown in the Volcano plot representations (**Figure 5.5**), only 3, 3 and 1 features were up-regulated when *C. mamane* was cultivated with C1 (**Figure 5.5a**), C2 (**Figure 5.5b**) and C4 (**Figure 5.5c**), respectively, indicating the few significantly up-regulated metabolites regardless the concentration used which is in line with the PLS-DA results (**Figure 5.4**). However, due to the low annotation rate, metabolites with the most important significant changes could not be putatively annotated.

Among the most significantly induced metabolites (**Table 5.2**), unknown features, at m/z 461.3239 at 11.468 min ($C_{26}H_{46}O_5$) and at m/z 174.1124 at 3.726 min ($C_8H_{15}NO_3$) were significantly up-regulated under concentration C1 and C4 of amastigotes, respectively. Similarly, the metabolite annotated as (\pm)-flavipucine was significantly up-regulated by concentration C2 of amastigotes. On the other side, the putatively annotated cyclic pentapeptide, previously isolated from a non-identified endophytic fungal strain (Li *et al.* 2004), was significantly down-regulated under concentration C1 and C2 of amastigotes

a) Volcano plot of treatment with C1 of amastigotes



b) Volcano plot of treatment with C2 of amastigotes



c) Volcano plot of treatment with C4 of amastigotes

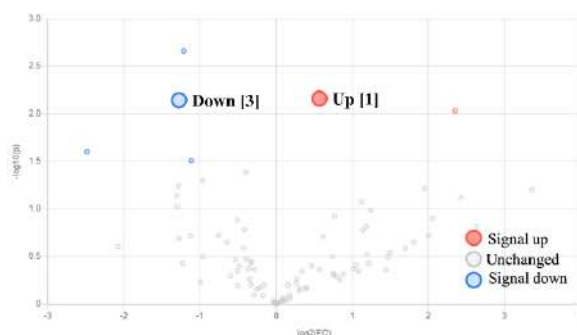


Figure 5.5. Volcano plot representations of *C. mamane* response to the treatment with heat-killed parasites at 3 different concentrations (C1, C2 and C4). Each dot represents a feature (m/z at Rt), the red and blue colors indicate a significant change (p -value < 0.05 and fold-change ratio > 2) of the features under treatment in up-regulating (red) or down-regulating (blue) their production in comparison with the untreated culture of *C. mamane*, while the numbers in brackets indicate the number of significant features observed.

Focusing on the TDKPs, the addition of the heat-killed parasites did not significantly induce the production of TDKPs, on the contrary, botryosulfuranol C was significantly down-regulated by concentration C1, C2 and C4 of amastigotes (**Figure 5.6**) while botryosulfuranol A was significantly down-regulated by concentration C4, both compared to the untreated culture of *C. mamane*. The TDKP botryosulfuranol D was almost undetectable as shown in **Figure 5.6**.

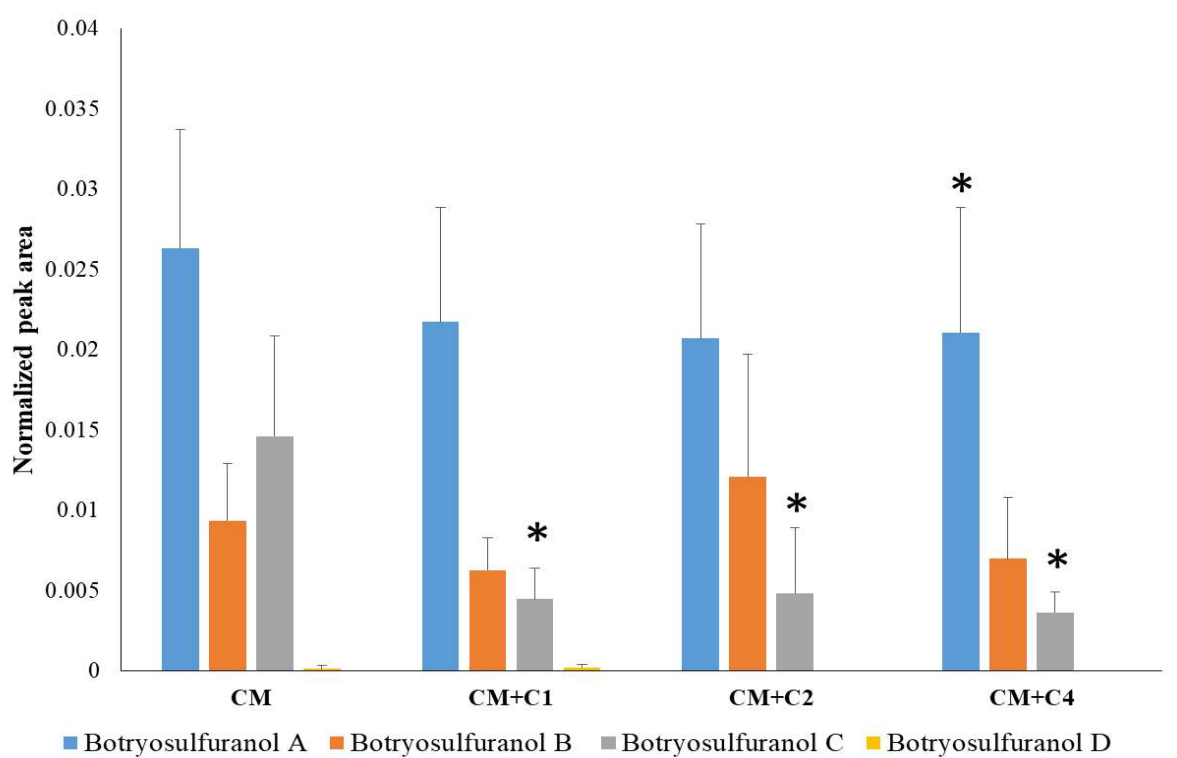


Figure 5.6. Normalized peak area of the TDKPs botryosulfuranols A, B, C and D detected under the addition of heat-killed *Leishmania* amastigotes at 3 different concentrations (C1, C2 and C4) in comparison to the untreated culture of *C. mamane* (CM). Significant changes (T-test with $p < 0.05$) are shown (*)

Table 5.2. Most important compounds up-regulated (↑) or down-regulated (↓) by the treatment heat-killed parasites in *C. mamane* culture. Up-regulation or down-regulation is shown with a significant (*) or not significant (N.S.) fold-change (FC) value. Significant values with *p*-value < 0.05 and fold-change ratio > 2

| ID | Rt min | Observed <i>m/z</i> | Adduct | Formula | Δ mass (ppm) | Putative identification | Ontology | <i>C. mamane</i> and <i>C. mamane</i> and | | <i>C. mamane</i> and <i>C. mamane</i> and | | | |
|----|--------|---------------------|-----------------------------------|--|--------------|--|-----------------------|---|---------|---|---------|--------------------------|-------|
| | | | | | | | | C1 amastigotes Log2 (FC) | Trend | C2 amastigotes Log2 (FC) | Trend | C4 amastigotes Log2 (FC) | Trend |
| 2 | 7.42 | 385.1033 | [M+H] ⁺ | C ₁₉ H ₁₆ N ₃ O ₇ | 0.78 | Unknown | Unknown | N.S. | -3.1775 | ↓* | -1.2925 | ↓ | |
| 4 | 6.07 | 392.2272 | [M+H] ⁺ | C ₂₂ H ₃₃ NO ₃ S | 4.61 | Unknown | Unknown | 2.8625 | ↑* | 1.2277 | ↑ | 2.0559 | ↑ |
| 7 | 11.47 | 461.3239 | [M+Na] ⁺ | C ₂₆ H ₄₆ O ₅ | 0.33 | Unknown | Unknown | 2.8714 | ↑* | 3.4472 | ↑ | 3.3639 | ↑ |
| 8 | 3.73 | 174.1124 | [M+H] ⁺ | C ₈ H ₁₅ NO ₃ | 0.68 | Unknown | Unknown | N.S. | -1.2187 | ↓ | 2.353 | ↑* | |
| 16 | 7.39 | 455.0332 | [M+Na] ⁺ | C ₁₉ H ₁₆ N ₂ O ₆ S ₂ | 2.12 | Botryosulfuranol C | Thiodiketopiperazines | N.S. | N.S. | N.S. | -1.2105 | ↓* | |
| 31 | 6.59 | 602.1285 | [M+NH ₄] ⁺ | C ₃₁ H ₂₆ O ₁₂ | 1.26 | Unknown | Unknown | N.S. | -1.1415 | ↓ | -2.4837 | ↓* | |
| 36 | 9.31 | 600.4116 | [M+H] ⁺ | C ₃₃ H ₅₃ N ₅ O ₅ | 0.54 | Cyclo-(L-Phe-L-Leu-L-Leu2-L-Leu3-L-Ile)* | Cyclopeptides | -2.4823 | ↓* | -2.4747 | ↓* | -1.3075 | ↓ |
| 38 | 5.40 | 193.0859 | [M+H] ⁺ | C ₁₁ H ₁₂ O ₃ | 0.21 | Unknown | Unknown | N.S. | 1.5046 | ↑* | 1.2445 | ↑ | |
| 50 | 5.68 | 238.1071 | [M+H] ⁺ | C ₁₂ H ₁₅ NO ₄ | 1.19 | (±)-Flavipucine | Tetrahydropyridines | 1.2189 | ↑ | 3.7314 | ↑* | N.S. | |
| 57 | 0.88 | 256.1182 | [M+H] ⁺ | C ₁₂ H ₁₇ NO ₅ | 0.98 | Unknown | Unknown | 2.5871 | ↑ | 2.6041 | ↑* | 1.2185 | ↑ |
| 69 | 9.37 | 279.2315 | [M+H] ⁺ | C ₁₈ H ₃₀ O ₂ | 1.24 | Unknown | Unknown | N.S. | N.S. | N.S. | -1.1146 | ↓* | |
| 71 | 7.36 | 285.1698 | [M+H] ⁺ | C ₁₅ H ₂₄ O ₅ | 0.52 | Unknown | Unknown | 2.1424 | ↑* | 1.5908 | ↑ | 1.4941 | ↑ |

*unknown amino acids sequence

Molecular network analysis

To better visualize chemical relationships based on acquired MS-MS data, and to focus on derivatives of annotated metabolites, a molecular networking was performed. Our analysis displayed a molecular network that contained 93 nodes and 12 spectral families with 31 connected nodes while the rest of the nodes showed no link to others (self-loop). The size of the nodes represents the total average of the detected peak areas for each compound in all the conditions while the colors depend on their detection in each condition (**Figure 5.7**).

The metabolite annotated as flavipucine (or isomers), which corresponds to one of the peaks induced by the addition of the parasites, was found to be linked to other two isomers. Moreover, two clusters were observed containing metabolites putatively annotated as cyclopentapeptides (**Figure 5.7**).

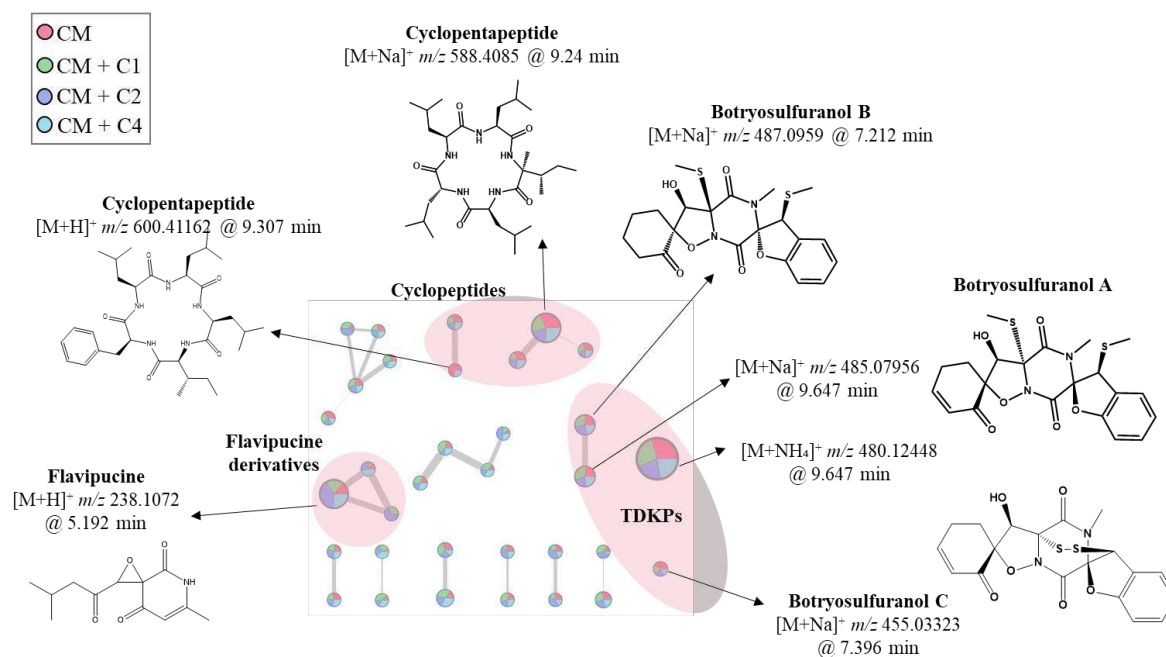


Figure 5.7. Molecular networking corresponding to the extracts of *C. mamane* treated with heat-killed parasites at 3 different concentrations (C1, C2, C4) based on features (m/z at R_t to its normalized peak area) detected in LC-MS chromatograms. Visualization of botryosulfuranols A, B and C and major peaks flavipucine and a cyclic peptide

Focusing on the cluster containing the thiodiketopiperazines, botryosulfuranol A and B were linked with a cosine value of 0.9 (data not shown) (**Figure 5.7**) but no other metabolites appeared to be linked in the same cluster. In another cluster, two metabolites putatively annotated as eicosanoids (m/z 295.22626 at 7.313 min and m/z 295.22632 at 7.719 min) were previously found to be produced by *Fusarium oxysporum* along with other jasmonates and related compounds (Miersch *et al.* 1999). This fungal

species has also been described in the literature as an endophyte isolated from different plants, including medicinal ones (do Nascimento *et al.* 2012; Ben Rhouma *et al.* 2020).

All putatively identified compounds are detailed in **Table 5.3**. The TDKP botryosulfuranol D does not possess an ID number because it was not considered for the metabolomics analyses due to its low detection.

Table 5.3. Putative identified compounds detected under the addition of heat-killed parasites in the culture of *C. mamane*

| ID | Rt (min) | <i>m/z</i> | Adduct | Formula | Δ mass (ppm) | Putative identification | Ontology |
|----|----------|------------|-----------------------------------|--|---------------------|---|-----------------------|
| 49 | 6.22 | 238.1069 | [M+H] ⁺ | C ₁₂ H ₁₅ NO ₆ | 1.9 | (±)-Flavipucine | Tetrahydropteridines |
| 61 | 5.19 | 238.1072 | [M+H] ⁺ | C ₁₂ H ₁₅ NO ₆ | 0.77 | (±)-Flavipucine | Tetrahydropteridines |
| 50 | 5.68 | 238.1071 | [M+H] ⁺ | C ₁₂ H ₁₅ NO ₆ | 1.19 | (±)-Flavipucine | Tetrahydropteridines |
| 43 | 8.92 | 574.3934 | [M+Na] ⁺ | C ₂₉ H ₅₃ N ₅ O ₅ | 0.8 | Cyclo-(L-Leu-L-Leu-D-L-leu-L-Leu-L-Leu-L-Val)* | Cyclopeptides |
| 26 | 9.24 | 588.4085 | [M+Na] ⁺ | C ₃₀ H ₅₅ N ₅ O ₅ | 1.75 | Cyclo-(L-Ile-L-Leu-L-Leu-L-Leu-L-Leu)* | Cyclopeptides |
| 27 | 9.07 | 566.4275 | [M+H] ⁺ | C ₃₀ H ₅₅ N ₅ O ₅ | 0.17 | Cyclo-(L-Ile-L-Leu-L-Leu-L-Leu-L-Leu)* | Cyclopeptides |
| 36 | 9.30 | 600.4116 | [M+H] ⁺ | C ₃₃ H ₅₃ N ₅ O ₅ | 0.54 | Cyclo-(L-Phe-L-Leu-L-Leu2-L-Leu3-L-Ile)* | Cyclopeptides |
| 78 | 7.31 | 295.2262 | [M+H] ⁺ | C ₁₈ H ₃₀ O ₃ | 1.73 | 3-Oxo-2-(2-enteny)cyclopentaneoctanoic acid;OPC-8:0 | Eicosanoids |
| 79 | 7.71 | 295.2263 | [M+H] ⁺ | C ₁₈ H ₃₀ O ₃ | 1.52 | 3-Oxo-2-(2-enteny)cyclopentaneoctanoic acid;OPC-8:0 | Eicosanoids |
| 18 | 7.21 | 487.0959 | [M+Na] ⁺ | C ₂₁ H ₂₄ N ₂ O ₆ S ₂ | 1.8 | Botryosulfuranol B | Thiodiketopiperazines |
| 16 | 7.39 | 455.0332 | [M+Na] ⁺ | C ₁₉ H ₁₆ N ₂ O ₆ S ₂ | 2.12 | Botryosulfuranol C | Thiodiketopiperazines |
| 21 | 6.94 | 480.1244 | [M+NH ₄] ⁺ | C ₂₁ H ₂₂ N ₂ O ₆ S ₂ | 2.65 | Botryosulfuranol A | Thiodiketopiperazines |
| - | 7.09 | 465.0231 | [M+H] ⁺ | C ₁₉ H ₁₆ N ₂ O ₆ S ₃ | 2.56 | Botryosulfuranol D | Thiodiketopiperazines |

*amino acid sequence not known

Antileishmanial activity of crude extracts against amastigotes of L. infantum

In order to know if there is a correlation between the concentration of TDKPs in the ethyl acetate extracts of the *C. mamane* culture and their antileishmanial activity, *in vitro* assays were performed on axenic amastigotes of *L. infantum* and the half-maximal effective concentration *Leishmania* growth (EC_{50}) was calculated for each extract while the IC_{50} value for the amphotericin B is 0.06 μ M (**Table 5.4**).

As observed in the chemical profiles (**Figure 5.3**) and the down-regulation of TDKPs when *C. mamane* was cultivated with heat-killed *Leishmania* (**Figure 5.6**), the crude extracts of the untreated culture of *C. mamane* present the best activity, being twice as high as the most active crude extract of the culture treated with *L. infantum* at concentration C2 (CM+C2) (EC_{50} =0.807 μ g/mL). Thus, these results tend to show a correlation between the concentration of TDKPs and the antileishmanial activity, but also that no other antileishmanial compounds appear to have been induced by the heat-killed parasites.

Table 5.4. Antileishmanial activity of the crude extracts of *C. mamane* (CM) cultivated under the addition of heat-killed leishmanial parasites at 3 different concentrations (C1, C2 and C4)

| Samples | EC_{50} (μg/mL) | | |
|-----------------------|--|---------|------|
| CM | 0.423 | \pm | 0.04 |
| CM + C1 | 1.217 | \pm | 0.79 |
| CM + C2 | 0.807 | \pm | 0.10 |
| CM + C4 | 1.193 | \pm | 0.89 |
| Amphotericin B | 0.06 | μ M | |

5.2.2 Co-culture with pathogen bacteria

General observations

We have previously observed that the addition of the *quorum*-sensing molecule N-butyryl-DL-homoserine lactone (HSL) into *C. mamane* culture, induced the production of the TDKPs botryosulfuranol C (see **Chapter 4**). In addition to the previous experiment with heat-killed *Leishmania* parasites and to hopefully increase antimicrobial metabolites and TDKPs production, living pathogenic bacteria were added into *C. mamane* culture. Two gram-negative bacteria, *P. aeruginosa* and *E. coli* and one gram-positive bacterium, *S. aureus*, were used for this purpose as observed in **Figure 5.8**. Also, as in this experiment the incubation was performed without agitation, the mycelium covers the surface of the culture media, contrary to what observed in **Figure 5.1**, where the mycelium has a round shape due to the agitation during the incubation.

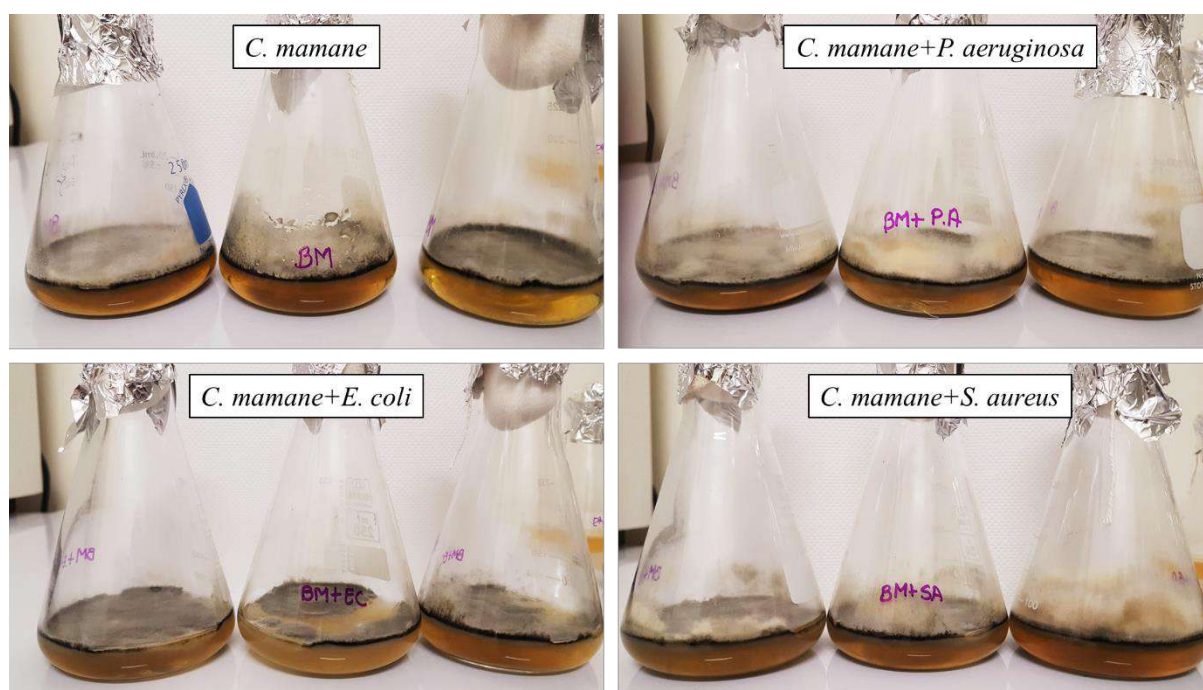


Figure 5.8. Culture of *C. mamane* in axenic conditions and in co-culture with pathogenic gram-negative bacteria, *E. coli* and *P. aeruginosa*, and gram-positive bacterium, *S. aureus*

The general chemical profiles of the axenic culture of *C. mamane* exhibit the presence of the TDKPs, whose extracted-ion chromatograms (XIC) are shown in **Figure 5.9**. To compare the general chemical profiles of the axenic culture of *C. mamani* and the ones of the axenic culture of each pathogenic bacteria against their respective co-culture, their chromatograms are shown in **Figure 5.10**, **Figure 5.11** and **Figure 5.12**.

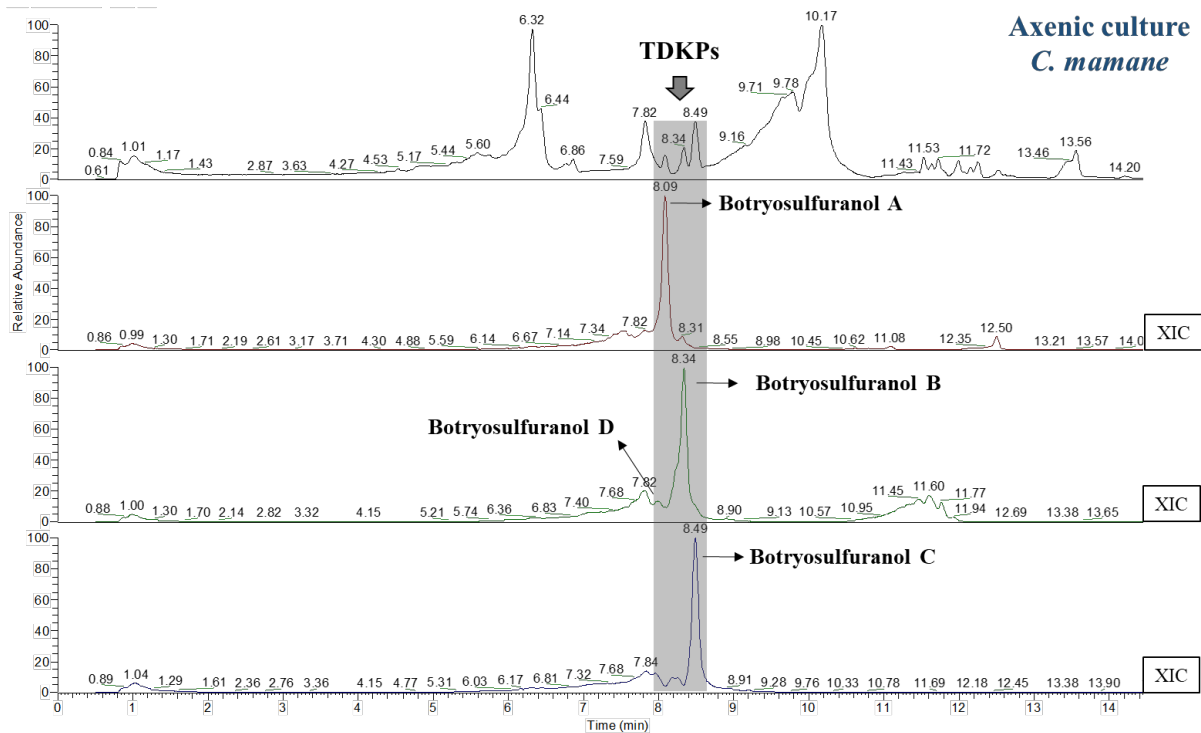


Figure 5.9. UHPLC-(+)ESI-HRMS base peak chromatograms from crude extracts of *C. mamane* culture with extracted-ion chromatograms showing the presence of TDKPs, botryosulfuranols A, B, C and D

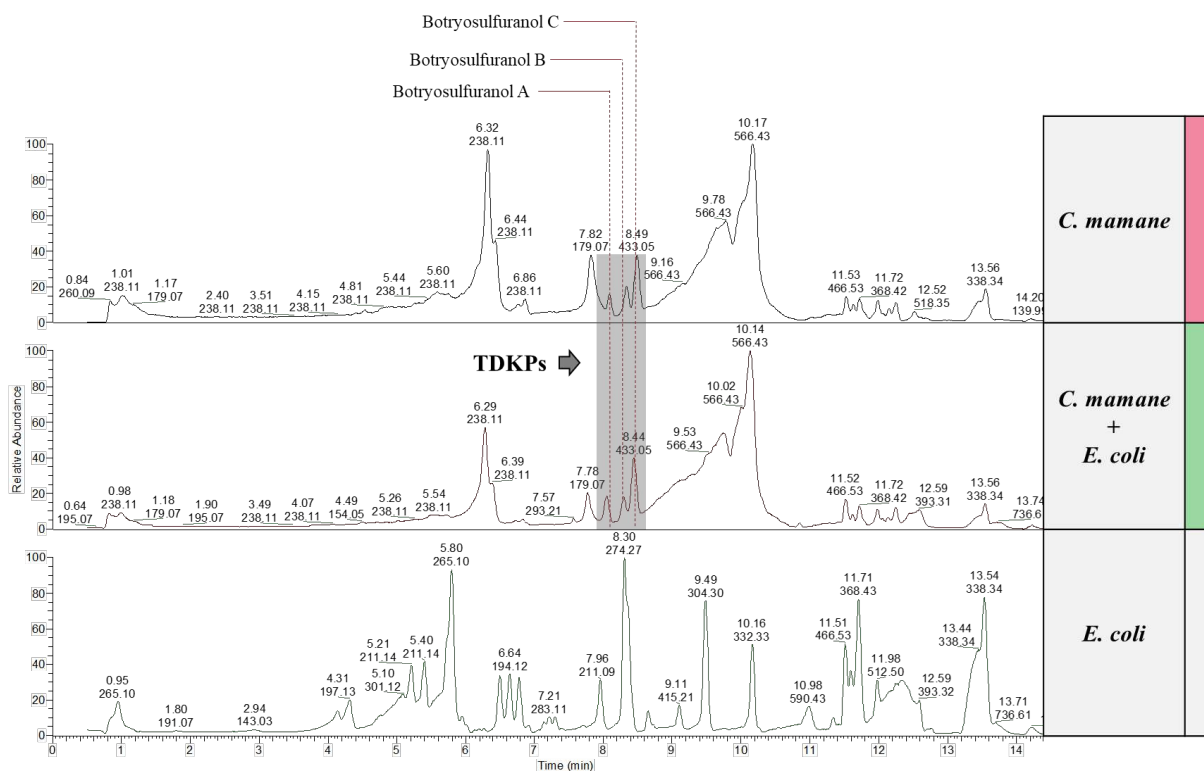


Figure 5.10. UHPLC-(+)ESI-HRMS base peak chromatograms of crude extracts of *C. mamane* and *E. coli* culture in axenic and co-culture conditions, showing the presence of the TDKPs

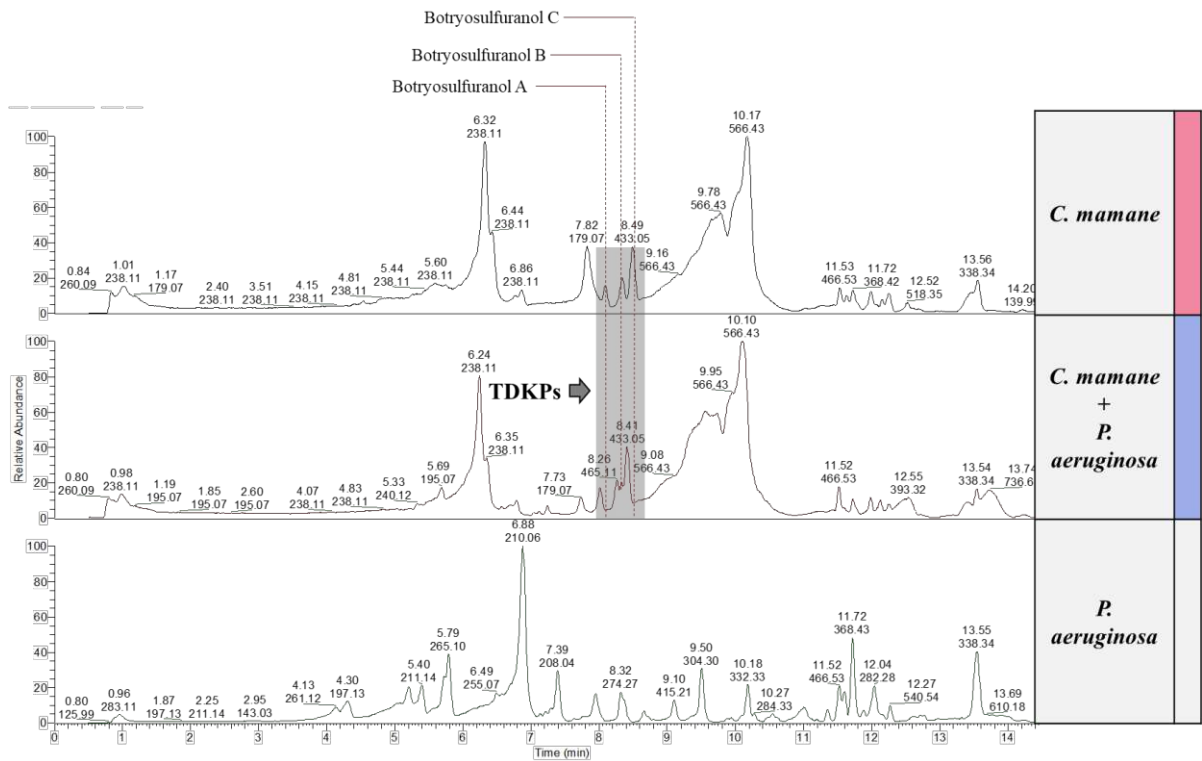


Figure 5.11. UHPLC-ESI-HRMS base peak chromatograms of crude extracts of *C. mamane* and *P. aeruginosa* culture in axenic and co-culture conditions, showing the presence of the TDKPs

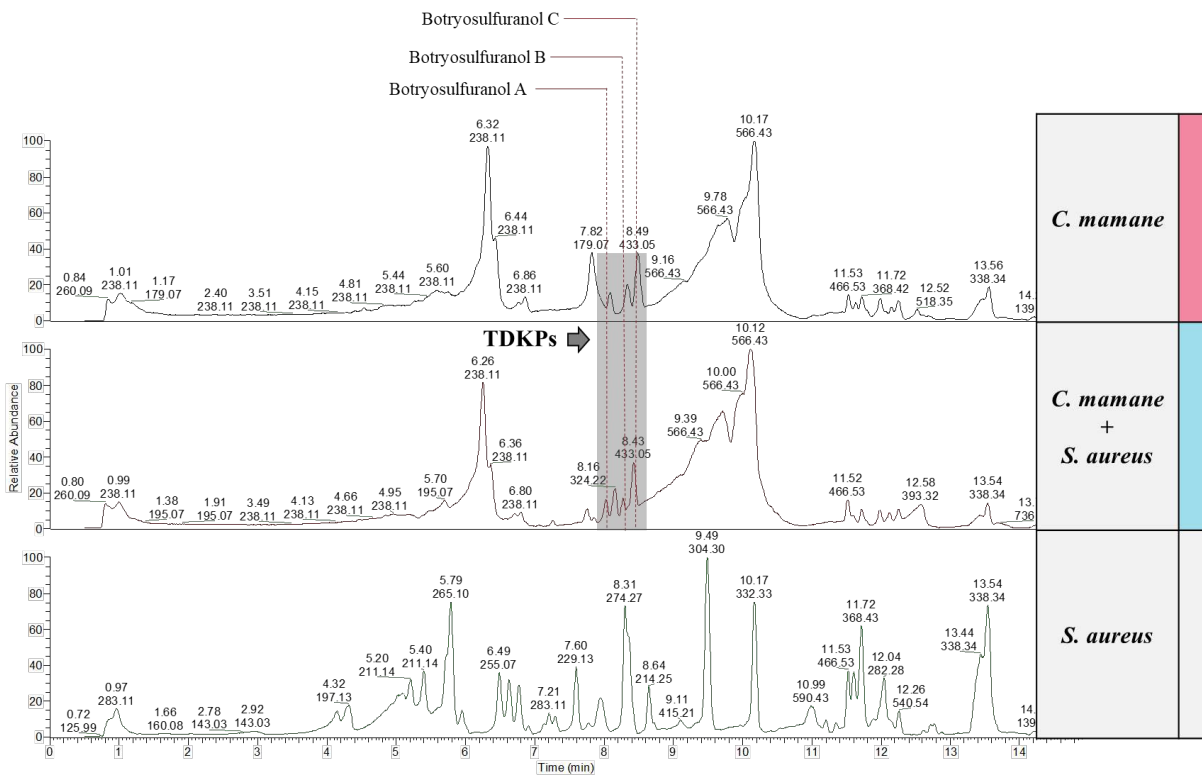


Figure 5.12. UHPLC-ESI-HRMS base peak chromatograms of crude extracts of *C. mamane* and *S. aureus* culture in axenic and co-culture conditions, showing the presence of the TDKPs

UHLC-(+)ESI-HRMS profiles of all the 12 crude extracts from this study afforded 328 features (m/z -RT pairs) in PI mode, which were considered for the metabolomics and statistical analysis. Some of the putatively annotated metabolites correspond to diketopiperazines (DKPs), cyclic peptides, cyclohexenones, benzenoids, hydroxyridines, etc.

Multivariate classification (PLS-DA)

A supervised PLS-DA analysis carried out on these data indicated the response of *C. mamane* in presence of the 3 living pathogenic bacteria, *E. coli* (EC), *P. aeruginosa* (PA) and *S. aureus* (SA) (**Figure 5.13**). Separate clusters appear on the PLS-DA score plot (32.4 % of total variability). The component 1 (X-axis) explained 20 % of the variability, allowing a slight discrimination mainly of the cluster corresponding to the co-culture of *C. mamane* with *S. aureus* from the rest conditions. Component 2 (Y-axis) explained 12.4 % of the variability with overlapping clusters, reflecting the few changes of the general metabolome of *C. mamane* when co-cultured with these pathogenic bacteria.

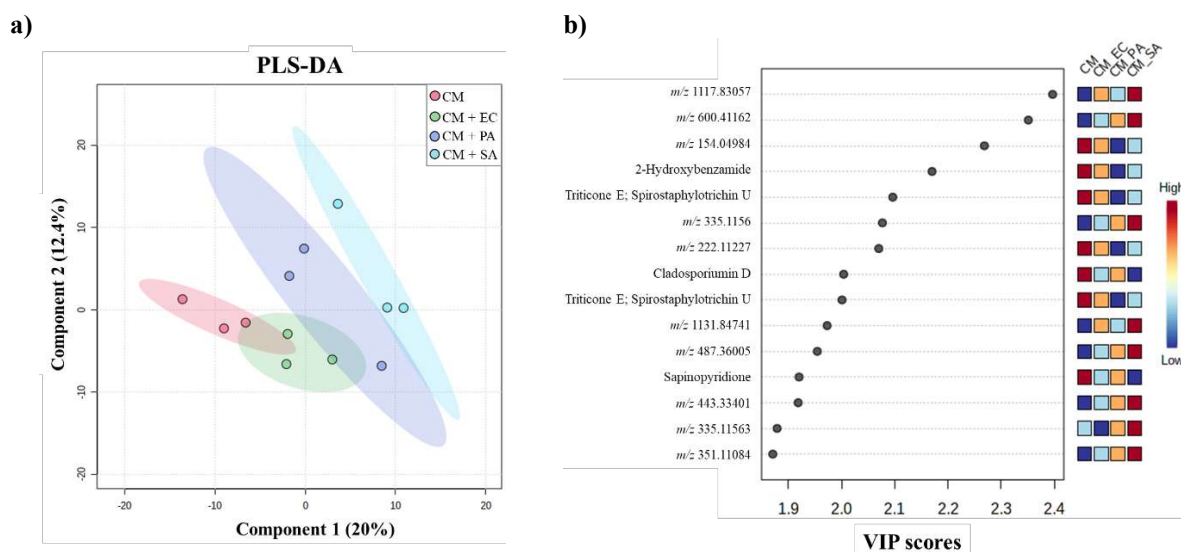


Figure 5.13. a) Partial least squares discriminant analysis (PLS-DA) obtained from LC-(+)ESI-HRMS profiles corresponding to the extracts of *C. mamane* (CM) co-cultivated with *E. coli* (EC), *P. aeruginosa* (PA) and *S. aureus* (SA); based on features (m/z at Rt to its normalized peak area) detected in LC-MS chromatograms; (b) Variable importance in projection (VIP) score of the top 15 compounds from the PLS-DA analysis corresponding to each condition

The **Figure 5.13** also presents the top 15 compounds that contribute to the differences between these clusters while some of them were putatively identified. For instance, feature m/z 238.1069 at 7.28 min was putatively identified as sapinopyridione, a structurally closely compound related to flavipucine, both isolated from an endophytic fungal strain of *Phoma* sp. (Loesgen *et al.* 2011). The metabolite annotated as 2-hydroxybenzamide was previously isolated from a strain of *Streptomyces* sp. (Shaaban

et al. 2017) while the metabolite annotated as 4-hydroxybenzamide was previously isolated from an endophytic fungal strain of *Colletotrichum gloeosporioides*, which exhibited a potent antifungal activity against two pathogenic strains of *Cladosporium sp* (Chapla *et al.* 2014).

Moreover, the metabolite annotated as cladosporium D, is a tetramic acid derivative previously isolated from a deep-sea derived strain of *Cladosporium sp.* with no reported biological activity (Huang *et al.* 2018). Features *m/z* 298.1287 at 5.7 and 6.8 min were putatively identified as spirostaphylotrichin U, one of the three major compounds obtained from an endophytic fungal strain of *Bipolaris sp.*, whose crude extract exhibited a weak antileishmanial activity (de Almeida *et al.* 2018). One of these two metabolites could also correspond to triticone E, a compound previously isolated from an endophytic fungal strain of *Curvularia lunata* which exhibited a good antibacterial activity against *E. coli*. Both molecules have previously shown to be inseparable by chromatographic methods (Hilario *et al.* 2020). These metabolites are detailed in **Table 5.5**.

Table 5.5. Top 15 features that significantly contribute to the difference in the PLS-DA analysis from LC-HRMS/MS data in *C. mamane* co-culture with *E. coli*, *P. aeruginosa* and *S. aureus*

| ID | Rt (min) | <i>m/z</i> | Adduct | Formula | Δ mass (ppm) | Putative identification | Ontology | VIP scores |
|-----|----------|------------|-----------------------------------|---|---------------------|-------------------------------------|--------------------|------------|
| 632 | 9.82 | 1117.8306 | [M+H] ⁺ | C ₅₆ H ₁₀₀ N ₂₀ O ₄ | 0.31 | Unknown | Unknown | 2.397 |
| 551 | 10.20 | 600.4116 | [M+NH ₄] ⁺ | C ₃₃ H ₅₀ N ₄ O ₅ | 0.54 | Unknown | Unknown | 2.351 |
| 26 | 6.87 | 154.0498 | [M+H] ⁺ | C ₇ H ₇ NO ₃ | 0.19 | Unknown | Unknown | 2.268 |
| 11 | 5.81 | 138.0549 | [M+H] ⁺ | C ₇ H ₇ NO ₂ | 0.25 | 2-Hydroxybenzamide | Benzenoids | 2.170 |
| 241 | 5.77 | 298.1283 | [M+H] ⁺ | C ₁₄ H ₁₉ NO ₆ | 0.82 | Triticone E; Spirostaphylotrichin U | Cyclohexenones | 2.096 |
| 292 | 7.06 | 335.1156 | [M+H] ⁺ | C ₁₅ H ₁₈ N ₄ O ₃ S | 4.89 | Unknown | Unknown | 2.077 |
| 113 | 5.82 | 222.1123 | [M+H] ⁺ | C ₁₂ H ₁₅ NO ₃ | 0.9 | Unknown | Unknown | 2.070 |
| 169 | 5.81 | 254.1385 | [M+H] ⁺ | C ₁₃ H ₁₉ NO ₄ | 0.85 | Cladosporium in D | Carbonyl compounds | 2.004 |
| 240 | 6.88 | 298.1283 | [M+H] ⁺ | C ₁₄ H ₁₉ NO ₆ | 0.82 | Triticone E; Spirostaphylotrichin U | Cyclohexenones | 2.001 |
| 635 | 10.12 | 1131.8474 | [M+H] ⁺ | C ₅₀ H ₁₀₂ N ₂₆ O ₂ S | 0.14 | Unknown | Unknown | 1.973 |
| 451 | 11.59 | 487.3601 | [M+Na] ⁺ | C ₂₅ H ₅₂ O ₇ | 0.97 | Unknown | Unknown | 1.954 |
| 150 | 7.28 | 238.1074 | [M+H] ⁺ | C ₁₂ H ₁₅ NO ₄ | 0.11 | Sapinopyridione | Hydropyridines | 1.920 |
| 407 | 11.60 | 443.3340 | [M+Na] ⁺ | C ₂₄ H ₄₄ N ₄ O ₂ | 3.69 | Unknown | Unknown | 1.918 |
| 293 | 7.26 | 335.1156 | [M+H] ⁺ | C ₁₅ H ₁₈ N ₄ O ₃ S | 4.79 | Unknown | Unknown | 1.879 |
| 306 | 6.68 | 351.1108 | [M+H] ⁺ | C ₁₅ H ₁₈ N ₄ O ₄ S | 3.74 | Unknown | Unknown | 1.871 |

Differential production of metabolites (Volcano plot analysis)

Regarding more in details the metabolome changes of *C. mamane* in co-culture with bacteria, as shown in the Volcano plot representations (**Figure 5.14**), a total of 4, 5 and 6 features were up-regulated when *C. mamane* was co-cultivated with *E. coli* (**Figure 5.14a**), *P. aeruginosa* (**Figure 5.14b**) and *S. aureus* (**Figure 5.14c**), respectively, indicating the few significantly up-regulated metabolites which is in line with the PLS-DA results while a higher number of significantly down-regulated metabolites were observed (**Figure 5.14**). However, not all the metabolites with the most important significant changes could not be putatively annotated (**Table 5.6**).

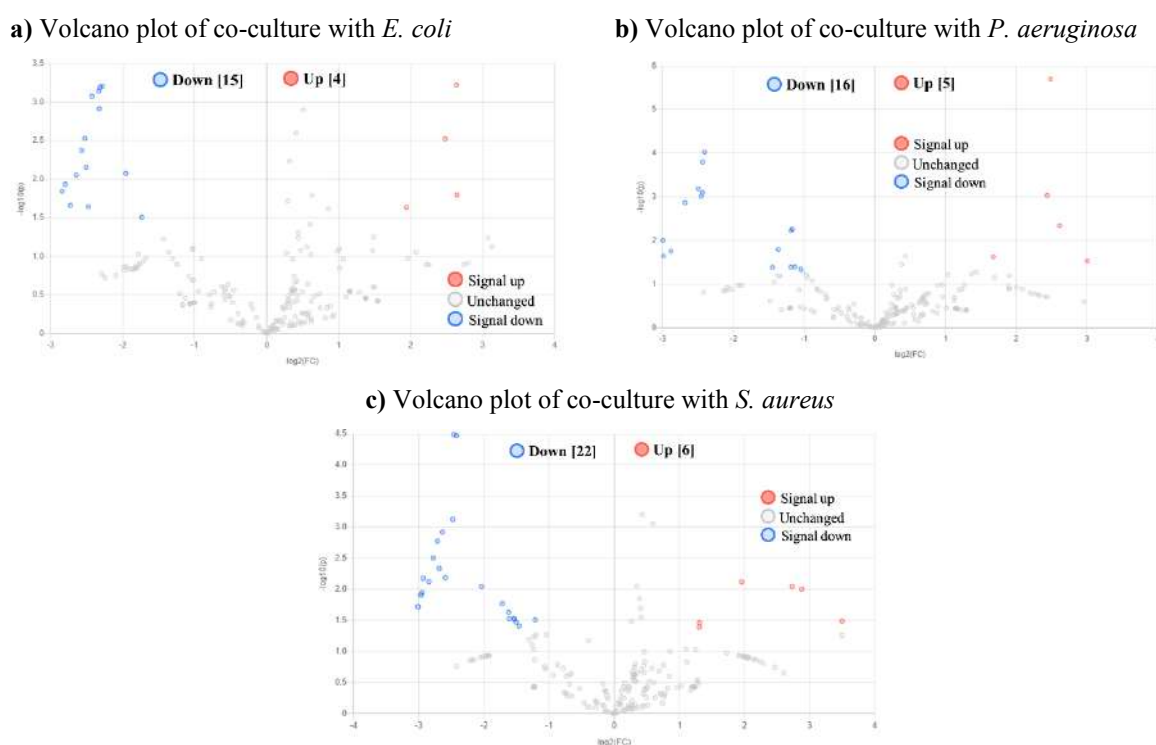


Figure 5.14. Volcano plot representations of co-culture of *C. mamane* with 3 different pathogenic bacteria (*E. coli*, *P. aeruginosa* and *S. aureus*). Each dot represents a feature (m/z at R_t), the red and blue colors indicate a significant change (p -value < 0.05 and fold-change ratio > 2) of the features under treatment in up-regulating (red) or down-regulating (blue) their production in comparison with the axenic culture of *C. mamane*, while the numbers in brackets indicate the number of significant features observed.

Among the most significantly induced compounds under co-culture with *E. coli*, we observed putatively identified diketopiperazine diphenylalazine B, previously isolated from entomopathogenic fungus *Epicoccum nigrum* (Guo *et al.* 2009). Two metabolites that were significantly up-regulated under the co-culture with *P. aeruginosa* and *S. aureus* were putatively identified as cyclic pentapeptides previously isolated from an endophytic fungal strain of *Cryptosporiopsis sp.*, which exhibited antifungal activity (Talontsi *et al.* 2012).

Moreover, of the 126 metabolites that were induced under the co-culture but lowly produced and undetected in the fungal or bacterial axenic cultures, were mainly produced under the co-culture with *E. coli*. Among these metabolites, two features (m/z 262.1436 at 5.99 min and m/z 262.1437 at 5.93 min) were putatively identified as bassiatin, a metabolite previously isolated from endophytic fungus *Fusarium proliferatum*, which exhibited moderate antibacterial activity (Jiang *et al.* 2019) (Table 5.7).

Regarding the TDKPs, the co-culture of *C. mamane* with *E. coli*, *P. aeruginosa* and *S. aureus* did not significantly alter their production (Figure 5.15) as it was also observed above in the chromatograms (Figure 5.10, Figure 5.11 and Figure 5.12). The TDKP botryosulfuranol D was almost undetectable as shown in Figure 5.15. Interestingly, the production of a metabolite, possessing the same chemical formula as botryosulfuranol C (m/z 433.05219 at 7.91 min), was significantly up-regulated under the co-culture with *E. coli* and *P. aeruginosa* while the production of a metabolite, possessing the same chemical formula as botryosulfuranol A (m/z 485.08069 at 7.95 min), was significantly up-regulated under the co-culture with *S. aureus*.

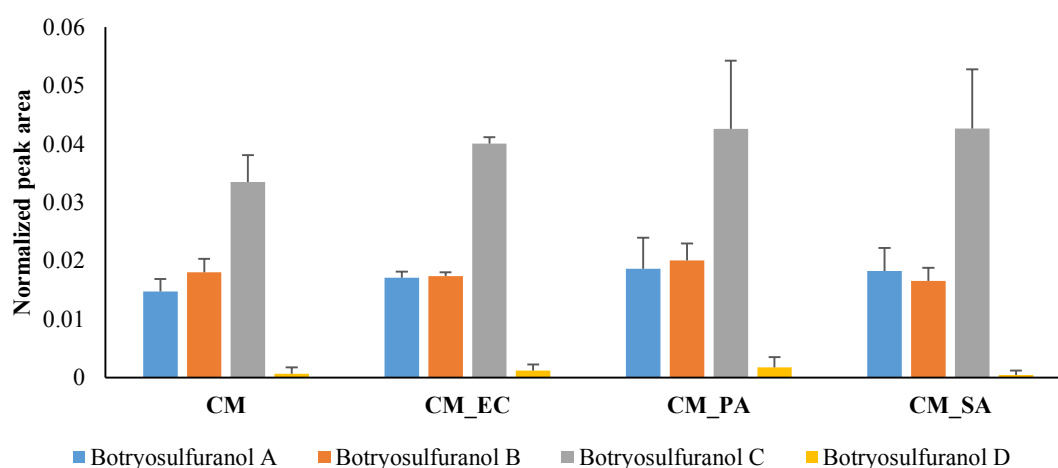


Figure 5.15. Normalized peak area of the TDKPs botryosulfuranol A, botryosulfuranol B, botryosulfuranol C and botryosulfuranol D detected under the co-culture with *E. coli* (EC), *P. aeruginosa* (PA) and *S. aureus* (SA) in comparison to the axenic culture of *C. mamane* (CM)

The most significantly induced metabolites produced under co-culture are detailed in Table 5.6.

Table 5.6. Metabolites up-regulated under the co-culture of *C. mamane* with *E. coli*, *P. aeruginosa* and *S. aureus* with significant values with *p*-value < 0.05 and fold-change (FC) ratio > 2 and compounds with not significant (N.S.) FC ratio.

| ID | Rt (min) | m/z | Adduct | Formula | Δ mass (ppm) | Putative identification | Ontology | <i>C. mamane</i> and <i>E. coli</i> | | |
|-----|----------|----------|-----------------------------------|---|--------------|--|-------------------|-------------------------------------|---|---------------------------------------|
| | | | | | | | | Log2 (FC) | <i>C. mamane</i> and <i>P. aeruginosa</i> | <i>C. mamane</i> and <i>S. aureus</i> |
| 165 | 7.74 | 247.0534 | [M+H] ⁺ | C ₁₂ H ₁₆ N ₂ O ₂ S | 0.55 | Unknown | Unknown | 2.48 | N.S. | N.S. |
| 277 | 7.88 | 323.1386 | [M+H] ⁺ | C ₁₉ H ₁₈ N ₂ O ₃ | 1.17 | Diphenylalazine B ₃ (+)- Diphenylalazine B | Diketopiperazines | 1.94 | N.S. | N.S. |
| 292 | 7.06 | 335.1156 | [M+H] ⁺ | C ₁₅ H ₁₈ N ₄ O ₃ S | 4.89 | Unknown | Unknown | N.S. | N.S. | 2.73 |
| 293 | 7.26 | 335.1156 | [M+H] ⁺ | C ₁₅ H ₁₈ N ₄ O ₃ S | 4.79 | Unknown | Unknown | N.S. | N.S. | 1.31 |
| 306 | 6.67 | 351.1108 | [M+H] ⁺ | C ₁₅ H ₁₈ N ₄ O ₄ S | 3.74 | Unknown | Unknown | N.S. | N.S. | 2.88 |
| 395 | 7.91 | 433.0521 | [M+H] ⁺ | C ₁₉ H ₁₆ N ₂ O ₆ S ₂ | 0.15 | Unknown | Unknown | 2.64 | 3.01 | N.S. |
| 445 | 7.95 | 485.0806 | [M+Na] ⁺ | C ₂₁ H ₂₂ N ₂ O ₆ S ₂ | 0.95 | Unknown | Unknown | N.S. | N.S. | 3.50 |
| 456 | 6.84 | 496.1167 | [M+H] ⁺ | C ₂₄ H ₂₁ N ₃ O ₇ S | 1.2 | Unknown | Unknown | N.S. | 2.49 | N.S. |
| 468 | 7.83 | 509.1046 | [M+H] ⁺ | C ₂₂ H ₂₄ N ₂ O ₈ S ₂ | 0.09 | Unknown | Unknown | N.S. | 2.62 | N.S. |
| 513 | 8.19 | 566.4273 | [M+H] ⁺ | C ₃₀ H ₅₅ N ₅ O ₅ | 0.55 | Cyclo-(L-Ile-L-Leu-L-Leu-L-Leu-L-Leu-L-Leu)* | Cyclic peptides | N.S. | N.S. | 1.31 |
| 517 | 9.96 | 566.4275 | [M+H] ⁺ | C ₃₀ H ₅₅ N ₅ O ₅ | 0.08 | Cyclo-(L-Ile-L-Leu-L-Leu-L-Leu-L-Leu-L-Leu-L-Leu)* | Cyclic peptides | N.S. | 2.44 | N.S. |
| 551 | 10.19 | 600.4116 | [M+NH ₄] ⁺ | C ₃₃ H ₅₀ N ₄ O ₅ | 0.54 | Unknown | Unknown | N.S. | 1.67 | 1.96 |
| 606 | 8.36 | 849.1001 | [M+H] ⁺ | C ₃₂ H ₄₁ N ₄ O ₉ PS ₆ | 0.77 | Unknown | Unknown | 2.63 | N.S. | N.S. |

*Unknown amino acids sequence of cyclic peptides

The mentioned putative identified metabolites are detailed in **Table 5.7** and in **Figure 5.16**.

Table 5.7. Putative identified compounds detected under the co-culture of *C. mamane* with *E. coli*, *P. aeruginosa* and *S. aureus*

| ID | Rt (min) | m/z | Adduct | Formula | Δ mass (ppm) | Putative identification | Ontology |
|-----|----------|----------|---------------------|--|---------------------|--|-----------------------|
| 277 | 7.88 | 323.1386 | [M+H] ⁺ | C ₁₉ H ₁₈ N ₂ O ₃ | 1.17 | Diphenylalazine B | Diketopiperazines |
| 421 | 8.03 | 463.0989 | [M+H] ⁺ | C ₂₁ H ₂₂ N ₂ O ₆ S ₂ | 0.63 | Botryosulfuranol A | Thiodiketopiperazines |
| 445 | 7.95 | 485.0806 | [M+Na] ⁺ | C ₂₁ H ₂₂ N ₂ O ₆ S ₂ | 0.95 | Unknown | Unknown |
| 426 | 8.28 | 465.1141 | [M+H] ⁺ | C ₂₁ H ₂₄ N ₂ O ₆ S ₂ | 1.47 | Botryosulfuranol B | Thiodiketopiperazines |
| 391 | 8.43 | 433.0516 | [M+H] ⁺ | C ₁₉ H ₁₆ N ₂ O ₆ S ₂ | 1.35 | Botryosulfuranol C | Thiodiketopiperazines |
| 395 | 7.91 | 433.0521 | [M+H] ⁺ | C ₁₉ H ₁₆ N ₂ O ₆ S ₂ | 0.15 | Unknown | Unknown |
| 425 | 8.17 | 465.0235 | [M+H] ⁺ | C ₁₉ H ₁₆ N ₂ O ₆ S ₃ | 1.77 | Botryosulfuranol D | Thiodiketopiperazines |
| 513 | 8.19 | 566.4273 | [M+H] ⁺ | C ₃₀ H ₅₅ N ₅ O ₅ | 0.55 | Cyclo-(L-Ile-L-Leu-L-Leu-L-Leu-L-Leu)* | Cyclic peptides |
| 517 | 9.97 | 566.4275 | [M+H] ⁺ | C ₃₀ H ₅₅ N ₅ O ₅ | 0.08 | Cyclo-(L-Ile-L-Leu-L-Leu-L-Leu-L-Leu)* | Cyclic peptides |
| 11 | 5.81 | 138.0549 | [M+H] ⁺ | C ₇ H ₇ NO ₂ | 0.25 | 2-Hydroxybenzamide | Benzenoids |
| 150 | 7.28 | 238.1074 | [M+H] ⁺ | C ₁₂ H ₁₅ NO ₄ | 0.11 | Sapinopyridione | Hydropyridines |
| 169 | 5.81 | 254.1384 | [M+H] ⁺ | C ₁₃ H ₁₉ NO ₄ | 0.85 | Cladosporiumin D | Carbonyl compounds |
| 240 | 6.88 | 298.1282 | [M+H] ⁺ | C ₁₄ H ₁₉ NO ₆ | 0.82 | Triticone E; Spirostaphylotrichin U | Cyclohexenones |
| 241 | 5.77 | 298.1282 | [M+H] ⁺ | C ₁₄ H ₁₉ NO ₆ | 0.82 | Triticone E; Spirostaphylotrichin U | Cyclohexenones |
| 187 | 5.99 | 262.1436 | [M+H] ⁺ | C ₁₅ H ₁₉ NO ₃ | 0.45 | Bassiatin | Alkaloids |
| 188 | 5.93 | 262.1437 | [M+H] ⁺ | C ₁₅ H ₁₉ NO ₃ | 0.23 | Bassiatin | Alkaloids |

*Unknown amino acids sequence of cyclic peptides

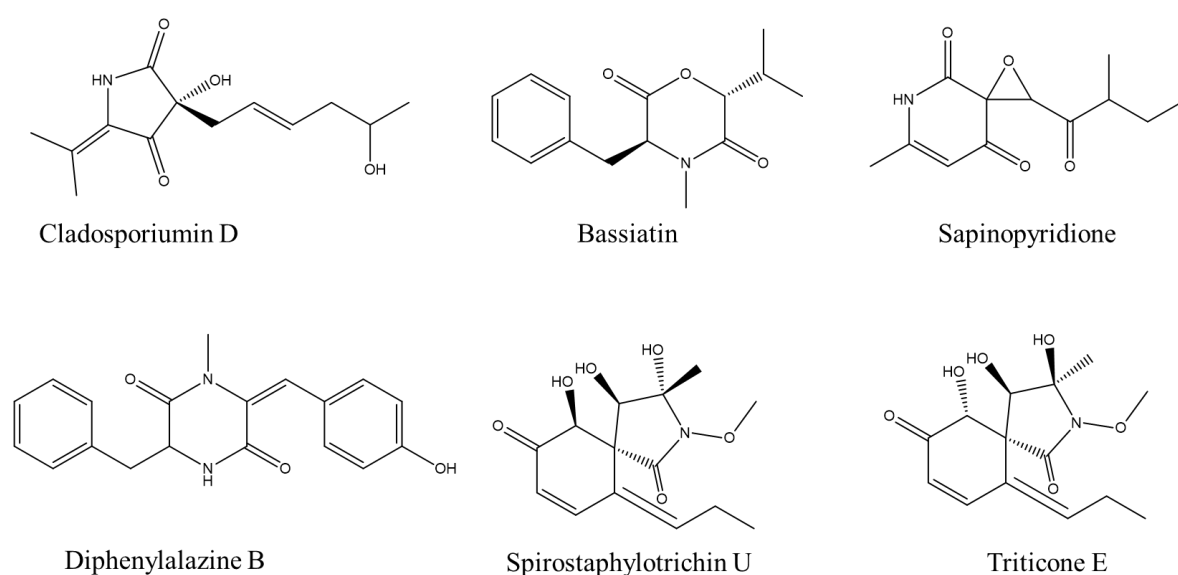


Figure 5.16. Chemical structure of some of the putatively identified compounds shown in Table 5.7

5.3 Discussions

5.3.1 Addition of thermally inactivated leishmanial parasites

Endophytic fungi have shown to naturally produce bioactive metabolites under standard laboratory culture conditions, representing an important resource of new drugs to combat antimicrobial resistance, including parasites (Hzounda Fokou *et al.* 2021) and bacteria (Deshmukh *et al.* 2014). Previously isolated TDKPs from *C. mamane* have shown a potent antileishmanial activity (unpublished data) and in order to induce their production and the production of other antiparasitic metabolites focusing on *Leishmania* parasites, we added heat-killed amastigotes of *Leishmania infantum* into the culture of *C. mamane*. It is important to mention that co-culture was not possible due to the very different culture conditions (culture media, temperature, oxygen levels, etc.) used for growing Leishmanial parasites and fungi. A preliminary work comparing the effects of heat-killed amastigotes versus promastigotes on *C. mamane* metabolism showed that the use of heat-killed amastigotes was more interesting in terms of production of TDKPs. Moreover, the difference observed between the addition of heat-killed promastigotes and amastigotes might be due to the expression of stage-specific molecules such as virulence factors expressed on the surface of the promastigotes of *Leishmania* species but minimally expressed in amastigotes forms, causing also different responses in the mammalian cells (Kima 2007).

In this study, the production of 12 metabolites were significantly influenced by the addition of heat-killed amastigotes of *L. infantum* at three different concentrations, with 7 of them showing an up-regulation, including flavipucine (and/or its isomers), while 5 metabolites showed a down-regulation, including a cyclic pentapeptide and a TDKP, botryosulfuranol C (**Table 5.2**). It has been described that during heat inactivation of cells, some cellular components might be exposed or solubilized, acting as inducers of antimicrobials compounds (Benitez *et al.* 2011; Liang *et al.* 2019). Specifically during wet heat inactivation, protein denaturation and membrane damage plays an important role (Smelt & Brul 2014). These exposed cellular components might have been recognized by *C. mamane*, triggering a response that induced the production of these 7 metabolites that included flavipucine (or isomers). Flavipucine has shown to be a cytotoxic compound, potent bactericide and strong antifungal (Loesgen *et al.* 2011; Kusakabe *et al.* 2019). This same metabolite was present in a fraction obtained from the crude extract of a *Botryosphaeria dothidea* strain, which exhibited cytotoxicity but no antiplasmodial activity (Kumarihamy *et al.* 2020). In the case of the down-regulated cyclopentapeptide, similar molecules have been observed to be used as chemical communication molecules between neighboring endophytic fungi and bacteria, highlighting a possible ecological function of these molecules (Wang *et al.* 2015) besides their different biological activities (Wang *et al.* 2017).

Moreover, the decrease in the production of botryosulfuranol C under the addition of heat-killed amastigotes in comparison to the untreated culture of *C. mamane* suggests that its production might be influenced by the cellular components from the living *Leishmania* parasites (degraded by temperature) or that their presence at different concentrations somehow represses the biosynthetic pathway of the TDKPs. This is reflected in the bioactivity results obtained against *L. infantum*, where the EC₅₀ values of the crude extracts of *C. mamane* cultivated with C1, C2 and C4 are higher (0.8 – 1.2 µg/mL) than the EC₅₀ value of the crude extract of the untreated culture of *C. mamane* (0.4 µg/mL).

Furthermore, molecular networking allowed to identify a cluster containing two eicosanoids, which have been reported to possess putative signaling functions in fungi, related to quorum sensing processes especially in pathogenic fungi (Shea & Del Poeta 2006).

Although our results show the down-regulation of the TKDPs, meaning the contrary to what we expected, and that no other antileishmanial metabolites might have been produced, considering the weaker bioactivity observed for the extract of the *C. mamane* culture with the heat-killed parasites, we might consider the addition of living parasites in conditions that favor the growth of the fungus but not the parasite.

5.3.2 Co-culture with pathogenic bacteria

Our second strategy used to induce the production of antimicrobial compounds consisted in co-cultivation of *C. mamane* with living pathogenic bacteria. These co-cultures led to the detection of 126 metabolites in co-culture conditions (undetected in axenic conditions) as well as the significant up-regulation of a few metabolites produced by *C. mamane*. In natural environments, the coexistence of different endophytic strain communities can be explained by the maintenance of low population densities through the production of metabolites that inhibit microbial growth without generating damage to the host plant (Chagas & Pupo 2018).

Among the metabolites induced under co-culture conditions, the production of metabolite bassiatin could suggest that this metabolite could play a role in regulating *E. coli* density due to the low detection of this compound. The diketopiperazine, diphenylalazine B and two metabolites possessing the same chemical formula as botryosulfuranol A and C, were the most significantly up-regulated in comparison to the axenic culture of *C. mamane*. In a study, 9 known diketopiperazines that were lowly produced by *Penicillium* sp. under axenic conditions were induced under the co-culture with *Bacillus* sp. (Yu *et al.* 2017). Similarly, glionitrin B appeared to be produced by *Aspergillus fumigatus* as a minor diketopiperazine, whose production was dramatically increased during long-term co-culture with *Sphingomonas* sp. (Park *et al.* 2011). Despite the fact that TDKPs botryosulfuranols A, B, C and D did

not show significant variations in their production during co-culture with *E. coli*, *P. aeruginosa* and *S. aureus*, co-culture with other microorganisms might be an interesting and promising method in the context of the search for this chemical class of compounds.

Moreover, the production of a cyclopentapeptide, a metabolite that is probably involved in chemical communication between neighboring microorganisms as mentioned before, was up-regulated in *C. mamane* when co-cultured with *P. aeruginosa* and *S. aureus*. Along with this, a cyclotetrapeptide was previously obtained from the co-culture of two marine mangrove fungi, *Phomopsis* sp. and *Alternaria* sp., exhibiting moderate antifungal activities (Huang *et al.* 2014).

It has been described in the literature that the *quorum*-sensing mechanisms differ between gram-negative and gram-positive bacteria: the acyl-homoserin lactones are predominantly involved in the communication processes within the gram-negative bacteria while modified oligopeptides are mainly used by gram-positive bacteria (Verbeke *et al.* 2017). Therefore, this might explain the different metabolites significantly induced in *C. mamane* under co-culture with gram-negative bacteria, *E. coli* and *P. aeruginosa* or with gram-positive bacterium, *S. aureus*. These results set a departure for further experiments of co-culture between *C. mamane* and these 3 bacteria, taking also into consideration other culture parameters, such as time of incubation and microorganisms concentrations. In addition, the biological test for the extracts will be performed against these same pathogenic bacterial strains.

5.4 Conclusions

The culture of *C. mamane* in presence of heat-killed promastigotes of *Leishmania infantum* attenuated the production of the TDKPs by this fungus. The addition of heat-killed amastigotes at different concentrations did not induce their production either. We can hypothesize that this might be due to heat degradation of the cellular components of the amastigotes of *Leishmania*. Moreover, the down-regulation of botryosulfuranol C was reflected in the bioactivity test against the amastigotes of *L. infantum*. The crude extract of the untreated culture of *C. mamane* exhibited a lower EC₅₀ value than the treated one, confirming that TDKPs are responsible of the antileishmanial activity of *C. mamane*.

Interestingly, cyclopentapeptides were down-regulated by heat-killed amastigotes of *L. infantum* but up-regulated in co-culture with *P. aeruginosa* and *S. aureus*, highlighting its possible role in chemical communication between neighboring living microorganisms.

Co-culture of *C. mamane* with pathogenic gram-negative bacterium, *E. coli*, also resulted in the up-regulation of diketopiperazines diphenylalazine B as also observed in other fungi-bacteria co-culture experiments. Similarly, the co-culture of *C. mamane* with *E. coli* led to the induction of bassiatin production, not detected under axenic conditions. Other parameters not analyzed in this study might have affected to the metabolites productions, such as the growing rate, development of the endophytic fungus or population density that induced specific metabolites.

Finally, the addition of heat-killed parasites or co-culture with living bacteria might results in the activation of other cryptic genes in *C. mamane* considering the potential of this endophytic fungi to produce bioactive compounds such as the TDKPs. Co-culture of *C. mamane* with heat-killed bacteria and the evaluation of antibacterial activity of the crude extracts will complement the results obtained with these experiments.

GENERAL DISCUSSIONS, CONCLUSIONS AND PERSPECTIVES OF THE THESIS

This work focused on the study of the metabolome variations under different culture-based approaches of an endophytic fungal strain of *Cophinforma mamane* (Botryosphaeriaceae), isolated from *Bixa orellana* (Bixaceae), a medicinal plant from Peru. This doctoral thesis is a work that continues the research carried out by Ph.D. Fatima Barakat and Ph.D. Asih Triastuti in the framework of their doctoral work, which allowed the study of the metabolome of this fungus (Triastuti *et al.* 2019, 2021) and led to the isolation of 4 antiparasitic compounds, botryosulfuranols A, B, C (Barakat *et al.* 2019) and D (Barakat 2018) (unpublished data).

Our main objectives were: 1) to achieve a better understanding about the production of the TDKP botryosulfuranols A-D and other DKPs and 2) to induce the production of anti-infective metabolites.

In order to carry out this work, different approaches were followed. A time-series study was performed to analysis the metabolome changes over time while the OSMAC approach was applied in order to observe the influence of the culture media and the presence of light on the global chemical profile of *C. mamane*. In an attempt to induce lowly-expressed or silenced genes, different chemical elicitors were added into the fungal culture, including 3 epigenetic modifiers that act at the chromosome level (histone proteins) and a *quorum*-sensing (QS) molecule (homoserin lactone). Moreover, to specifically stimulate the biosynthetic pathways of alkaloids, including the TDKPs botryosulfuranols A-D, the amino acids L-tryptophan and L-phenylalanine were added as biosynthetic precursors into the fungal culture. Finally, in the context of the search for new anti-infectives, *C. mamane* was co-cultured with 3 different pathogenic bacterial strains. Also, a methodology using heat-killed *Leishmania* parasites for culturing *C. mamane* was developed in order to induce the production of antileishmanial metabolites, including the TDKPs, botryosulfuranols A-D.

General modulation of the chemical diversity of *C. mamane*

The methodologies used allowed us to identify different chemical groups produced by *C. mamane* in mostly all the conditions such as diketopiperazines, cyclic peptides, long and medium-chain fatty acids, eicosanoids, followed by sphingolipids, terpenoids, quinones, coumarins, hydroxyridines and benzenoids as the least frequent ones. Among the applied methodologies, the addition of epigenetic modifiers was the one that most altered the general metabolism of *C. mamane*, specifically the addition of 5-azacytidine (a DNA methyltransferase inhibitor) both in the up- and down-regulation of metabolites. Also, culturing *C. mamane* in ISP2 culture medium during 10 days under light conditions seemed to alter the general metabolism, both in the up-regulation and down-regulation of many metabolites, in comparison to the other methodologies used.

Besides the approaches regarding the variations of the culture media and culture conditions, the addition of dried host plant parts or crude extracts of the host plant to the culture medium of the endophytic fungi, may be another interesting method for simulating natural environment conditions that might trigger different biosynthetic pathways. Preliminary studies of the addition of crude extracts of *Bixa orella* and dried and ground leaves in the culture medium of *C. mamane* were carried out but they need to be further investigated, which will be also considered for future experiments.

One of the main results, focusing on putatively annotated metabolites, led us to the finding that this endophytic fungus produces a group of metabolites identified as cyclopentapeptides. These metabolites were observed, in most conditions, as the major peaks in the chemical chromatograms, appearing during an early stage (D4-D8) and decreasing rapidly after this period, as observed during the time-variations study. However, in a previous study of the dynamics in co-culture between *C. mamane* and *Fusarium solani*, the production of cyclopentapeptides by both fungi was maintained over time after 10 days of co-culture in MEA (Triastuti *et al.* 2021). Moreover, the cyclopentapeptides production was down-regulated under the presence of heat-killed *Leishmania* amastigotes but significantly induced under the presence of living bacteria, including *P. aeruginosa* and *S. aureus*. In previous studies, fungal cyclopentapeptides have exhibited diverse biological activities against other fungi, bacteria, virus and parasites with potential applications (Wang *et al.* 2017). However, their ecological role have been less discussed and in this regard, some authors have suggested their role in interspecies communication, such as in the case of the cyclopentapeptides produced by the endophytic fungus *Fusarium decemcellulare* (Li *et al.* 2016) or the hexocyclopeptides produced by endophytic fungus *Fusarium solani* (Wang *et al.* 2015). The down-regulation of the cyclopentapeptides production in *C. mamane* under the presence of heat-killed parasites but their up-regulation under the presence of living bacteria, reinforces their potential role as communication molecules between different microbial species but further research is needed in order to understand the triggering mechanisms as it is known for bacterial *quorum*-sensing process.

Modulation of the production of TDKPs botryosulfuranols A-D

The methodologies applied in this doctoral thesis had also the objective to induce the production of 4 TDKPs, previously isolated from *C. mamane* (Barakat *et al.* 2019). One interesting result is the short-time frame production observed during the time-variations study for botryosulfuranol C, a TDKP that possesses a di-sulfur bridge on its structure that might play an important role on its biological activity (Jiang & Guo 2011; Barakat *et al.* 2019). It is suggested that the producer organism could activate defense mechanisms that involves the opening of the di-sulfur bridge to avoid its toxicity as observed for the biosynthesis of the DKP gliotoxin produced by *Aspergillus fumigatus* (Scharf *et al.* 2014) which might explain the short time-frame production of botryosulfuranol C observed in *C. mamane*. Regarding

botryosulfuranol D, which is one of the most active TDKPs in comparison to botryosulfuranols A-C, exhibited a low production under the different methodologies and that was mostly undetected, indicating a different production pattern with a crucial step on its biosynthetic pathway influenced by the culture conditions and other factors.

Moreover, culturing *C. mamane* in ISP2 during 6 days in dark conditions seemed to exert a delaying effect on TDKPs production during the OSMAC approach study. It is known that the coding genes of these type of compounds respond to different environmental signals including light (Brakhage 2013) while a large proportion of the fungal genome respond to different light intensities and wavelengths (Yu & Fischer 2019). This finding highlights that light can influence the production of these metabolites in *C. mamane* and that it is an important factor to consider when culturing in laboratory conditions and during large-scale fermentations in bioreactors at the industrial level. Further experiments involving different light wavelengths when culturing *C. mamane* will be considered.

Regarding the addition of chemical modulators, the DNMTi AZA down-regulated the production of botryosulfuranols A, B and C, indicating that the inhibition of the DNA methylation has a negative effect on their production, either directly or indirectly along their biosynthetic pathway. However, the addition of HSL, which is a QS molecule involved in the communication of gram-negative bacteria with other species, significantly induced the production of botryosulfuranol C. It is also important to mention that HSL not only significantly induced the production of this TDKP but also the production of other putatively identified DKPs, including diphenylalazine B and bisdethiobis(methylthio)gliotoxin, highlighting that these types of metabolites might be involved in interspecies communication. Their role as QS molecules was also suggested for the DKPs produced during bacterial interactions between *Cronobacter sakazakii* and *Bacillus cereus* (Bofinger *et al.* 2017) but also for the DKPs, emestrin A and B, produced by *Aspergillus fumigatus* in presence of HSL and during co-culture with *Streptomyces bulli* (Rateb *et al.* 2013). In addition to this, the addition of heat-killed parasites into *C. mamane* culture significantly down-regulated the production of botryosulfuranol C while the addition of living bacteria did not significantly affect its production under the tested conditions. Longer fermentation periods and other bacterial and fungal species will be performed for the co-culture experiments in order to verify the influence of living microorganism on TDKPs production, considering the results obtained under the addition of the QS molecule, HSL and their potential role in microbial communication.

Furthermore, contrary to what was expected with the addition of two DKPs bioprecursors, L-phe and L-tryptophan, the production of botryosulfuranols A-D was down-regulated in comparison to the untreated culture, even though these metabolites are biogenetically derived from two phenylalanine residues (Barakat *et al.* 2019). More generally, the addition of these two amino acids did not seem to induce other DKPs derived from at least one phenylalanine, tyrosine or tryptophan (Welch & Williams 2014). Further investigations are needed in order to understand the negative effects that these amino acids exert on the

production of TDKPs while the addition of different amino acids at different concentrations will be also tested.

Induction of the production of anti-infective metabolites

The general alteration of the *C. mamane* metabolome observed under the addition of heat-killed parasites and the co-culture with living pathogenic bacteria seemed to significantly up- and down-regulate metabolites but no chemical family of metabolites could be identified to be specifically influenced under each condition. However, co-culturing fungus-fungus or fungus-bacterium has previously shown to induce metabolites that are not produced under axenic conditions or that are lowly detected (Kamdem *et al.* 2018) as a results of the diverse complex interactions regarding the competition for nutrients or antagonistic interactions in shared environments (Deshmukh *et al.* 2018). Different pathogenic bacterial strains with different resistant profiles as well as the co-culture with previously isolated endophytes from *Bixa orellana*, kept in the fungal collection of the UMR 152 laboratory, will be considered for future co-culture experiments.

Furthermore, we will contemplate the addition of living *Leishmania* parasites into the culture of *C. mamane* in order to evaluate the difference with heat-killed parasites, where mutual responses can potentially induce the production of anti-infective metabolites. The main limitations of this methodology will include the pH and the nutrients of the culture medium and the temperature and time of incubation, which will might need an appropriate culture chamber or a different dispositive to carry it out.

Moreover, metabolomics itself represents a strategy that facilitates and accelerates the laborious processes of natural product isolation and helps to avoid the re-isolation of known compounds by providing MS/MS information useful for structural elucidation and comparison with available databases (Demarque *et al.* 2020). However, it presents some limitations and challenges due to its inherent complexity, from samples preparation and data acquisition to reproducibility and interpretation issues (Marshall & Powers 2017). Furthermore, the absence of a consensus in structural data from different platforms and the low availability of free databases can generate low putative annotation rates (Van Santen *et al.* 2019), therefore, dereplication studies continue to be a very important part to complement this type of work. Despite of the limitations, metabolomics has unveiled information that otherwise would have remained hidden and this relatively novel approach used in the discovery of natural products has shown to effectively reduce large data sets into much smaller ones that potentially contain novel or active metabolites (Caesar *et al.* 2019). Different and better methodologies regarding the UHPL-HRMS analysis when considering specific metabolites as well as more replicates will be considered for future experiments with *C. mamane* and the metabolites produced by this this endophytic strain.

Conclusions

The results obtained under an untargeted metabolomics analysis provide us direct and useful information about what is being produced by cells during their metabolism, in this case, what *C. mamane* is producing at a specific time under a specific culture condition, giving us insights about its physiological status and cellular activity.

Integrating this information with other omics techniques such as genomics, proteomics and transcriptomics, which falls on the systems biology research field, will offer a more holistic understanding about microorganisms and even communities, especially for endophytic microorganism, representing at the same time an ongoing challenge (Pinu *et al.* 2019). In this sense, understanding the physiological responses of *C. mamane* to environmental changes and different culture conditions can lead to specific pathway activation or manipulation. This is useful for the search of specific metabolites, mainly to determine the optimal cultures conditions, including the optimal incubation time for their production and extraction, as is the case with the TDKPs.

In addition to this and besides the further experiments needed, the isolation of the most significant induced metabolites and the metabolites chemically related to the known TDKPs revealed by the molecular networking, will also be the focus of future work, as well as the understanding of their biological mechanisms and potential ecological roles in nature. Thanks to a pre-maturation project, financially supported by the Occitanie region, a highly concentrated TDKP fraction has been obtained as a result of a collaboration with Critt Bioindustries, who took over the cultivation of the fungus on a larger scale. This will allow further investigation for the *in vivo* biological activity of the TDKPs.

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Chapter 1

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Chapter 2

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Chapter 5

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ANNEXE 1 - TIME-SERIES STUDY

| ID | Alignment | Average Rt.min | Average Mz | Adduct type | Formula | Structure | Total score | Theoretical mass | Mass error |
|----|-----------|----------------|------------|------------------------|------------|---|-------------|------------------|------------|
| 1 | 1006 | 5.082 | 297.18481 | [M+H] ⁺ | C20H24O2 | 17 α -Ethinylestradiol | 5.2195 | 296.17763 | 0.0001065 |
| 2 | 101 | 3.117 | 135.1012 | [M+H] ⁺ | GH14O3 | | | 134.0942943 | 0.0003708 |
| 3 | 1010 | 8.379 | 297.24203 | [M+H+H2O] ⁺ | C18H34O4 | Octadecanedioic acid | 5.1112 | 314.2457096 | 0.0004213 |
| 4 | 1032 | 5.2 | 300.17975 | [M+H] ⁺ | C15H25NO5 | | | 299.1732729 | 0.0008494 |
| 5 | 1036 | 3.437 | 300.20148 | [M+NH4] ⁺ | C12H26O7 | | | 282.1678532 | 0.0001787 |
| 6 | 1041 | 7.447 | 301.28476 | [M+H] ⁺ | C17H36N2O2 | | | 300.2776784 | 0.0001548 |
| 7 | 1044 | 5.092 | 302.15915 | [M+H] ⁺ | C14H23NO6 | | | 301.1525375 | 0.0007139 |
| 8 | 1055 | 8.734 | 304.2988 | [M+H] ⁺ | | | | | |
| 9 | 1072 | 0.946 | 308.14929 | [M+H] ⁺ | C16H21NO5 | | | 307.1419728 | -5.08E-05 |
| 10 | 1073 | 5.578 | 308.14932 | [M+H] ⁺ | C16H21NO5 | | | 307.1419728 | -5.08E-05 |
| 11 | 108 | 0.879 | 140.06798 | [M+Na] ⁺ | C5H11NO2 | L-Valine | 5.4877 | 117.0789786 | 0.0001993 |
| 12 | 1081 | 5.735 | 309.08722 | [M+H] ⁺ | C17H12N2O4 | | | 308.0797069 | -0.0002167 |
| 13 | 1086 | 8.875 | 309.24249 | [M+H] ⁺ | C19H32O3 | androstane-3 β eta,6 α lpha,17 β | 5.035 | 308.2351449 | -7.87E-05 |
| 14 | 1094 | 5.962 | 310.22198 | [M+NH4] ⁺ | C14H28O6 | | | 292.1885886 | 0.0004142 |
| 15 | 1102 | 11.079 | 311.16382 | [M+H] ⁺ | C20H22O3 | | | 310.1568946 | 0.000371 |
| 16 | 1111 | 9.248 | 312.22198 | [M+H] ⁺ | | | | | |
| 17 | 1114 | 10.195 | 312.35995 | [M+H] ⁺ | | | | | |
| 18 | 1335 | 2.83 | 344.17001 | [M+H] ⁺ | C16H25NO7 | | | 343.1631021 | 0.0003786 |
| 19 | 1121 | 8.34 | 313.32172 | [M+H] ⁺ | C19H40N2O | | | 312.3140639 | -0.0003596 |
| 20 | 1138 | 4.357 | 315.19589 | [M+H] ⁺ | C20H26O3 | 4-oxo-Retinoic acid | 5.3372 | 314.1881947 | -0.0004289 |
| 21 | 1139 | 3.811 | 315.19604 | [M+H] ⁺ | C20H26O3 | 4-oxo-Retinoic acid | 5.1338 | 314.1881947 | -0.0005289 |
| 22 | 1160 | 5.002 | 318.15482 | [M+H+H2O] ⁺ | C15H21N5O4 | | | 335.1593542 | 0.0012659 |
| 23 | 1162 | 8.39 | 318.29987 | [M+H] ⁺ | C18H39NO3 | Phytosphingosine | 5.1831 | 317.2929941 | 0.0003706 |
| 24 | 1169 | 5.219 | 319.66742 | [M+2H] ²⁺ | | | | | |
| 25 | 1183 | 5.526 | 322.16431 | [M+H+H2O] ⁺ | C17H25NO6 | | | 339.1681875 | 0.0005993 |
| 26 | 1808 | 4.829 | 428.16754 | [M+H] ⁺ | C18H25N3O9 | | | 427.1590794 | -0.0011442 |
| 27 | 1194 | 4.452 | 324.1806 | [M+H] ⁺ | C17H25NO5 | | | 323.1732729 | -5.06E-05 |
| 28 | 1075 | 5.298 | 308.14941 | [M+H+H2O] ⁺ | C16H23NO6 | | | 325.1525375 | -0.0001508 |
| 29 | 1085 | 5.403 | 309.22675 | [M+H] ⁺ | C15H32O6 | | | 308.2198887 | 0.0004652 |
| 30 | 1220 | 5.456 | 327.15518 | [M+H] ⁺ | C15H22N2O6 | | | 326.1477864 | -0.0001371 |
| 31 | 1231 | 5.102 | 328.1745 | [M+H] ⁺ | C16H25NO6 | | | 327.1681875 | 0.000964 |
| 32 | 1233 | 4.978 | 328.1756 | [M+H] ⁺ | C16H25NO6 | | | 327.1681875 | -0.000136 |
| 33 | 1245 | 8.343 | 329.31604 | [M+H] ⁺ | | | | | |
| 34 | 125 | 0.903 | 147.07593 | [M+NH4] ⁺ | C5H7NO3 | Pyroglutamic acid | 5.0851 | 129.0425931 | 0.0005186 |
| 35 | 1250 | 8.907 | 330.29791 | [M+H] ⁺ | | | | | |
| 36 | 1253 | 9.091 | 330.33633 | [M+H] ⁺ | C20H43NO2 | | | 329.3293796 | 0.0003561 |
| 37 | 1262 | 6.481 | 332.15277 | [M+H] ⁺ | | | | | |
| 38 | 1267 | 8.472 | 332.27905 | [M+H] ⁺ | C18H37NO4 | | | 331.2722587 | 0.0004351 |
| 39 | 1277 | 5.263 | 334.13181 | [M+H] ⁺ | C14H23NO6S | | | 333.1246085 | 8.49E-05 |
| 40 | 1289 | 5.199 | 336.14355 | [M+H] ⁺ | C17H21NO6 | | | 335.1368874 | 0.0005638 |
| 41 | 1293 | 10.706 | 336.32571 | [M+H+H2O] ⁺ | C22H43NO2 | | | 353.3293796 | 0.0003914 |
| 42 | 1294 | 8.6 | 337.10425 | [M+Na] ⁺ | C18H18O5 | | | 314.1154237 | 0.0004444 |

| | | | | | | | | |
|----|------|--------|-----------|-------------------------|--------------|--------------------------------|-------------|------------|
| 43 | 1310 | 10.657 | 340.39404 | [M++] ⁺ | C16H24N2O6 | | 340.1634365 | -0.0001871 |
| 44 | 1313 | 5.639 | 341.17093 | [M++] ⁺ | C16H23NO7 | | 341.1474521 | -7.15E-05 |
| 45 | 1324 | 6.071 | 342.15482 | [M++] ⁺ | C16H23NO7 | | 341.1474521 | -0.0003715 |
| 46 | 1325 | 5.881 | 342.15509 | [M++] ⁺ | C16H23NO7 | | 343.1631021 | 0.0005786 |
| 47 | 1334 | 4.991 | 344.16977 | [M++] ⁺ | C16H25NO7 | | 321.2164751 | -0.0009042 |
| 48 | 1338 | 5.429 | 344.20663 | [M+Na] ⁺ | C16H27N5O2 | | 343.1994876 | -0.0001359 |
| 49 | 1340 | 5.714 | 344.20688 | [M++] ⁺ | C17H29NO6 | | | |
| 50 | 1341 | 3.674 | 344.228 | [M++] ⁺ | | | | |
| 51 | 1347 | 4.525 | 345.20178 | [M++] ⁺ | C16H28N2O6 | | 344.1947366 | 0.0002131 |
| 52 | 1352 | 4.056 | 346.186 | [M++] ⁺ | C16H27NO7 | | 345.1787522 | 2.87E-05 |
| 53 | 1354 | 9.127 | 346.33084 | [M++] ⁺ | | | | |
| 54 | 1363 | 4.724 | 350.17105 | [M++] ⁺ | C17H23N3O5 | | 349.1637708 | -5.27E-05 |
| 55 | 1378 | 6.024 | 352.14249 | [M++] ⁺ | C14H25NO7S | | 351.1351731 | -5.04E-05 |
| 56 | 1379 | 6.228 | 352.14276 | [M+NH4] ⁺ | C15H18N4O3S | | 334.1099614 | 0.000987 |
| 57 | 139 | 1.192 | 151.09625 | [M+H] ⁺ | G6H14O4 | | 150.0892089 | 0.0001854 |
| 58 | 1303 | 11.938 | 339.32559 | [M++-H2O] ⁺ | C22H44O3 | 22-Hydroxydocosanoic acid | 5.1297 | 0.000157 |
| 59 | 1422 | 3.966 | 358.26956 | [2M+H] ⁺ | | | | |
| 60 | 1432 | 4.277 | 359.21732 | [M+H] ⁺ | C17H30N2O6 | | 358.2103867 | 0.0003631 |
| 61 | 1436 | 7.062 | 360.15591 | [M+NH4] ⁺ | C18H18N2O5 | | 342.1215717 | -0.0005028 |
| 62 | 144 | 1.182 | 153.0753 | [M+H] ⁺ | C5H12O5 | | 152.0684735 | 0.0004499 |
| 63 | 1463 | 9.647 | 365.13528 | [M+Na] ⁺ | C20H22O5 | | 342.1467238 | 0.0006445 |
| 64 | 1465 | 4.102 | 366.12152 | [M+H] ⁺ | C18H15N5O4 | | 365.112404 | -0.0018196 |
| 65 | 1474 | 4.548 | 368.181 | [M+H] ⁺ | C17H25N3O6 | | 367.1743355 | 0.000612 |
| 66 | 1477 | 11.043 | 368.42523 | [M+H] ⁺ | | | | |
| 67 | 1479 | 5.202 | 369.21307 | [M+NH4] ⁺ | C17H25N3O5 | | 351.1794209 | 0.0001465 |
| 68 | 1509 | 10.947 | 374.30295 | [M+Na] ⁺ | C22H41NO2 | | 351.3137296 | 5.03E-05 |
| 69 | 1514 | 8.111 | 375.19843 | [M+H] ⁺ | C22H30O3S | | 374.1915658 | 0.0004423 |
| 70 | 1521 | 8.515 | 376.30508 | [M+NH4] ⁺ | C20H38O5 | | 358.2719243 | 0.0006499 |
| 71 | 1531 | 10.566 | 377.32059 | [M+H] ⁺ | C28H40 | | 376.3130013 | -0.0003223 |
| 72 | 1538 | 6.089 | 378.19098 | [M++-2H2O] ⁺ | C20H31NO8 | | 413.204967 | 0.000114 |
| 73 | 1539 | 5.811 | 378.19113 | [M+H] ⁺ | C20H27NO6 | | 377.1838376 | 1.40E-05 |
| 74 | 1548 | 4.531 | 380.18088 | [M+H] ⁺ | C18H25N3O6 | | 379.1743355 | 0.000712 |
| 75 | 1563 | 5.021 | 382.1853 | [M+H] ⁺ | C19H27NO7 | | 381.1787522 | 0.0007287 |
| 76 | 1566 | 4.892 | 383.08353 | [M+H] ⁺ | C15H18N4O4S2 | | 382.0769471 | 0.0007235 |
| 77 | 1567 | 9.947 | 383.20413 | [M+Na] ⁺ | C18H32O7 | | 360.2148034 | -7.59E-05 |
| 78 | 1596 | 4.352 | 387.224 | [2M+Na] ⁺ | C8H14N4O | | 182.1167611 | -0.0009029 |
| 79 | 1603 | 3.867 | 388.25351 | [M+H] ⁺ | C21H33N5S | | 387.2456671 | -0.0005565 |
| 80 | 1618 | 4.804 | 392.19128 | [M+H] ⁺ | C17H29NO9 | | 391.1842315 | 0.000208 |
| 81 | 1620 | 10.146 | 392.328 | [M+H] ⁺ | C23H41N3O2 | | 391.3198776 | -0.000846 |
| 82 | 1632 | 5.496 | 394.18604 | [M+H] ⁺ | C20H27NO7 | | 393.1787522 | 2.87E-05 |
| 83 | 1642 | 11.644 | 395.3306 | [M+H] ⁺ | C28H42O | sta-5,7,22,24(28)-tetraen-3-be | 5.2604 | 0.0002424 |
| 84 | 1656 | 4.896 | 398.18042 | [M+H] ⁺ | C19H27NO8 | | 397.1736668 | 0.0005433 |
| 85 | 1658 | 10.972 | 398.233 | [M+H] ⁺ | C24H31NO4 | | 397.2253085 | -0.0004151 |
| 86 | 1679 | 9.483 | 402.35648 | [M+H] ⁺ | C23H47NO4 | | 401.350509 | 0.0012854 |
| 87 | 1681 | 5.006 | 403.20697 | [M+H] ⁺ | C22H30N2O3S | | 402.1977138 | -0.0020097 |

| | | | | | | | | | |
|-----|------|--------|-----------|-------------------------|--------------|--------------------------|--------|-------------|------------|
| 88 | 1597 | 7.605 | 387.27371 | [M+H] ⁺ | C21H38O6 | 6-deoxyerythronolide B | 5.3016 | 386.2668389 | 0.0004154 |
| 89 | 1692 | 11.063 | 407.27158 | [M+H] ⁺ | C23H38N2O2S | | | 406.2653995 | 0.0010759 |
| 90 | 1502 | 7.98 | 373.25739 | [2M+H] ⁺ | C10H18O3 | | | 186.1255944 | 0.0002584 |
| 91 | 1713 | 12.03 | 409.30969 | [M+H] ⁺ | C28H40O2 | | | 408.3028305 | 0.000407 |
| 92 | 1718 | 4.645 | 410.2287 | [M+H] ⁺ | C21H27N7O2 | | | 409.2226231 | 0.0011996 |
| 93 | 1724 | 12.363 | 411.32533 | [M+H] ⁺ | C28H42O2 | Gamma-Tocotrienol | 5.5606 | 410.3184806 | 0.000557 |
| 94 | 1726 | 5.351 | 412.20715 | [M+H] ⁺ | C23H29N3O2S | | 5.0592 | 411.1980482 | -0.0018754 |
| 95 | 1742 | 4.74 | 415.25558 | [M+H] ⁺ | C19H34N4O6 | | | 414.2478348 | -0.0004887 |
| 96 | 1771 | 5.863 | 422.1806 | [M+H] ⁺ | C22H23N5O4 | | | 421.1750042 | 0.0016807 |
| 97 | 1683 | 10.553 | 403.23276 | [M+H] ⁺ | C20H34O8 | | | 402.225368 | -0.0001555 |
| 98 | 1803 | 11.394 | 427.3208 | [M+H] ⁺ | C28H42O3 | | | 426.3133952 | -0.0001283 |
| 99 | 1817 | 11.528 | 429.26062 | [M+Na] ⁺ | C24H38O5 | 7-Ketodeoxycholic acid | 5.0478 | 406.2719243 | 0.000545 |
| 100 | 1837 | 8.263 | 432.23782 | [M+NH4] ⁺ | C24H30O6 | | | 414.2042387 | 0.0002642 |
| 101 | 1838 | 4.031 | 432.27994 | [M+H] ⁺ | C19H37N5O6 | | | 431.2743839 | 0.0017604 |
| 102 | 1850 | 10.009 | 436.35751 | [M+H] ⁺ | C30H45NO | | | 435.3501151 | -0.0001085 |
| 103 | 1855 | 8.266 | 437.19345 | [M+Na] ⁺ | C24H30O6 | | | 414.2042387 | -4.06E-05 |
| 104 | 1873 | 6.564 | 440.22745 | [M+H] ⁺ | C22H33NO8 | | | 439.220617 | 0.0004935 |
| 105 | 1878 | 8.267 | 441.2576 | [M+NH4] ⁺ | C22H33NO7 | | | 423.2257024 | 0.0019279 |
| 106 | 1879 | 8.051 | 441.25845 | [M+NH4] ⁺ | C22H33NO7 | | | 423.2257024 | 0.0010279 |
| 107 | 1891 | 6.701 | 442.24338 | [M+H] ⁺ | C22H35NO8 | | | 441.2362671 | 0.0001435 |
| 108 | 1893 | 6.611 | 442.24362 | [2M+NH4] ⁺ | C11H16O4 | | | 212.104859 | -0.0003025 |
| 109 | 1895 | 6.847 | 442.24414 | [M+H] ⁺ | C23H31N5O4 | | | 441.2376045 | 0.0007809 |
| 110 | 1901 | 11.542 | 443.20456 | [M+Na] ⁺ | C23H32O7 | | | 420.2148034 | -0.0005759 |
| 111 | 1934 | 12.117 | 449.30225 | [M+H-2H2O] ⁺ | | | | | |
| 112 | 1937 | 7.505 | 450.07834 | [M+NH4] ⁺ | C19H16N2O6S2 | Botryosulfuranol C | | | |
| 113 | 490 | 6.279 | 225.08673 | [M+H] ⁺ | C10H12N2O4 | Hydroxykyurenine | 5.4224 | 224.0797069 | 0.0002833 |
| 114 | 1948 | 9.471 | 452.32147 | [M+NH4] ⁺ | C23H38N4O4 | | | 434.2893057 | 0.0016312 |
| 115 | 1961 | 9.672 | 454.29193 | [M+H] ⁺ | C21H44NO7P | | | 453.2855394 | 0.0009158 |
| 116 | 1970 | 7.324 | 456.26086 | [M+H] ⁺ | C24H33N5O4 | | | 455.2532545 | -0.000369 |
| 117 | 1972 | 11.813 | 456.31046 | [M+H] ⁺ | C28H41NO4 | | | 455.3035588 | 0.0003352 |
| 118 | 1802 | 11.094 | 427.24567 | [M+Na] ⁺ | C24H36O5 | Lovastatin | 6.0218 | 404.2562743 | -0.000205 |
| 119 | 2017 | 11.949 | 463.28152 | [M+Na] ⁺ | C28H40O4 | chaxalactin A, (rel)- | 5.1483 | 440.2926598 | 0.0003805 |
| 120 | 2005 | 4.85 | 460.25354 | [M+H] ⁺ | C22H37NO9 | | | 459.2468318 | 0.0006082 |
| 121 | 2030 | 12.211 | 465.29791 | [M+H] ⁺ | C30H40O4 | | | 464.2926598 | 0.0020362 |
| 122 | 2063 | 8.211 | 475.25754 | [M+H] ⁺ | C29H34N2O4 | | | 474.2518576 | 0.001634 |
| 123 | 2064 | 7.686 | 475.25993 | [M+H] ⁺ | C29H34N2O4 | | | 474.2518576 | 0.000134 |
| 124 | 2073 | 8.651 | 476.27695 | [M+H] ⁺ | C23H42NO7P | | | 475.2698893 | 0.0002658 |
| 125 | 1392 | 4.173 | 355.18668 | [M+H] ⁺ | C15H31O7P | | | 354.18074 | 0.0013164 |
| 126 | 2083 | 9.212 | 478.29099 | [M+Na] ⁺ | C28H41NO4 | | | 455.3035588 | 0.0017795 |
| 127 | 2084 | 9.033 | 478.29358 | [M+H] ⁺ | C23H44NO7P | LysopE(18:2(9Z,12Z)/0:0) | 5.0817 | 477.2855394 | -0.0007842 |
| 128 | 2094 | 9.917 | 480.30856 | [M+H] ⁺ | C23H46NO7P | | | 479.3011894 | -0.0001341 |
| 129 | 2095 | 9.719 | 480.30933 | [M+H] ⁺ | C23H46NO7P | | | 479.3011894 | -0.0008341 |
| 130 | 210 | 5.471 | 171.14876 | [M+H] ⁺ | C9H18N2O | | | 170.1419132 | 0.0003896 |
| 131 | 2100 | 5.161 | 480.6525 | [M+H] ⁺ | | | | | |
| 132 | 1995 | 8.371 | 459.26364 | [M+H] ⁺ | C29H34N2O3 | | | 458.2569429 | 0.0006194 |

| | | | | | | | | | |
|-----|------|--------|-----------|-------------------------|--------------|----------------------|--------|-------------|------------|
| 133 | 2104 | 11.552 | 481.29141 | [M+Na] ⁺ | C28H42O5 | candelalide C | 5.5591 | 458.3032244 | 0.0010451 |
| 134 | 2105 | 11.401 | 481.29199 | [M+Na] ⁺ | C28H42O5 | | | 458.3032244 | 0.0004451 |
| 135 | 2143 | 7.325 | 487.0961 | [M+Na] ⁺ | C21H24N2O6S2 | Botrysosulfuranol B | | 464.1067095 | -0.0001698 |
| 136 | 2127 | 7.922 | 484.15665 | [M+NH4] ⁺ | C21H26N2O6S2 | | | 466.1232286 | 0.0004541 |
| 137 | 2146 | 12.194 | 487.23059 | [M+H] ⁺ | C27H34O8 | | | 486.225368 | 0.0020445 |
| 138 | 1520 | 4.877 | 376.19632 | [M+H] ⁺ | C18H25N5O4 | | | 375.1906543 | 0.0016307 |
| 139 | 2175 | 11.775 | 494.56531 | [M+H] ⁺ | | | | | |
| 140 | 2177 | 12.195 | 495.30795 | [M+Na] ⁺ | C29H44O5 | | | 472.3188745 | 9.52E-05 |
| 141 | 2180 | 9.704 | 496.33936 | [M+H] ⁺ | C24H50NO7P | LysopC(16:0) | 5.1632 | 495.3324896 | 0.000366 |
| 142 | 2183 | 10.671 | 496.3938 | [M+Na] ⁺ | | | | | |
| 143 | 1481 | 11.924 | 369.29749 | [M+Na] ⁺ | C20H42O4 | | | 346.3083098 | 3.05E-05 |
| 144 | 2223 | 10.619 | 506.39932 | [M+H] ⁺ | C34H51NO2 | | | 505.3919799 | -4.37E-05 |
| 145 | 2230 | 8.587 | 508.38324 | [M+H] ⁺ | C26H53NO8 | | | 507.3771177 | 0.0011941 |
| 146 | 1106 | 9.451 | 311.25812 | [M+H] ⁺ | C19H34O3 | | | 310.2507949 | -2.86E-05 |
| 147 | 2237 | 9.528 | 510.35696 | [M+H] ⁺ | C25H52NO7P | | | 509.3481396 | -0.0015839 |
| 148 | 2240 | 10.177 | 510.35889 | [M+H] ⁺ | C32H47NO4 | | | 509.350509 | -0.0011146 |
| 149 | 2247 | 10.639 | 512.37384 | [M+H] ⁺ | C32H49NO4 | | | 511.366159 | -0.0003645 |
| 150 | 1788 | 10.863 | 424.36337 | [M+NH4] ⁺ | C22H46O6 | | | 406.3294392 | -0.0001353 |
| 151 | 2253 | 10.605 | 514.38885 | [M+H] ⁺ | C32H51NO4 | | | 513.3818091 | 0.0001856 |
| 152 | 2245 | 7.145 | 512.15302 | [M+NH4] ⁺ | C26H22O10 | | | 494.1212969 | 0.0021225 |
| 153 | 2264 | 8.684 | 518.32184 | [M+H] ⁺ | C26H48NO7P | | | 517.3168395 | 0.002316 |
| 154 | 2265 | 8.516 | 518.32489 | [M+H] ⁺ | C26H48NO7P | | | 517.3168395 | -0.000784 |
| 155 | 2274 | 4.297 | 520.33252 | [M+H] ⁺ | | | | | |
| 156 | 2282 | 9.945 | 522.35565 | [M+H] ⁺ | C26H52NO7P | | | 521.3481396 | -0.0002839 |
| 157 | 2283 | 9.749 | 522.35583 | [M+H] ⁺ | C26H52NO7P | LysopC(18:1(9Z)) | 5.1043 | 521.3481396 | -0.0003839 |
| 158 | 2284 | 12.14 | 522.59662 | [M+H] ⁺ | C36H75N | | | 521.5899514 | 0.0006279 |
| 159 | 2285 | 12.288 | 522.59662 | [M+H] ⁺ | C36H75N | | | 521.5899514 | 0.0006279 |
| 160 | 229 | 0.884 | 175.05748 | [M+H] ⁺ | C3H6N6O3 | | | 174.0501381 | -8.55E-05 |
| 161 | 2329 | 6.966 | 534.23651 | [M+H-2H2O] ⁺ | C33H35N3O6 | | | 569.2525858 | 0.0022329 |
| 162 | 2085 | 10.956 | 479.27713 | [M+H] ⁺ | C30H38O5 | | | 478.2719243 | 0.0021008 |
| 163 | 2351 | 9.25 | 542.31927 | [M+Na] ⁺ | C26H50NO7P | LysopC(18:2(9Z,12Z)) | 5.315 | 519.3324896 | 0.0024103 |
| 164 | 2276 | 9.066 | 520.33826 | [M+H] ⁺ | C26H50NO7P | LysopC(18:2(9Z,12Z)) | 5.2432 | 519.3324896 | 0.001466 |
| 165 | 2134 | 7.053 | 485.08157 | [M+Na] ⁺ | C21H22N2O6S2 | Botrysosulfuranol A | | 462.0919284 | -0.0004509 |
| 166 | 2391 | 11.735 | 554.17578 | [M+NH4] ⁺ | C28H58O9 | | | 538.4080834 | -0.000491 |
| 167 | 2400 | 10.796 | 556.44238 | [M+NH4] ⁺ | C30H56O10 | | | 576.387348 | 0.0008598 |
| 168 | 2411 | 9.606 | 559.38324 | [M+H-2O] ⁺ | C7H12N2O2 | | | 156.0898776 | 0.0003983 |
| 169 | 242 | 1.471 | 179.0787 | [M+Na] ⁺ | | | | | |
| 170 | 2430 | 4.41 | 564.35864 | [M+H] ⁺ | | | | | |
| 171 | 244 | 4.248 | 179.11758 | [M+H-2O] ⁺ | C10H16N2O2 | | | 196.1211778 | 0.0002895 |
| 172 | 2385 | 9.038 | 552.41187 | [M+H] ⁺ | C29H53N5O5 | Cyclopentapeptide | 4.6903 | 551.4046698 | 4.62E-05 |
| 173 | 2482 | 6.521 | 580.28528 | [M+H] ⁺ | C32H41N3O5S | | | 579.2766924 | -0.0013311 |
| 174 | 2491 | 9.37 | 583.45184 | [M+NH4] ⁺ | C30H55N5O5 | Cyclopentapeptide | 4.3852 | 565.4203199 | 0.0023454 |
| 175 | 2508 | 12.255 | 589.1792 | [M+H] ⁺ | C31H28N2O10 | | | 588.1743951 | 0.0024715 |
| 176 | 1431 | 4.556 | 359.21725 | [2M+H] ⁺ | C9H13N3O | | | 179.105862 | 0.000576 |
| 177 | 2519 | 6.352 | 592.28479 | [M+H] ⁺ | | | | | |

| | | | | | | | | |
|-----|------|--------|-----------|----------------------|-------------|---------------------|-------------|------------|
| 178 | 2542 | 10.77 | 600.46887 | [M+NH4] ⁺ | C30H62O10 | | 582.4342982 | -0.0007763 |
| 179 | 2556 | 10.686 | 604.43671 | [M+H] ⁺ | | | | |
| 180 | 2609 | 6.432 | 622.3338 | [M+H] ⁺ | | | | |
| 181 | 2627 | 11.813 | 628.49951 | [M+NH4] ⁺ | | | | |
| 182 | 2042 | 10.758 | 467.31326 | [M+Na] ⁺ | C28H44O4 | | 444.3239599 | -0.0001194 |
| 183 | 2704 | 11.098 | 658.51001 | [M+H] ⁺ | | | | |
| 184 | 273 | 4.207 | 185.11708 | [M+H] ⁺ | | | | |
| 185 | 2741 | 11.784 | 672.52515 | [M+NH4] ⁺ | C10H16O3 | Streptenol C | 184.1099444 | 0.0001208 |
| 186 | 2778 | 10.713 | 688.52112 | [M+NH4] ⁺ | | | | |
| 187 | 2783 | 10.643 | 690.3974 | [M+H] ⁺ | C41H55NO8 | | 689.3927677 | 0.0026442 |
| 188 | 2785 | 10.462 | 690.39868 | [M+H] ⁺ | C41H55NO8 | | 689.3927677 | 0.0013442 |
| 189 | 2788 | 11.514 | 690.61444 | [M+H] ⁺ | | | | |
| 190 | 2829 | 12.021 | 704.19318 | [M+NH4] ⁺ | C31H30N2O16 | | 686.1595329 | 0.0001584 |
| 191 | 2861 | 3.161 | 718.33728 | [M+H] ⁺ | C28H47N9O13 | | 717.3293326 | -0.000691 |
| 192 | 2886 | 10.686 | 732.54749 | [M+NH4] ⁺ | C49H66N2O2 | | 714.5124294 | -0.0012451 |
| 193 | 291 | 4.216 | 188.12796 | [M+H] ⁺ | C9H17NO3 | | 187.1208434 | 0.0001199 |
| 194 | 2942 | 3.148 | 759.32196 | [M+NH4] ⁺ | C40H43N3O11 | Polyethylene glycol | 741.2897592 | 0.0015847 |
| 195 | 2946 | 11.726 | 760.57776 | [M+NH4] ⁺ | | | | |
| 196 | 296 | 3.361 | 190.04977 | [M+H] ⁺ | C10H7NO3 | Kynurenic acid | 189.0425931 | 6.95E-05 |
| 197 | 2978 | 10.658 | 776.57416 | [M+NH4] ⁺ | | | | |
| 198 | 298 | 3.719 | 190.10757 | [M+NH4] ⁺ | C8H12O4 | | 172.0735589 | -0.0002156 |
| 199 | 3007 | 10.75 | 785.50165 | [M+Na] ⁺ | C40H74O13 | | 762.5129424 | 0.0005631 |
| 200 | 3009 | 10.504 | 785.50232 | [M+Na] ⁺ | C40H74O13 | | 762.5129424 | -0.0001369 |
| 201 | 2962 | 3.407 | 771.24805 | [M+H] ⁺ | C38H42O17 | | 770.2421999 | 0.0014763 |
| 202 | 3025 | 11.19 | 799.51904 | [M+H] ⁺ | C43H70N6O6S | Symprostatin 1 | 798.507755 | -0.0039686 |
| 203 | 3029 | 9.776 | 801.49646 | [M+Na] ⁺ | C40H74O14 | | 778.507857 | 0.0005777 |
| 204 | 304 | 5.112 | 191.29794 | [2M+Na] ⁺ | | | | |
| 205 | 3043 | 11.698 | 804.604 | [M+H] ⁺ | | | | |
| 206 | 3056 | 11.494 | 813.53479 | [2M+H] ⁺ | C23H38N2O2S | | 406.2653995 | 0.0013634 |
| 207 | 308 | 5.503 | 193.0858 | [M+H] ⁺ | C11H12O3 | | 192.0786442 | 0.0001207 |
| 208 | 3166 | 11.381 | 899.57178 | [M+Na] ⁺ | | | | |
| 209 | 3230 | 11.074 | 943.59753 | [M+Na] ⁺ | | | | |
| 210 | 3249 | 11.968 | 953.61761 | [M+H] ⁺ | C52H86O15 | | 952.6123221 | 0.0019986 |
| 211 | 3268 | 11.192 | 969.61432 | [M+H] ⁺ | C53H84N4O12 | | 968.6085741 | 0.0015506 |
| 212 | 3271 | 11.596 | 971.62817 | [M+H] ⁺ | C53H86N4O12 | | 970.6242242 | 0.0033006 |
| 213 | 3281 | 10.803 | 987.62225 | [M+Na] ⁺ | C63H84N2O6 | | 964.6329384 | -0.0001409 |
| 214 | 3282 | 10.658 | 987.62299 | [M+H] ⁺ | C63H86N4O13 | | 986.6191388 | 0.0034153 |
| 215 | 2363 | 10.037 | 544.36346 | [M+H] ⁺ | C32H49NO6 | | 543.3559883 | -0.0002353 |
| 216 | 370 | 2.89 | 201.07533 | [M+H] ⁺ | C9H12O5 | | 200.0684735 | 0.0004499 |
| 217 | 374 | 1.479 | 202.15491 | [M+H] ⁺ | C9H19N3O2 | | 201.1477268 | 0.0001033 |
| 218 | 378 | 4.078 | 203.12743 | [M+H] ⁺ | C10H18O4 | | 202.1205091 | 0.0003855 |
| 219 | 392 | 0.911 | 206.04558 | [M+Na] ⁺ | C5H13NO4S | | 183.0565289 | 0.0001496 |
| 220 | 401 | 3.695 | 208.10773 | [M+H] ⁺ | C10H13N3O2 | | 207.1007767 | 0.0003531 |
| 221 | 407 | 1.794 | 210.07588 | [M+H] ⁺ | C10H11NO4 | | 209.0688078 | 0.0001843 |
| 222 | 410 | 3.958 | 210.1698 | [M+NH4] ⁺ | | | | |

| | | | | | | | | | |
|-----|------|--------|-----------|-------------|------------|--|-------------------------------|-------------|------------|
| 223 | 428 | 7.016 | 213.112 | [M++]+ | C11H16O4 | | | 212.104859 | 0.0001354 |
| 224 | 429 | 5.244 | 213.11209 | [M++]+ | C11H16O4 | | | 212.104859 | 3.54E-05 |
| 225 | 450 | 3.031 | 216.12267 | [M++]+ | C10H17NO4 | | | 215.115758 | 0.0003345 |
| 226 | 451 | 5.372 | 216.15926 | [M++]+ | C11H21NO3 | | | 215.1521435 | 0.00012 |
| 227 | 455 | 3.765 | 217.09698 | [M++]+ | C12H12N2O2 | | | 216.0898776 | 0.0001541 |
| 228 | 456 | 0.979 | 217.10439 | [M++]+ | | | | | |
| 229 | 1080 | 0.834 | 309.05576 | [M++]+ | | | | | |
| 230 | 476 | 5.612 | 222.11243 | [M++]+ | C12H15NO3 | | | 221.1051933 | 6.98E-05 |
| 231 | 481 | 3.515 | 223.15347 | [M++]+ | C10H22O5 | | | 222.1467238 | 0.0005003 |
| 232 | 489 | 5.366 | 224.18565 | [M+NH4]+ | C10H22O4 | | | 206.1518092 | -6.53E-05 |
| 233 | 400 | 4.268 | 208.09625 | [M++H2O]+ | C11H15NO4 | | | 225.100108 | 0.0005197 |
| 234 | 502 | 3.378 | 227.09137 | [M++]+ | C11H14O5 | | | 226.0841235 | 0 |
| 235 | 504 | 2.325 | 227.10255 | [M++]+ | C10H14N2O4 | | | 226.0953569 | 3.34E-05 |
| 236 | 505 | 6.592 | 227.10274 | [M++]+ | C10H14N2O4 | | Porphobilinogen | 226.0953569 | -6.66E-05 |
| 237 | 510 | 7.076 | 228.1954 | [M++]+ | C13H25NO2 | | | 227.188529 | 0.0004055 |
| 238 | 513 | 10.264 | 228.23228 | [M++]+ | C14H29NO | | | 227.2249145 | -0.000109 |
| 239 | 518 | 4.282 | 229.10693 | [M++]+ | C11H16O5 | | | 228.097736 | 0.0001501 |
| 240 | 535 | 4.58 | 230.13834 | [M++]+ | C11H19NO4 | | | 229.1314081 | 0.0003845 |
| 241 | 539 | 5.895 | 230.17487 | [M++]+ | C12H23NO3 | | | 229.1677936 | 0.00017 |
| 242 | 541 | 7.472 | 230.24719 | [M++]+ | C14H31NO | | | 229.2405646 | 0.0006411 |
| 243 | 542 | 7.896 | 230.24767 | [M++]+ | C14H31NO | | | 229.2405646 | 0.0001411 |
| 244 | 558 | 7.506 | 232.11191 | [M++-2H2O]+ | C17H17NO2 | | | 267.1259288 | 0.0001759 |
| 245 | 561 | 2.857 | 232.15396 | [M++]+ | C11H21NO4 | | | 231.1470582 | 0.0003346 |
| 246 | 563 | 1.004 | 232.15428 | [M++]+ | C11H21NO4 | | Butyrylcarnitine | 231.1470582 | 3.46E-05 |
| 247 | 599 | 3.369 | 240.18019 | [M+NH4]+ | C10H22O5 | | | 222.1467238 | 0.0003494 |
| 248 | 1249 | 5.381 | 330.19138 | [M++]+ | C16H27NO6 | | | 329.1838376 | -0.000286 |
| 249 | 612 | 10.653 | 242.24786 | [M++]+ | C15H31NO | | | 241.2405646 | -5.89E-05 |
| 250 | 1429 | 4.965 | 359.18097 | [M++]+ | C17H22N6O3 | | | 358.1753386 | 0.001615 |
| 251 | 501 | 4.351 | 227.09108 | [M++]+ | C11H14O5 | | | 226.0841235 | 3.00E-04 |
| 252 | 627 | 5.427 | 244.15398 | [M++]+ | C12H21NO4 | | Tiglylcarnitine | 243.1470582 | 0.0003346 |
| 253 | 628 | 4.946 | 244.15401 | [M++]+ | C12H21NO4 | | | 243.1470582 | 0.0003346 |
| 254 | 497 | 6.391 | 226.17963 | [M++H2O]+ | C13H25NO3 | | | 243.1834437 | 0.0005554 |
| 255 | 1699 | 11.721 | 408.30835 | [M+Na]+ | C22H43NO4 | | | 385.3192089 | 0.0001296 |
| 256 | 631 | 11.854 | 244.19066 | [M+NH4]+ | C13H22O3 | | | 226.1568946 | 2.01E-05 |
| 257 | 632 | 4.897 | 244.25784 | [M++]+ | | | | | |
| 258 | 646 | 2.001 | 246.13303 | [M++]+ | C11H19NO5 | | | 245.1263227 | 0.0005992 |
| 259 | 648 | 3.732 | 246.16965 | [M++]+ | C12H23NO4 | | | 245.1627082 | 0.0003847 |
| 260 | 649 | 3.343 | 247.11732 | [M++]+ | C11H18O6 | | | 246.1103383 | 0.0003147 |
| 261 | 791 | 1.273 | 270.09491 | [M+Na]+ | C10H17NO6 | | | 247.1055873 | -9.20E-05 |
| 262 | 550 | 5.653 | 231.123 | [M++]+ | C11H18O5 | | | 230.1154237 | -0.0002999 |
| 263 | 659 | 0.985 | 249.08444 | [M+Na]+ | C10H14N2O4 | | Porphobilinogen | 226.0953569 | 0.0001776 |
| 264 | 662 | 7.049 | 250.17824 | [M++]+ | | | | | |
| 265 | 663 | 3.902 | 250.18004 | [M++]+ | C15H23NO2 | | c-1H-pyrrol-1-yl)-2-methyldec | 5.0916 | 0.0001554 |
| 266 | 664 | 4.772 | 251.18512 | [M++]+ | C12H26O5 | | | 250.1780239 | 0.0002004 |
| 267 | 689 | 3.111 | 256.17514 | [M+NH4]+ | C10H22O6 | | | 238.1416384 | 0.000364 |

| | | | | | | | | | |
|-----|------|--------|-----------|-------------------------|------------|-------------------------------|-------------|------------|--|
| 268 | 690 | 11.333 | 256.26358 | [M+H-H2O] ⁺ | | | | | |
| 269 | 705 | 5.025 | 258.16992 | [M+H] ⁺ | | | | | |
| 270 | 623 | 2.021 | 244.11798 | [M+H-H2O] ⁺ | C13H23NO4 | | 257.1627082 | 8.47E-05 | |
| 271 | 747 | 3.568 | 264.1225 | [M+H] ⁺ | C11H19NO6 | | 261.1212373 | -5.09E-05 | |
| 272 | 660 | 7.38 | 249.20563 | [M+H] ⁺ | C14H17NO4 | | 263.115758 | 0.0005345 | |
| 273 | 384 | 0.937 | 204.12315 | [M+H] ⁺ | C13H28O4 | | 248.1987594 | 0.0004358 | |
| 274 | 794 | 11.625 | 270.27914 | [M+H] ⁺ | C9H17NO4 | L-Acetylcarnitine | 203.115758 | -0.0001655 | |
| 275 | 802 | 5.978 | 272.1492 | [M+H] ⁺ | C17H35NO | | 269.2718647 | 4.12E-05 | |
| 276 | 803 | 5.706 | 272.1499 | [M+H] ⁺ | C13H21NO5 | | 271.1419728 | 4.92E-05 | |
| 277 | 804 | 2.317 | 272.16043 | [M+H] ⁺ | C12H21N3O4 | | 271.1532062 | -0.0006508 | |
| 278 | 815 | 4.92 | 274.16464 | [M+H] ⁺ | C13H23NO5 | | 273.1576228 | 8.26E-05 | |
| 279 | 937 | 4.607 | 290.13904 | [M+H] ⁺ | C16H22O4 | | 289.1314081 | 0.0002993 | |
| 280 | 818 | 7.424 | 274.27344 | [M+H] ⁺ | C16H19NO4 | | | -0.0003155 | |
| 281 | 822 | 0.927 | 275.21188 | [2M+H] ⁺ | | | | | |
| 282 | 1279 | 6.407 | 334.27359 | [M+H] ⁺ | C21H35NO2 | | 333.2667794 | 0.0004558 | |
| 283 | 841 | 11.247 | 278.21164 | [M+H] ⁺ | C17H27NO2 | | 277.2041791 | -0.0001444 | |
| 284 | 845 | 3.581 | 279.13367 | [M+H] ⁺ | C14H18N2O4 | | 278.1266571 | 0.0002335 | |
| 285 | 846 | 9.962 | 279.1593 | [M+H] ⁺ | C16H22O4 | [1-hydroxy-1-methylethyl]-8-(| 278.1518092 | -0.0002144 | |
| 286 | 386 | 0.886 | 205.06834 | [M+Na] ⁺ | G6H14O6 | Galactitol | 182.0790382 | -4.11E-05 | |
| 287 | 864 | 11.681 | 282.22156 | [M+H] ⁺ | C20H27N | | 281.2143499 | 2.63E-05 | |
| 288 | 865 | 11.49 | 282.27933 | [M+H] ⁺ | C18H35NO | Oleamide | 281.2718647 | -0.0001588 | |
| 289 | 871 | 8.06 | 283.19049 | [M+H] ⁺ | C16H26O4 | | 282.1831093 | -0.0001142 | |
| 290 | 882 | 4.902 | 284.14944 | [M+H] ⁺ | C14H21NO5 | | 283.1419728 | -0.0001508 | |
| 291 | 885 | 12.431 | 284.29486 | [M+H] ⁺ | C18H37NO | Octadecanamide | 283.2875148 | -0.0001087 | |
| 292 | 886 | 12.314 | 284.29501 | [M+H] ⁺ | C18H37NO | | 283.2875148 | -0.0002087 | |
| 293 | 1333 | 7.409 | 343.29544 | [M+Na] ⁺ | | | | | |
| 294 | 913 | 10.489 | 287.22189 | [M+H] ⁺ | C16H30O4 | Hexadecanedioic acid | 286.2144094 | -0.0002141 | |
| 295 | 1263 | 5.028 | 332.17004 | [M+H] ⁺ | C15H25NO7 | | 331.1631021 | 0.0003786 | |
| 296 | 923 | 7.711 | 288.29123 | [M+H] ⁺ | | | | | |
| 297 | 938 | 6.935 | 290.15945 | [M+H] ⁺ | C13H23NO6 | | 289.1525375 | 0.0003139 | |
| 298 | 1560 | 6.279 | 381.18106 | [M+H-2H2O] ⁺ | C22H28N2O6 | | 416.1947366 | -0.0002163 | |
| 299 | 96 | 10.724 | 134.04448 | [M+H] ⁺ | C4H7NO4 | L-Aspartic acid | 133.0375077 | 0.0002842 | |
| 300 | 960 | 5.119 | 292.15359 | [M+H] ⁺ | C16H21NO4 | | 291.1470582 | 0.0007346 | |
| 301 | 961 | 4.655 | 292.15393 | [M+H-2H2O] ⁺ | C16H25NO6 | | 327.1681875 | 0.0004346 | |
| 302 | 962 | 4.924 | 292.15445 | [M+H] ⁺ | C16H21NO4 | | 291.1470582 | -6.54E-05 | |
| 303 | 993 | 7.837 | 295.22675 | [M+H-H2O] ⁺ | C18H32O4 | 8,13-DihODE | 312.2300595 | 7.13E-05 | |

ANNEXE 2 - OSMAC APPROACH

| ID | Alignment | Average Rt.min. | Average M/z | Adduct: type | Formula | Structure | Theoretical mass | Mass error |
|----|-----------|-----------------|-------------|--------------------------------------|---|------------------------------------|------------------|------------|
| 1 | 108 | 0.902 | 123.0555 | [M+H] ⁺ | C ₆ H ₆ N ₂ O | Niacinamide | 122.0480128 | -0.0002107 |
| 2 | 113 | 1.269 | 124.03941 | [M+H] ⁺ | C ₆ H ₅ N ₂ O | Nicotinic acid | 123.0320284 | -9.51E-05 |
| 3 | 114 | 1.051 | 124.0395 | [M+H] ⁺ | C ₆ H ₅ N ₂ O | Nicotinic acid | 123.0320284 | -0.0001951 |
| 4 | 1143 | 2.783 | 201.12352 | [M+H] ⁺ | C ₉ H ₁₆ N ₂ O ₃ | | 200.1160924 | -0.0001312 |
| 5 | 115 | 9.706 | 124.08679 | [M+H] ⁺ | C ₆ H ₉ N ₃ | | 123.0796473 | 0.0001237 |
| 6 | 1159 | 4.939 | 202.14467 | [M+H] ⁺ | C ₁₀ H ₁₉ N ₃ O | | 201.1364935 | -0.0009301 |
| 7 | 1162 | 1.011 | 202.1803 | [M+H] ⁺ | C ₁₁ H ₂₃ N ₂ O | | 201.172879 | -0.0001446 |
| 8 | 1189 | 5.323 | 204.11406 | [M+H] ⁺ | C ₁₁ H ₁₃ N ₃ O | | 203.105862 | -0.0009615 |
| 9 | 1234 | 4.914 | 207.15929 | [M+H] ⁺ | C ₁₀ H ₂₂ O ₄ | | 206.1518092 | -0.0002144 |
| 10 | 1241 | 4.595 | 208.097 | [M+H] ⁺ | C ₁₁ H ₁₃ N ₃ O | N-Acetyl-L-phenylalanine | 207.0895433 | -0.0001803 |
| 11 | 1300 | 5.447 | 211.05466 | [M+H] ⁺ | C ₉ H ₁₀ N ₂ O ₂ S | | 210.0462986 | -0.001125 |
| 12 | 1301 | 7.21 | 211.08641 | [M+H] ⁺ | C ₁₃ H ₁₀ N ₂ O | | 210.0793129 | 0.0001894 |
| 13 | 1307 | 4.409 | 211.14413 | [M+NH ₄] ⁺ | C ₁₁ H ₁₅ N ₂ O | | 193.1102787 | 4.30E-06 |
| 14 | 1309 | 1.055 | 211.14424 | [M+NH ₄] ⁺ | C ₁₁ H ₁₅ N ₂ O | | 193.1102787 | -9.57E-05 |
| 15 | 1325 | 1.406 | 212.1032 | [M+H] ⁺ | C ₉ H ₁₃ N ₃ O | | 211.0956913 | -0.0002323 |
| 16 | 1358 | 2.328 | 214.08952 | [M+H] ⁺ | C ₁₀ H ₁₅ N ₂ O ₂ S | | 213.0823497 | 0.0001262 |
| 17 | 1359 | 0.824 | 214.08969 | [M+H] ⁺ | C ₁₀ H ₁₅ N ₂ O ₂ S | N-Butylbenzenesulfonamide | 213.0823497 | -7.38E-05 |
| 18 | 1394 | 6.55 | 216.15955 | [M+H] ⁺ | C ₁₁ H ₂₁ N ₃ O | | 215.1521435 | -8.00E-05 |
| 19 | 1422 | 4.181 | 218.1297 | [M+H] ⁺ | C ₁₂ H ₁₅ N ₃ O | | 217.1215121 | -0.0009114 |
| 20 | 1175 | 0.964 | 203.04686 | [M+H] ⁺ | C ₇ H ₁₀ N ₂ O ₃ S | | 202.0412132 | 0.0015896 |
| 21 | 1456 | 2.619 | 220.1187 | [M+H] ⁺ | C ₉ H ₁₇ N ₅ O | Pantothenic acid | 219.1106726 | -0.0007509 |
| 22 | 1476 | 3.976 | 221.13161 | [M+H] ⁺ | C ₁₂ H ₁₆ N ₂ O ₂ | | 220.1211778 | -0.0031458 |
| 23 | 1550 | 4.658 | 227.05357 | [2M+NH ₄] ⁺ | | | | |
| 24 | 1553 | 2.38 | 227.1032 | [M+H] ⁺ | C ₁₀ H ₁₄ N ₂ O ₄ | Porphobilinogen | 226.0953569 | -0.0005666 |
| 25 | 1558 | 4.14 | 227.13882 | [M+H] ⁺ | C ₁₁ H ₁₈ N ₂ O ₃ | | 226.1317424 | 0.0002189 |
| 26 | 1561 | 5.156 | 227.1393 | [M+H] ⁺ | C ₁₁ H ₁₈ N ₂ O ₃ | | 226.1317424 | -0.0002811 |
| 27 | 1577 | 2.948 | 228.13412 | [M+NH ₄] ⁺ | C ₁₀ H ₁₄ N ₂ O ₃ | | 210.1004423 | 0.0001679 |
| 28 | 1578 | 3.171 | 228.13455 | [M+H] ⁺ | C ₁₀ H ₁₇ N ₃ O ₃ | γ-cedipptide A; (-)-Cordycepidpept | 227.1269914 | -0.0003321 |
| 29 | 1582 | 7.216 | 228.18906 | [M+H] ⁺ | | | | |
| 30 | 1586 | 10.899 | 228.23224 | [M+H] ⁺ | C ₁₄ H ₂₉ N ₃ O | | 227.2249145 | -9.00E-06 |
| 31 | 1610 | 6.017 | 230.17619 | [M+H] ⁺ | C ₁₂ H ₂₃ N ₃ O | | 229.1677936 | -0.00113 |
| 32 | 1620 | 1.01 | 231.08386 | [M+H-2H ₂ O] ⁺ | C ₁₁ H ₁₄ N ₄ O ₄ | | 266.1015049 | 0.003752 |
| 33 | 1357 | 1.268 | 214.08939 | [M+H] ⁺ | C ₁₀ H ₁₅ N ₂ O ₂ S | | 213.0823497 | 0.0002262 |
| 34 | 1633 | 3.471 | 232.14743 | [M+H] ⁺ | C ₁₃ H ₁₇ N ₃ O | | 231.1371622 | -0.0029614 |
| 35 | 1695 | 4.388 | 238.10045 | [M+H] ⁺ | C ₁₁ H ₁₅ N ₃ O ₅ | | 237.0935831 | 0.0004596 |
| 36 | 1698 | 6.899 | 238.12596 | [M+H] ⁺ | C ₉ H ₁₉ N ₆ O | | 237.1212373 | 0.0025138 |
| 37 | 1699 | 5.494 | 238.14235 | [M+Na] ⁺ | C ₁₄ H ₂₁ N ₃ O | | 215.1521435 | -0.0009358 |
| 38 | 1726 | 5.365 | 240.12415 | [M+H] ⁺ | C ₁₂ H ₁₇ N ₄ O | | 239.115758 | -0.0010655 |
| 39 | 1727 | 6.256 | 240.12418 | [M+H] ⁺ | C ₁₂ H ₁₇ N ₄ O | | 239.115758 | -0.0011655 |
| 40 | 1728 | 4.432 | 240.12427 | [M+FA+H] ⁺ | C ₁₁ H ₁₅ N ₂ O | | 193.1102787 | -0.0012658 |
| 41 | 1730 | 2.404 | 240.18028 | [M+H] ⁺ | C ₁₄ H ₂₅ N ₅ O | | 239.1170708 | -0.0022527 |
| 42 | 1741 | 3.029 | 241.11806 | [M+FA+H] ⁺ | C ₁₀ H ₁₄ N ₂ O ₂ | | 194.1055277 | 0.0001831 |

| | | | | | | | |
|----|------|--------|-----------|-------------------------|-------------|-------------|------------|
| 43 | 1763 | 10.972 | 242.24785 | [M+H] ⁺ | C15H31NO | 241.2405646 | 4.11E-05 |
| 44 | 1777 | 3.837 | 243.13385 | [M+H] ⁺ | C11H18N2O4 | 242.1266571 | 3.35E-05 |
| 45 | 1778 | 4.206 | 243.13406 | [M+H] ⁺ | C11H18N2O4 | 242.1266571 | -0.0001665 |
| 46 | 1805 | 1.008 | 245.09517 | [2M+H] ⁺ | CGH6N2O | 122.0480128 | 0.0037767 |
| 47 | 1809 | 4.633 | 245.12862 | [M+H] ⁺ | C14H16N2O2 | 244.1211778 | -0.0001458 |
| 48 | 181 | 1.727 | 131.07019 | [M+H] ⁺ | CGH10O3 | 130.0629942 | 7.06E-05 |
| 49 | 1810 | 4.836 | 245.12866 | [2M+Na] ⁺ | CGH9NO | 111.0684139 | -0.0016 |
| 50 | 1815 | 4.317 | 245.15002 | [M+H] ⁺ | C11H20N2O4 | 244.1423071 | -0.0004164 |
| 51 | 1830 | 4.221 | 246.16295 | [M+H] ⁺ | C14H19N3O | 245.1528122 | -0.0028113 |
| 52 | 1257 | 4.809 | 209.11734 | [M+H-H2O] ⁺ | C12H18O4 | 226.1205091 | -7.92E-05 |
| 53 | 1874 | 3.598 | 249.12344 | [M+H-H2O] ⁺ | C13H18N2O4 | 266.1266571 | -3.12E-05 |
| 54 | 1887 | 11.023 | 251.04663 | [M+H] ⁺ | C11H10N2O3S | 250.0412132 | 0.0018896 |
| 55 | 1890 | 4.899 | 251.18639 | [M+H] ⁺ | C12H26O5 | 250.1780239 | -0.0010996 |
| 56 | 1891 | 8.192 | 251.2005 | [M+H] ⁺ | C16H26O2 | 250.1932801 | 5.65E-05 |
| 57 | 1898 | 6.578 | 252.06348 | [M+H] ⁺ | C15H9NO3 | 251.0582431 | 0.0020196 |
| 58 | 1931 | 4.736 | 254.13995 | [M+H-2H2O] ⁺ | C14H19N5O2 | 289.1538748 | 2.19E-05 |
| 59 | 1716 | 3.214 | 239.14981 | [M+H] ⁺ | C11H18N4O2 | 238.1429758 | 0.0004523 |
| 60 | 1968 | 11.504 | 256.26346 | [M+H] ⁺ | C16H33NO | 255.2562147 | -8.90E-06 |
| 61 | 1977 | 5.642 | 257.08105 | [M+H-H2O] ⁺ | C15H14O5 | 274.0841235 | -0.0002647 |
| 62 | 198 | 3.297 | 133.07629 | [M+H] ⁺ | C8H8N2 | 132.0687483 | -0.0002753 |
| 63 | 1793 | 6.514 | 244.19188 | [M+H] ⁺ | C13H25NO3 | 243.1834437 | -0.0011799 |
| 64 | 2020 | 0.64 | 259.14716 | [M+H] ⁺ | C15H18N2O2 | 258.1368278 | -0.0030957 |
| 65 | 2021 | 1.255 | 259.14725 | [M+H] ⁺ | C15H18N2O2 | 258.1368278 | -0.0030957 |
| 66 | 1946 | 5.565 | 255.0654 | [M+H] ⁺ | C15H10O4 | 254.0579088 | -0.0002147 |
| 67 | 2035 | 4.913 | 260.11304 | [M+H] ⁺ | C11H17NO6 | 259.1055873 | -0.0001363 |
| 68 | 2050 | 5.696 | 261.15994 | [2M+Na] ⁺ | | | |
| 69 | 2063 | 3.408 | 262.11862 | [M+NH4] ⁺ | C10H16N2O3S | 244.0881634 | 0.0033889 |
| 70 | 2073 | 4.699 | 263.09497 | [M+H-H2O] ⁺ | C14H16O6 | 280.0946882 | -0.0036 |
| 71 | 2075 | 3.83 | 263.1026 | [M+H] ⁺ | C13H14N2O4 | 262.0953569 | 3.34E-05 |
| 72 | 2106 | 4.797 | 265.09744 | [M+FA+H] ⁺ | C15H10N2 | 218.0843983 | -0.0002462 |
| 73 | 2147 | 1.279 | 268.1044 | [2M+NH4] ⁺ | | | |
| 74 | 2148 | 1.024 | 268.10443 | [2M+NH4] ⁺ | | | |
| 75 | 1889 | 5.701 | 251.18571 | [M+H] ⁺ | C12H26O5 | 250.1780239 | -0.0003996 |
| 76 | 2179 | 3.281 | 270.12576 | [M+H] ⁺ | C15H15N3O2 | 269.1164267 | -0.0020968 |
| 77 | 2184 | 11.802 | 270.27939 | [M+H] ⁺ | C17H35NO | 269.2718647 | -0.0002588 |
| 78 | 2200 | 7.481 | 271.18848 | [M+Na] ⁺ | C14H24N4 | 248.2000968 | 0.0008175 |
| 79 | 2213 | 3.126 | 272.14883 | [M+H] ⁺ | C13H21NO5 | 271.1419728 | 0.0004492 |
| 80 | 2222 | 7.945 | 272.25833 | [M+H] ⁺ | C16H33NO2 | 271.2511293 | 0.0001057 |
| 81 | 2238 | 5.269 | 273.18225 | [M+H] ⁺ | C18H24O2 | 272.17763 | 0.0026065 |
| 82 | 2251 | 10.467 | 274.15094 | [M+NH4] ⁺ | C10H16N4O4 | 256.117155 | 8.05E-05 |
| 83 | 2271 | 3.355 | 275.04855 | [M+H] ⁺ | C13H10N2O3S | 274.0412132 | -0.0001104 |
| 84 | 2273 | 4.045 | 275.15244 | [2M+H] ⁺ | | | |
| 85 | 2291 | 4.849 | 276.12207 | [M+Na] ⁺ | C13H19NO4 | 253.1314081 | -0.0014712 |
| 86 | 2310 | 3.892 | 277.11804 | [M+H] ⁺ | C9H16N4O6 | 276.1069842 | -0.0037393 |
| 87 | 2313 | 4.162 | 277.15475 | [M+H] ⁺ | C15H20N2O3 | 276.1473925 | -0.000131 |

5,7-Dihydroxyflavone

Tuberonic acid

±-L-Proj)-(-)-cyclo-(L-Phe-L-Pro); N
Ketoleucine

| | | | | | | | | |
|-----|------|--------|-----------|------------------------|-------------|------------------------------------|-------------|------------|
| 88 | 2314 | 4.255 | 277.15524 | [M+H] ⁺ | C15H20N2O3 | | 276.1473925 | -0.000531 |
| 89 | 2320 | 6.95 | 277.21613 | [M+H] ⁺ | C18H28O2 | Stearidonic acid | 276.2089301 | 0.0001066 |
| 90 | 2322 | 7.068 | 277.21622 | [2M+H] ⁺ | C9H14O | 2,4-Nonadienal | 138.1044651 | -0.000271 |
| 91 | 2363 | 7.3 | 279.22345 | [M+H-H2O] ⁺ | C20H28N2 | | 296.2252489 | -0.0014393 |
| 92 | 2373 | 11.738 | 279.49023 | [M+Na] ⁺ | | | | |
| 93 | 2397 | 4.588 | 281.06018 | [M+H] ⁺ | C12H12N2O4S | | 280.0517779 | -0.0011457 |
| 94 | 2421 | 1.629 | 282.12009 | [M+H] ⁺ | C11H15N5O4 | | 281.112404 | -0.0004196 |
| 95 | 2424 | 11.553 | 282.2793 | [M+H] ⁺ | C18H35NO | Oleamide | 281.2718647 | -0.0001588 |
| 96 | 2443 | 5.232 | 283.16556 | [M+H] ⁺ | C14H22N2O4 | | 282.1579572 | -0.0003664 |
| 97 | 2449 | 7.876 | 283.22818 | [2M+NH4] ⁺ | | | | |
| 98 | 2463 | 4.82 | 284.13953 | [M+H] ⁺ | C16H17N3O2 | amide F1-L-Prolyl-L-tryptophan an | 283.1320768 | -0.0001468 |
| 99 | 2468 | 3.091 | 284.20679 | [M+H] ⁺ | C13H25N5O2 | | 283.200825 | 0.0013015 |
| 100 | 2470 | 12.432 | 284.29462 | [M+H] ⁺ | C18H37NO | | 283.2875148 | 0.0001913 |
| 101 | 2490 | 5.625 | 285.07614 | [M+H] ⁺ | C16H12O5 | ixy-2-(4-methoxyphenyl)-4H-chrc | 284.0684735 | -0.0003501 |
| 102 | 2511 | 8.487 | 286.22845 | [M+NH4] ⁺ | C18H24N2 | | 268.1939488 | -0.0007257 |
| 103 | 2522 | 7.675 | 287.08673 | [M+Na] ⁺ | C14H16O5 | | 264.0997736 | 0.0022943 |
| 104 | 2524 | 5.752 | 287.09161 | [M+H] ⁺ | C16H14O5 | Sakuranetin | 286.0841235 | -2.00E-04 |
| 105 | 2526 | 4.123 | 287.1713 | [M+H] ⁺ | C17H22N2O2 | | 286.1681279 | 0.0041044 |
| 106 | 2528 | 6.032 | 287.19803 | [M+H] ⁺ | C19H26O2 | | 286.1932801 | 0.0025565 |
| 107 | 2533 | 6.287 | 287.22189 | [M+H] ⁺ | C16H30O4 | Hexadecanedioic acid | 286.2144094 | -0.0002141 |
| 108 | 2538 | 3.842 | 288.07162 | [M+NH4] ⁺ | C7H14N2O5S2 | | 270.0344135 | -0.0033609 |
| 109 | 2076 | 3.996 | 263.13934 | [M+H] ⁺ | C14H18N2O3 | | 262.1317424 | -0.0002811 |
| 110 | 2546 | 1.106 | 288.18036 | [M+FA+H] ⁺ | C13H23NO3 | | 241.1677936 | 0.000149 |
| 111 | 2547 | 0.636 | 288.18045 | [M+FA+H] ⁺ | C13H23NO3 | | 241.1677936 | 4.90E-05 |
| 112 | 2550 | 8.581 | 288.25351 | [M+H] ⁺ | C16H33NO3 | | 287.2460439 | -0.0001796 |
| 113 | 2587 | 5.164 | 291.01718 | [M+H] ⁺ | C14H10O3S2 | | 290.0071362 | -0.0027874 |
| 114 | 2618 | 6.075 | 292.14584 | [M+H] ⁺ | C11H21N3O6 | | 291.1430354 | 0.0045118 |
| 115 | 2632 | 5.977 | 293.06055 | [M+H] ⁺ | C13H12N2O4S | | 292.0517779 | -0.0014457 |
| 116 | 2634 | 3.294 | 293.11298 | [M+H] ⁺ | C14H16N2O5 | | 292.1059216 | 0.0001981 |
| 117 | 2645 | 7.934 | 293.21091 | [M+H] ⁺ | C18H28O3 | .10,15-phytyldienoic acid;2-Cycloj | 292.2038448 | 0.0002212 |
| 118 | 2646 | 7.46 | 293.21094 | [M+H-H2O] ⁺ | C18H30O4 | | 310.2144094 | 0.0002212 |
| 119 | 2648 | 6.69 | 293.21158 | [M+H] ⁺ | C18H28O3 | .10,15-phytyldienoic acid;2-Cycloj | 292.2038448 | -0.0004788 |
| 120 | 2649 | 8.029 | 293.21222 | [M+H] ⁺ | C18H28O3 | | 292.2038448 | -0.0010788 |
| 121 | 265 | 9.176 | 140.2084 | [M+H] ⁺ | | | | |
| 122 | 1200 | 3.66 | 205.09691 | [M+H] ⁺ | C11H12N2O2 | L-Tryptophan | 204.0898776 | 0.0002541 |
| 123 | 4114 | 5.92 | 379.09863 | [M+H] ⁺ | C17H18N2O6S | | 378.0885573 | -0.0027663 |
| 124 | 3060 | 7.786 | 313.23727 | [M+H-H2O] ⁺ | C18H34O5 | 9,12,13-TRIHOME | 330.2406242 | 3.60E-05 |
| 125 | 2687 | 8.236 | 295.19067 | [M+H] ⁺ | C17H26O4 | | 294.1831093 | -0.0003142 |
| 126 | 2697 | 6.546 | 295.22705 | [M+H-H2O] ⁺ | C18H32O4 | 7S,8S-Dihydroxylinoleic acid | 312.2300595 | -0.0003287 |
| 127 | 2712 | 3.123 | 296.13513 | [M+H] ⁺ | C11H21NO8 | | 295.1267166 | -0.0011069 |
| 128 | 2742 | 7.294 | 297.2337 | [M+H] ⁺ | C20H28N2 | | 296.2252489 | -0.0011747 |
| 129 | 2747 | 10.425 | 297.24249 | [M+NH4] ⁺ | | | | |
| 130 | 2748 | 10.278 | 297.24255 | [M+H] ⁺ | C18H32O3 | 12,13-EHOME | 296.2351449 | -0.0001787 |
| 131 | 2749 | 10.101 | 297.24258 | [M+NH4] ⁺ | | | | |
| 132 | 1892 | 8.475 | 251.20079 | [M+H] ⁺ | C16H26O2 | | 250.1932801 | -0.0002435 |

| | | | | | | | | |
|-----|------|--------|-----------|-------------------------|-------------|---------------------------------|-------------|------------|
| 133 | 2778 | 9.039 | 299.03537 | [M+H] ⁺ | C16H10O4S | | 298.0299798 | 0.0018563 |
| 134 | 2806 | 5.444 | 300.17084 | [M+H] ⁺ | C17H21N3O2 | | 299.1633769 | -0.0001466 |
| 135 | 1596 | 3.319 | 229.11824 | [M+H] ⁺ | C10H16N2O4 | | 228.111007 | 8.34E-05 |
| 136 | 2812 | 11.072 | 300.29013 | [M+H] ⁺ | C18H37NO2 | 3-Dehydrospinganine | 299.2824294 | -0.0003941 |
| 137 | 2824 | 4.207 | 301.11844 | [M+H] ⁺ | C16H16N2O4 | | 300.111007 | -0.0001166 |
| 138 | 2826 | 10.066 | 301.14114 | [M+Na] ⁺ | C16H22O4 | Borpinol C | 278.1518092 | -7.01E-05 |
| 139 | 1772 | 5.132 | 243.08777 | [M+H] ⁺ | C12H10N4O2 | | 242.0803756 | -0.000148 |
| 140 | 2872 | 7.541 | 304.17554 | [M+Na] ⁺ | C18H23N3 | | 281.1891977 | 0.0029184 |
| 141 | 2886 | 5.658 | 305.14957 | [M+H] ⁺ | C16H20N2O4 | | 304.1423071 | -1.64E-05 |
| 142 | 2888 | 8.468 | 305.16916 | [M+H] ⁺ | C14H20N6O2 | | 304.1647739 | 0.0028503 |
| 143 | 2922 | 10.506 | 307.22659 | [M+H] ⁺ | C19H30O3 | ihydromonacolin L,Dihydromona | 306.2194948 | 0.0001713 |
| 144 | 2925 | 9.108 | 307.22791 | [M+H-H2O] ⁺ | C19H32O4 | | 324.2300595 | -0.0011287 |
| 145 | 2926 | 8.117 | 307.24841 | [M+H] ⁺ | C17H30N4O | | 306.2419616 | 0.000838 |
| 146 | 2169 | 9.783 | 269.20844 | [M+Na] ⁺ | C14H30O3 | | 246.2194948 | 0.0003155 |
| 147 | 2946 | 11.847 | 308.29416 | [M+H] ⁺ | C20H37NO | | 307.2875148 | 0.0005913 |
| 148 | 2954 | 5.21 | 309.11432 | [M+H] ⁺ | C19H16O4 | | 308.104859 | -0.0021646 |
| 149 | 1737 | 0.909 | 241.06812 | [M+Na] ⁺ | C9H14O6 | | 218.0790382 | 0.0001589 |
| 150 | 2968 | 5.524 | 309.22733 | [M+H] ⁺ | C16H28N4O2 | | 308.2212261 | 0.0012026 |
| 151 | 2969 | 9.025 | 309.24274 | [M+H] ⁺ | C19H32O3 | | 308.2351449 | -0.0002787 |
| 152 | 2980 | 4.95 | 310.15564 | [M+H] ⁺ | C18H19N3O2 | | 309.1477268 | -0.0005967 |
| 153 | 2281 | 8.455 | 275.20059 | [M+H-H2O] ⁺ | C18H28O3 | | 292.2038448 | -4.35E-05 |
| 154 | 2996 | 4.782 | 311.13907 | [M+H] ⁺ | C18H18N2O3 | | 310.1317424 | -8.11E-05 |
| 155 | 2997 | 5.917 | 311.13956 | [M+H] ⁺ | C18H18N2O3 | | 310.1317424 | -0.0005811 |
| 156 | 30 | 2.178 | 109.02826 | [M+H-H2O] ⁺ | C6H6O3 | 1,2,3-Trihydroxybenzene | 126.0316941 | 0.0001058 |
| 157 | 3001 | 8.006 | 311.18524 | [M+FA+H] ⁺ | C16H24O3 | | 264.1725446 | 0.0001001 |
| 158 | 3007 | 7.934 | 311.22122 | [M+H] ⁺ | C18H30O4 | | 310.2144094 | 0.0004859 |
| 159 | 2650 | 6.369 | 293.21255 | [M+H-2H2O] ⁺ | C18H32O5 | Corchoriaty acid F | 328.2249741 | -0.0014788 |
| 160 | 3010 | 9.605 | 311.2485 | [M+H] ⁺ | C21H30N2 | | 310.240899 | -0.0003246 |
| 161 | 3011 | 11.204 | 311.29449 | [M+H] ⁺ | C20H38O2 | Eicosenoic acid | 310.2871805 | -4.31E-05 |
| 162 | 3025 | 5.813 | 312.23853 | [M+H] ⁺ | C20H29N3 | | 311.2361479 | -0.0001756 |
| 163 | 3026 | 9.165 | 312.24356 | [M+H] ⁺ | C18H28O2 | Stearidonic acid | 276.2089301 | -0.0011934 |
| 164 | 2327 | 9.064 | 277.21744 | [M+H] ⁺ | C19H41N3 | | 311.3300483 | 0.0027248 |
| 165 | 3036 | 8.954 | 312.33459 | [M+H] ⁺ | C18H30O3 | -enteny)cyclopentaneoctanoic ac | 294.2194948 | -2.87E-05 |
| 166 | 2694 | 9.764 | 295.22678 | [M+H] ⁺ | C18H32O4 | S(S),8(R)-DihODE | 312.2300595 | -0.000164 |
| 167 | 3061 | 5.839 | 313.23752 | [M+H] ⁺ | C11H15N5O4S | | 313.084475 | -0.0002486 |
| 168 | 3072 | 1.273 | 314.09198 | [M+H] ⁺ | C32H37NO12 | | 627.2315756 | -9.65E-05 |
| 169 | 3074 | 5.891 | 314.1192 | [M+H]2 ⁺ | C18H30O2 | Crepennic acid | 278.2245802 | -0.0004433 |
| 170 | 2370 | 9.641 | 279.23227 | [M+H] ⁺ | C20H26O3 | | 314.1881947 | 0.0008711 |
| 171 | 3099 | 8.439 | 315.19464 | [M+H] ⁺ | C18H34O4 | -Epoxy-18-hydroxy-octadecanoic | 314.2457096 | -0.000114 |
| 172 | 3105 | 8.424 | 315.25314 | [M+H] ⁺ | C18H34O4 | -Epoxy-18-hydroxy-octadecanoic | 314.2457096 | -0.000214 |
| 173 | 3106 | 8.115 | 315.25323 | [M+H] ⁺ | C16H17N3O4 | | 315.121906 | -0.0002175 |
| 174 | 3111 | 4.598 | 316.12936 | [M+Na] ⁺ | C18H30O3 | -enteny)cyclopentaneoctanoic ac | 294.2194948 | -0.0001845 |
| 175 | 3133 | 9.745 | 317.20886 | [M+Na] ⁺ | C20H32O5 | Prostaglandin D2 | 352.2249741 | 0.0013212 |
| 176 | 3134 | 9.066 | 317.20984 | [M+H-2H2O] ⁺ | C9H18O2 | | 158.1306798 | -0.0004562 |
| 177 | 3136 | 9.027 | 317.26895 | [2M+H] ⁺ | | | | |

| | | | | | | | | |
|-----|------|--------|-----------|------------------------|-------------|----------------------------|-------------|------------|
| 178 | 3142 | 9.989 | 318.3002 | [M+H] ⁺ | C18H39NO3 | Phytosphingosine | 317.2929941 | 7.06E-05 |
| 179 | 3148 | 7.988 | 319.05911 | [M+Na] ⁺ | C17H12O5 | | 296.0684735 | -0.0014058 |
| 180 | 3152 | 9.062 | 319.18497 | [M+H] ⁺ | C15H22N6O2 | | 318.1804239 | 0.0027004 |
| 181 | 3184 | 10.163 | 321.1666 | [M+H] ⁺ | C18H24O5 | | 320.1623739 | 0.0030503 |
| 182 | 3192 | 3.696 | 322.1084 | [M+H] ⁺ | C11H19N3O6S | | 321.0994563 | -0.0016672 |
| 183 | 3203 | 4.356 | 322.16608 | [2M+NH4] ⁺ | CSH12O5 | | 152.0684735 | 0.002062 |
| 184 | 3236 | 7.313 | 324.20779 | [M+H] ⁺ | C20H25N3O | | 323.1997624 | -0.0007611 |
| 185 | 3240 | 10.526 | 324.28967 | [M+H] ⁺ | C20H37NO2 | | 323.2824294 | 5.90E-06 |
| 186 | 3277 | 3.912 | 326.17255 | [M+H] ⁺ | C15H23N3O5 | | 325.1637708 | -0.0014527 |
| 187 | 5519 | 11.219 | 528.24475 | [M+NH4] ⁺ | C25H34O11 | | 510.2101119 | -0.0008625 |
| 188 | 3294 | 9.347 | 327.0784 | [M+H] ⁺ | C13H14N2O8 | | 326.0750154 | 0.0038919 |
| 189 | 3298 | 7.173 | 327.13373 | [M+H] ⁺ | C13H18N4O6 | | 326.1226343 | -0.0037893 |
| 190 | 2280 | 7.156 | 275.20038 | [M+H] ⁺ | C18H26O2 | | 274.1932801 | 0.0001565 |
| 191 | 3322 | 12.077 | 328.32086 | [M+H] ⁺ | C20H41NO2 | | 327.3137296 | 0.000106 |
| 192 | 3370 | 6.948 | 331.18076 | [M+H] ⁺ | C11H22N8O4 | | 330.1764012 | 0.0028776 |
| 193 | 3374 | 7.429 | 331.18979 | [M+Na] ⁺ | C18H28O4 | | 308.1987594 | -0.0018199 |
| 194 | 3379 | 5.495 | 331.21088 | [M+Na] ⁺ | C21H28N2 | | 308.2252489 | 0.0035696 |
| 195 | 3387 | 7.068 | 331.24973 | [M+H] ⁺ | C18H34O5 | 9,12,13-TriHOME | 330.2406242 | -0.0017994 |
| 196 | 340 | 1.808 | 144.0479 | [M+H] ⁺ | G6H9NO5 | | 143.0404849 | -0.0001386 |
| 197 | 3413 | 7.154 | 333.20343 | [M+Na] ⁺ | C18H30O4 | | 310.2144094 | 0.0002301 |
| 198 | 3419 | 8.427 | 333.20557 | [M+H] ⁺ | C20H28O4 | | 332.1987594 | 0.0004358 |
| 199 | 3422 | 8.101 | 333.26566 | [M+H] ⁺ | C18H36O5 | | 332.2562743 | -0.0021493 |
| 200 | 2952 | 5.782 | 309.08728 | [M+H] ⁺ | C17H12N2O4 | | 308.0797069 | -0.0003167 |
| 201 | 3432 | 8.776 | 334.23602 | [M+NH4] ⁺ | C20H28O3 | | 316.2038448 | 0.0016703 |
| 202 | 3442 | 6.001 | 335.16052 | [M+H] ⁺ | C17H22N2O5 | | 334.1528718 | -0.0003517 |
| 203 | 3466 | 4.192 | 336.15674 | [M+H] ⁺ | C21H21NO3 | | 335.1521435 | 0.00272 |
| 204 | 3475 | 10.811 | 336.32596 | [M+H-H2O] ⁺ | C22H43NO2 | | 353.3293796 | 9.14E-05 |
| 205 | 3480 | 8.688 | 337.1066 | [M+H] ⁺ | C20H16O5 | | 336.0997736 | 0.0004501 |
| 206 | 3493 | 4.003 | 338.18298 | [M+H] ⁺ | C15H23N5O4 | Kyotorphin | 337.1750042 | -0.0007193 |
| 207 | 3529 | 4.413 | 340.18719 | [M+NH4] ⁺ | C16H22N2O5 | | 322.1528718 | -0.0005026 |
| 208 | 354 | 8.997 | 146.06042 | [M+H] ⁺ | C9H7NO | 1H-Indole-3-carboxaldehyde | 145.0527638 | -0.0003597 |
| 209 | 3542 | 3.347 | 340.28519 | [M+H] ⁺ | C20H37NO3 | | 339.277344 | -0.0005795 |
| 210 | 3548 | 9.774 | 341.09424 | [M+H] ⁺ | C14H16N2O8 | | 340.0906655 | 0.0037419 |
| 211 | 3563 | 5.066 | 342.15503 | [M+H] ⁺ | C16H23NO7 | | 341.1474521 | -0.0002715 |
| 212 | 3564 | 4.607 | 342.20267 | [M+H] ⁺ | C16H27N3O5 | | 341.195071 | -0.0003526 |
| 213 | 357 | 2.271 | 146.11732 | [M+H] ⁺ | C7H15NO2 | | 145.1102787 | 0.0002552 |
| 214 | 3576 | 4.143 | 343.13126 | [M+Na] ⁺ | C21H20O3 | | 320.1412445 | -0.0008348 |
| 215 | 3595 | 6.319 | 344.06665 | [M+H] ⁺ | C16H13N3O4S | | 343.0626769 | 0.0032533 |
| 216 | 478 | 3.73 | 154.0498 | [M+H] ⁺ | C7H7NO3 | 3-Hydroxyanthranilic acid | 153.0425931 | 6.95E-05 |
| 217 | 3648 | 10.533 | 347.21936 | [M+Na] ⁺ | C19H32O4 | | 324.2300595 | -0.0001198 |
| 218 | 3660 | 6.678 | 348.27493 | [M+NH4] ⁺ | C18H34O5 | 9,12,13-TriHOME | 330.2406242 | -0.0004503 |
| 219 | 3677 | 8.933 | 349.23547 | [M+Na] ⁺ | C19H34O4 | | 326.2457096 | -0.0005697 |
| 220 | 3687 | 11.665 | 350.3056 | [M+H] ⁺ | C22H39NO2 | | 349.2980795 | -0.0002441 |
| 221 | 3757 | 0.575 | 354.03375 | [M+H] ⁺ | | | | |
| 222 | 3765 | 8.82 | 354.28537 | [M+H] ⁺ | C23H35N3 | | 353.2830981 | 0.0049746 |

| | | | | | | | | |
|-----|------|--------|-----------|-------------------------|-------------|----------------------------------|-------------|------------|
| 223 | 3767 | 11.663 | 354.33728 | [M+H] ⁺ | C22H43NO2 | | 353.3293796 | -0.0006439 |
| 224 | 3778 | 10.175 | 355.10962 | [M+H] ⁺ | C19H18N2O3S | | 354.1038134 | 0.0014899 |
| 225 | 3787 | 11.39 | 355.28201 | [M+Na] ⁺ | C19H40O4 | | 332.2926598 | -0.0001195 |
| 226 | 3799 | 5.021 | 356.21817 | [M+H] ⁺ | C22H29NO3 | | 355.2147438 | 0.0038202 |
| 227 | 3800 | 5.147 | 356.21841 | [M+H] ⁺ | C22H29NO3 | | 355.2147438 | 0.0036202 |
| 228 | 3811 | 4.437 | 357.08032 | [M+H] ⁺ | C9H17N4O9P | nino-6-(5'-phosphoribitylamino)l | 356.0733148 | 0.0002912 |
| 229 | 3822 | 12.119 | 357.33694 | [M+H] ⁺ | C22H44O3 | 22-Hydroxydocosanoic acid | 356.3290453 | -0.0005783 |
| 230 | 3828 | 6.825 | 358.14145 | [M+NH4] ⁺ | C15H20N2O5S | | 340.1092927 | 0.0017183 |
| 231 | 3830 | 4.238 | 358.19763 | [M+H] ⁺ | C16H27N3O6 | | 357.1899856 | -0.000338 |
| 232 | 3834 | 6.668 | 358.29547 | [M+H] ⁺ | C20H39NO4 | | 357.2879087 | -0.0003148 |
| 233 | 3845 | 5.273 | 359.18158 | [M+H] ⁺ | C21H26O5 | | 358.1780239 | 0.0037004 |
| 234 | 3846 | 10.212 | 359.21951 | [M+Na] ⁺ | C20H32O4 | 15(S)-HPETE | 336.2300595 | -0.0002198 |
| 235 | 3848 | 8.826 | 359.24075 | [M+H] ⁺ | C15H30N6O4 | | 358.2328534 | -0.0006701 |
| 236 | 2369 | 10.733 | 279.23218 | [M+H] ⁺ | C18H30O2 | Crepentenic acid | 278.2245802 | -0.0003433 |
| 237 | 3906 | 0.947 | 365.10635 | [M+H-2H2O] ⁺ | C18H24O8S | | 400.1191887 | -0.0010642 |
| 238 | 3908 | 9.788 | 365.13675 | [M+Na] ⁺ | C20H22O5 | Brosimacutin C | 342.1467238 | -0.0007555 |
| 239 | 395 | 9.88 | 149.01721 | [M+H] ⁺ | C7H4N2S | | 148.0095191 | -0.0004044 |
| 240 | 3952 | 5.4 | 369.20248 | [M+H] ⁺ | C19H24N6O2 | | 368.196074 | 0.0008505 |
| 241 | 3969 | 10.575 | 370.29538 | [M+NH4] ⁺ | C21H36O4 | | 352.2613596 | -0.0002148 |
| 242 | 4010 | 7.925 | 373.25845 | [2M+H] ⁺ | C10H18O3 | | 186.1255944 | -0.0002916 |
| 243 | 4011 | 7.713 | 373.25861 | [M+H] ⁺ | C20H36O6 | 8,8a-Deoxyoleanolide | 372.2511889 | -0.0001347 |
| 244 | 4014 | 8.612 | 373.25873 | [2M+H] ⁺ | C10H18O3 | | 186.1255944 | -0.0003916 |
| 245 | 2811 | 9.383 | 300.29013 | [M+H] ⁺ | C18H37NO2 | 3-Dehydrospinganine | 299.2824294 | -0.0003941 |
| 246 | 4031 | 10.13 | 375.1207 | [M+Na] ⁺ | C21H20O5 | | 352.1310737 | -0.0004056 |
| 247 | 4034 | 8.175 | 375.19971 | [M+H] ⁺ | C19H26N4O4 | | 374.1954053 | 0.0029818 |
| 248 | 4084 | 11.093 | 377.07898 | [M+Na] ⁺ | C15H18N2O6S | | 354.088573 | -0.001222 |
| 249 | 4104 | 11.57 | 378.21072 | [M+H] ⁺ | C24H27NO3 | | 377.1990937 | -0.0043298 |
| 250 | 4107 | 11.395 | 378.22128 | [M+H] ⁺ | C20H31N3O2S | | 377.2136982 | -0.0003253 |
| 251 | 4118 | 9.662 | 379.2485 | [M+Na] ⁺ | C20H36O5 | | 356.2562743 | -0.003005 |
| 252 | 1893 | 6.804 | 251.20102 | [M+H] ⁺ | C16H26O2 | | 250.1932801 | -0.0004435 |
| 253 | 4156 | 10.054 | 383.2041 | [M+Na] ⁺ | C18H32O7 | | 360.2148034 | -7.59E-05 |
| 254 | 4179 | 6.522 | 384.12085 | [M+NH4] ⁺ | C16H18N2O6S | | 366.0885573 | 0.0015828 |
| 255 | 2231 | 6.101 | 273.07623 | [M+H] ⁺ | C15H12O5 | Naringenin | 272.0684735 | -0.0004501 |
| 256 | 3897 | 12.278 | 363.25323 | [M+H] ⁺ | C22H34O4 | | 362.2457096 | -0.000214 |
| 257 | 4202 | 10.765 | 385.29224 | [M+H] ⁺ | C22H40O5 | | 384.2875744 | 0.0026508 |
| 258 | 4223 | 3.958 | 388.25531 | [M+NH4] ⁺ | C16H34O9 | | 370.2202827 | -0.0011918 |
| 259 | 4244 | 5.421 | 390.20255 | [M+H] ⁺ | C20H27N3O5 | | 389.195071 | -0.0001526 |
| 260 | 4305 | 7.789 | 395.28067 | [M+H-2H2O] ⁺ | C23H40O6 | | 412.282489 | -0.0014992 |
| 261 | 4307 | 11.796 | 395.33066 | [M+NH4] ⁺ | C23H39NO3 | | 377.2929941 | -0.0038803 |
| 262 | 432 | 0.843 | 151.03549 | [M+H] ⁺ | C8H6O3 | | 150.0316941 | 0.0034705 |
| 263 | 4340 | 10.928 | 398.16537 | [M+H] ⁺ | C16H23N5O7 | | 397.1597481 | 0.0016245 |
| 264 | 4349 | 7.686 | 399.10123 | [M+H] ⁺ | C20H18N2O5S | | 398.0936427 | -0.0002809 |
| 265 | 4094 | 11.682 | 377.22998 | [M+H] ⁺ | C22H32O5 | | 376.2249741 | 0.0022506 |
| 266 | 6216 | 6.736 | 600.13391 | [M+H] ⁺ | C28H25NO14 | | 599.1275045 | 0.0008809 |
| 267 | 441 | 5.713 | 151.11162 | [M+H-2H2O] ⁺ | C10H16O2 | | 168.1150298 | 0.0001415 |

| | | | | | | | |
|-----|------|--------|-----------|-------------------------|---------------|-------------|------------|
| 268 | 4420 | 12.114 | 406.32959 | [M+NH4] ⁺ | C25H40O3 | 388.2977451 | 0.0019707 |
| 269 | 4519 | 7.447 | 417.05743 | [M+H] ⁺ | C23H12O8 | 416.0532173 | 0.0030938 |
| 270 | 4344 | 11.125 | 398.23254 | [M+H] ⁺ | C24H31NO4 | 397.2253085 | 8.49E-05 |
| 271 | 4543 | 11.819 | 420.32501 | [M+H] ⁺ | C24H41N3O3 | 419.3147922 | -0.0029314 |
| 272 | 4576 | 8.545 | 421.25754 | [M+Na] ⁺ | C22H38O6 | 398.2668389 | -0.0014404 |
| 273 | 462 | 0.838 | 153.03275 | [M+FA+H] ⁺ | | | |
| 274 | 4640 | 11.369 | 427.32059 | [M+H] ⁺ | C28H42O3 | 426.3133952 | 7.17E-05 |
| 275 | 465 | 4.493 | 153.05447 | [M+Na] ⁺ | G6H10O3 | 130.0629942 | -0.0022851 |
| 276 | 4653 | 5.305 | 429.2139 | [M+FA+H] ⁺ | C20H30O7 | 382.1991533 | -0.0019912 |
| 277 | 3159 | 11.049 | 319.2847 | [M+H] ⁺ | C18H38O4 | 318.2770097 | -0.0004139 |
| 278 | 4685 | 4.124 | 432.28 | [M+NH4] ⁺ | C18H38O10 | 414.2464974 | 0.000323 |
| 279 | 4692 | 4.638 | 433.11285 | [M+H] ⁺ | C21H20O10 | 432.1056468 | 2.33E-05 |
| 280 | 4732 | 9.997 | 437.20865 | [M+H] ⁺ | C25H28N2O5 | 436.199822 | -0.0015016 |
| 281 | 4737 | 9.936 | 437.26855 | [M+H-H2O] ⁺ | C28H38O5 | 454.2719243 | 3.61E-05 |
| 282 | 4787 | 10.244 | 441.37299 | [M+H-H2O] ⁺ | C30H50O3 | 458.3759955 | -0.0002928 |
| 283 | 4795 | 6.598 | 442.3381 | [2M+NH4] ⁺ | C11H20N2O2 | 212.1524779 | 6.64E-05 |
| 284 | 4825 | 4.105 | 447.12918 | [M+H] ⁺ | C22H22O10 | 446.1212969 | -0.0006266 |
| 285 | 4839 | 6.332 | 448.10406 | [M+NH4] ⁺ | C11H20NAQ10P2 | 430.0654661 | -0.0048083 |
| 286 | 4843 | 4.687 | 449.10339 | [M+FA+H] ⁺ | C12H23N2O9PS | 402.0861879 | -0.0044566 |
| 287 | 4896 | 7.674 | 455.03632 | [M+Na] ⁺ | C19H16N2O6S2 | 432.0455 | |
| 288 | 4864 | 4.954 | 451.11911 | [M+H] ⁺ | C21H22O11 | 450.1162115 | 0.004388 |
| 289 | 3287 | 12.31 | 326.30573 | [M+H] ⁺ | C20H39NO2 | 325.2980795 | -0.0003441 |
| 290 | 488 | 1.527 | 155.08113 | [M+H] ⁺ | C7H10N2O2 | 154.0742276 | 0.000404 |
| 291 | 4880 | 6.337 | 453.07443 | [M+Na] ⁺ | C21H18O10 | 430.0899968 | 0.0048175 |
| 292 | 489 | 0.939 | 155.08136 | [M+H-2H2O] ⁺ | C7H14N2O4 | 190.0953569 | 0.000104 |
| 293 | 4962 | 10.989 | 463.12997 | [M+H] ⁺ | C25H22N2O5S | 462.1249428 | 0.0022193 |
| 294 | 4966 | 12.078 | 463.28217 | [M+Na] ⁺ | C28H44O4 | 440.2926598 | -0.0003195 |
| 295 | 5004 | 11.661 | 465.29758 | [M+H] ⁺ | C30H44O4 | 464.2926598 | 0.0023362 |
| 296 | 4863 | 6.516 | 451.06314 | [M+FA+H] ⁺ | C18H16N2O5S2 | 404.0500636 | -0.0002809 |
| 297 | 507 | 1.458 | 156.13872 | [M+H] ⁺ | C9H17NO | 155.1310142 | -0.0004094 |
| 298 | 5070 | 12.371 | 471.36475 | [M+Na] ⁺ | C32H48O | 448.3705162 | -0.0049631 |
| 299 | 5084 | 7.675 | 473.24554 | [M+H] ⁺ | C21H36NAO6S | 472.2355559 | -0.0026677 |
| 300 | 5108 | 4.262 | 476.30917 | [M+H] ⁺ | C21H41N5O7 | 475.3005987 | -0.0013249 |
| 301 | 4961 | 7.191 | 463.09897 | [M+H] ⁺ | C21H41N5O7 | 462.092 | |
| 302 | 5155 | 8.451 | 481.24847 | [M+H-2H2O] ⁺ | C21H22N2O6S2 | 516.2624223 | 6.93E-05 |
| 303 | 5157 | 11.493 | 481.2926 | [M+Na] ⁺ | C31H36N2O5 | 458.3032244 | -0.0001549 |
| 304 | 4817 | 11.628 | 445.33109 | [M+H-2H2O] ⁺ | C28H48O6 | 480.3450893 | 0.0001363 |
| 305 | 5202 | 7.443 | 487.09891 | [M+Na] ⁺ | C21H24N2O6S2 | 464.1077 | |
| 306 | 5169 | 12.398 | 482.28357 | [M+H] ⁺ | C19H39N5O9 | 481.2747778 | -0.0015457 |
| 307 | 4358 | 11.334 | 399.30832 | [M+Na] ⁺ | C22H40N4O | 376.3202119 | 0.0001326 |
| 308 | 5236 | 10.206 | 490.20792 | [M+H] ⁺ | C25H31NO9 | 489.1998816 | -0.000742 |
| 309 | 525 | 7.393 | 158.15375 | [M+H] ⁺ | C9H19NO | 157.1466642 | 0.0002407 |
| 310 | 5274 | 5.873 | 496.11917 | [M+H] ⁺ | C21H25N3O7S2 | 495.1133921 | 0.00014686 |
| 311 | 4673 | 5.105 | 431.09268 | [M+FA+H] ⁺ | C20H16O8 | 384.0845175 | 0.00045729 |
| 312 | 5277 | 9.679 | 496.34018 | [M+H] ⁺ | C24H50NO7P | 495.3324896 | -0.000434 |

LysoPC(16:0)

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|-----|------|--------|-----------|-------------------------|-------------|-------------------------------------|-------------|------------|
| 313 | 3946 | 5.415 | 369.09732 | [M++H2O]+ | C20H1808 | | 386.1001675 | -0.0004207 |
| 314 | 5293 | 6.161 | 498.13516 | [M+H] ⁺ | C24H23N3O7S | | 497.1256711 | -0.0022525 |
| 315 | 5308 | 6.319 | 500.15057 | [M+H] ⁺ | C24H25N3O7S | | 499.1413211 | -0.0020024 |
| 316 | 5315 | 5.106 | 501.0784 | [M+Na] ⁺ | C25H18O10 | | 478.0899968 | 0.0008175 |
| 317 | 5320 | 12.007 | 501.37582 | [M+Na] ⁺ | C27H50N4O3 | | 478.3882915 | 0.0017122 |
| 318 | 5334 | 5.427 | 503.09399 | [M+Na] ⁺ | C25H20O10 | | 480.1056468 | 0.0008675 |
| 319 | 5387 | 11.971 | 510.43738 | [M+H] ⁺ | | | | |
| 320 | 5474 | 9.913 | 522.3551 | [M+H] ⁺ | C26H52N07P | | 521.3481396 | 0.0003161 |
| 321 | 5493 | 8.38 | 524.38031 | [M+H] ⁺ | | | | |
| 322 | 5510 | 11.284 | 526.43134 | [M+NH4] ⁺ | | | | |
| 323 | 5518 | 11.419 | 528.22955 | [M+NH4] ⁺ | C21H34O14 | | 510.1948558 | -0.0009187 |
| 324 | 5577 | 6.02 | 538.11572 | [M+NH4] ⁺ | C23H20O14 | | 520.0853053 | 0.00034309 |
| 325 | 5607 | 10.139 | 541.37335 | [M++H2O] ⁺ | C31H50N4O5 | | 558.3781207 | 0.0014325 |
| 326 | 5613 | 11.612 | 542.24475 | [M+NH4] ⁺ | C27H32N4O5S | | 524.2093411 | -0.0016333 |
| 327 | 5628 | 8.413 | 544.36353 | [M+H] ⁺ | C32H49N06 | | 543.3559883 | -0.0002353 |
| 328 | 5646 | 10.484 | 546.39984 | [M+NH4] ⁺ | C29H52O8 | | 528.3662186 | 0.0002442 |
| 329 | 5673 | 10.666 | 550.38348 | [M+Na] ⁺ | | | | |
| 330 | 5694 | 9.027 | 552.30884 | [M+H] ⁺ | C31H41N3O6 | | 551.299536 | -0.0019875 |
| 331 | 5695 | 9.305 | 552.39417 | [M+H] ⁺ | | | | |
| 332 | 5698 | 9.176 | 552.41449 | [M+H] ⁺ | C30H55N5O5 | clo-(L-Leu-L-Leu-D-Leu-L-Leu-L-V | 552.41449 | |
| 333 | 2983 | 12.331 | 310.3103 | [M+H] ⁺ | C20H39NO | | 309.3031649 | 0.0001413 |
| 334 | 5583 | 8.782 | 538.39624 | [M+H] ⁺ | C33H51N3O3 | | 537.3930425 | 0.0041189 |
| 335 | 5735 | 11.663 | 556.27661 | [M+NH4] ⁺ | C27H38O11 | | 538.241412 | -0.0013624 |
| 336 | 5738 | 10.902 | 556.44263 | [M+NH4] ⁺ | C28H58O9 | | 538.4080834 | -0.000691 |
| 337 | 5851 | 9.173 | 566.42755 | [M+H] ⁺ | C30H55N5O5 | | 565.4203199 | -3.70E-06 |
| 338 | 5879 | 6.172 | 568.10803 | [M++H2O] ⁺ | | | | |
| 339 | 5955 | 9.118 | 574.39636 | [M+H] ⁺ | C31H51N5O5 | | 573.3890197 | -0.0001038 |
| 340 | 5897 | 9.088 | 569.5553 | [M+2H] ²⁺ | | | | |
| 341 | 5908 | 11.258 | 570.45654 | [M+NH4] ⁺ | C36H56O4 | | 552.4178603 | -0.0048142 |
| 342 | 5915 | 8.005 | 571.42065 | [M+NH4] ⁺ | C33H51N3O4 | | 553.3879571 | 0.0010827 |
| 343 | 5924 | 9.723 | 572.43719 | [M+NH4] ⁺ | C36H50N4O | | 554.3984622 | -0.0049122 |
| 344 | 5953 | 9.435 | 574.32751 | [M+H-H2O] ⁺ | C34H45N3O6 | | 591.3308362 | 4.79E-05 |
| 345 | 5967 | 8.407 | 575.37909 | [M+H-H2O] ⁺ | C30H56O11 | | 592.3822626 | -0.0001256 |
| 346 | 6022 | 8.36 | 582.4248 | [2M+NH4] ⁺ | C20H26O | | 282.1983655 | 0.0026039 |
| 347 | 6024 | 8.309 | 582.42578 | [M+H] ⁺ | C35H55N3O4 | | 581.4192572 | 0.0007337 |
| 348 | 6057 | 11.953 | 584.47516 | [M+NH4] ⁺ | | | | |
| 349 | 6074 | 9.036 | 586.39642 | [M+H] ⁺ | C31H55N09 | | 585.3876823 | -0.0014412 |
| 350 | 6112 | 11.62 | 588.40979 | [M+H] ⁺ | C32H53N5O5 | | 587.4046698 | 0.0021462 |
| 351 | 6113 | 9.509 | 588.40997 | [M+Na] ⁺ | C30H55N5O5 | | 565.4203199 | -0.0004594 |
| 352 | 6138 | 10.453 | 590.42639 | [M+NH4] ⁺ | C31H56O9 | yclo-(L-Ile-L-Leu-L-Leu-L-Leu-L-Leu | 572.3924334 | -0.0001411 |
| 353 | 6689 | 8.634 | 684.49341 | [M+NH4] ⁺ | C35H62N4O8 | | 666.4567649 | -0.0028095 |
| 354 | 6192 | 8.948 | 597.36298 | [M+Na] ⁺ | C31H50N4O6 | | 574.3730353 | -0.000744 |
| 355 | 6195 | 9.119 | 597.46997 | [M+H] ⁺ | C35H64O7 | | 596.4652044 | 0.0024808 |
| 356 | 6196 | 9.78 | 597.47241 | [M+H] ⁺ | C35H64O7 | isoannacin A | 596.4652044 | 8.08E-05 |
| 357 | 1947 | 6.544 | 255.07693 | [M+H-2H2O] ⁺ | C14H14N2O5 | N-(1H-Indol-3-ylacetyl)aspartic ac | 290.0902715 | -0.0004814 |

| | | | | | | | | |
|-----|------|--------|-----------|-------------------------|-------------|----------------------------------|-------------|------------|
| 358 | 6209 | 11.85 | 599.42877 | [M+Na] ⁺ | C35H6006 | | 576.438986 | -0.0005897 |
| 359 | 6215 | 6.642 | 600.1156 | [M+H] ⁺ | | | | |
| 360 | 6222 | 9.491 | 600.41187 | [M+H] ⁺ | C33H53N4O5 | 1o-(L-Phe-L-Leu1-L-Leu2-L-Leu3-L | 582.3781207 | 4.63E-05 |
| 361 | 6226 | 10.872 | 600.46863 | [M+NH4] ⁺ | C30H62O10 | | 582.4342982 | -0.0004763 |
| 362 | 6262 | 5.789 | 606.06964 | [M+H] ⁺ | | | | |
| 363 | 6291 | 11.36 | 609.41333 | [M+Na] ⁺ | C36H58O6 | | 586.4233396 | -0.0007397 |
| 364 | 6309 | 11.822 | 611.42841 | [2M+Na] ⁺ | C18H30O3 | | 294.2194948 | -0.0003691 |
| 365 | 6334 | 12.31 | 613.44336 | [M+Na] ⁺ | C36H62O6 | | 590.4546397 | 0.0004604 |
| 366 | 6345 | 11.37 | 614.48389 | [M+NH4] ⁺ | C31H64O10 | | 596.4499482 | -0.0001262 |
| 367 | 635 | 4.135 | 169.07651 | [M+H] ⁺ | C11H8N2 | | 168.0687483 | -0.0004753 |
| 368 | 3156 | 9.64 | 319.22437 | [M+Na] ⁺ | C18H32O3 | 12,13-EPOME | 296.2351449 | -3.44E-05 |
| 369 | 6358 | 10.143 | 616.40588 | [M+H] ⁺ | C33H53N5O6 | | 615.3995844 | 0.0009609 |
| 370 | 636 | 2.11 | 169.0968 | [M+H] ⁺ | C8H12N2O2 | Pyridoxamine | 168.0898776 | 0.0003541 |
| 371 | 6364 | 7.488 | 617.40875 | [M+NH4] ⁺ | C37H49N3O4 | | 599.372307 | -0.0026674 |
| 372 | 2140 | 9.403 | 267.17209 | [M+H-2H2O] ⁺ | C19H26O3 | | 302.1881947 | 0.0022418 |
| 373 | 6219 | 7.796 | 600.38171 | [M+H] ⁺ | C37H49N3O4 | | 599.372307 | -0.0021165 |
| 374 | 6403 | 9.491 | 622.39368 | [M+Na] ⁺ | C33H53N5O5 | | 599.4046698 | 0.0001905 |
| 375 | 6426 | 8.092 | 626.45209 | [M+H] ⁺ | C37H59N3O5 | | 625.445472 | 0.0006484 |
| 376 | 6432 | 10.433 | 627.42352 | [M+Na] ⁺ | C36H60O7 | | 604.4339043 | -0.000375 |
| 377 | 6439 | 11.924 | 628.50037 | [M+NH4] ⁺ | | | | |
| 378 | 6448 | 10.877 | 629.43903 | [M+H] ⁺ | C34H56N6O5 | | 628.4312189 | -0.0005047 |
| 379 | 6473 | 10.418 | 634.45282 | [M+NH4] ⁺ | C33H60O10 | | 616.4186481 | -0.0003263 |
| 380 | 5453 | 12.112 | 518.34778 | [M+H] ⁺ | C30H47N06 | | 517.3403382 | -0.0001853 |
| 381 | 2473 | 12.278 | 284.29468 | [M+H] ⁺ | C18H37NO | Octadecanamide | 283.2875148 | 9.13E-05 |
| 382 | 6517 | 10.844 | 644.49554 | [M+NH4] ⁺ | C38H62N2O5 | | 626.4658731 | 0.0041986 |
| 383 | 6539 | 10.7 | 648.46869 | [M+NH4] ⁺ | C31H54N10O4 | | 630.4329502 | -0.0019242 |
| 384 | 5849 | 9.433 | 566.33722 | [M+H] ⁺ | C30H47N09 | | 565.3250821 | -0.0048415 |
| 385 | 6561 | 12.417 | 653.43866 | [M+Na] ⁺ | C38H62O7 | | 630.4495543 | 7.50E-05 |
| 386 | 6583 | 11.203 | 658.51031 | [M+NH4] ⁺ | C33H68O11 | | 640.476163 | -0.0003115 |
| 387 | 659 | 3.038 | 171.11255 | [M+H] ⁺ | C8H14N2O2 | | 170.1055277 | 0.0003041 |
| 388 | 660 | 3.332 | 171.11267 | [M+H] ⁺ | C8H14N2O2 | | 170.1055277 | 0.0001041 |
| 389 | 661 | 5.599 | 171.14389 | [M+NH4] ⁺ | | | | |
| 390 | 6633 | 11.607 | 671.45013 | [M+H] ⁺ | C36H58N6O6 | | 670.4417836 | -0.00104 |
| 391 | 6658 | 11.89 | 677.48187 | [M+Na] ⁺ | | | | |
| 392 | 6660 | 10.39 | 678.47961 | [M+NH4] ⁺ | C41H60N2O5 | | 660.450223 | 0.0044486 |
| 393 | 6694 | 12.172 | 685.4339 | [M+Na] ⁺ | C42H63O4P | | 662.4463971 | 0.0017178 |
| 394 | 6707 | 10.81 | 688.52167 | [M+NH4] ⁺ | | | | |
| 395 | 6725 | 12.395 | 691.49268 | [M+Na] ⁺ | | | | |
| 396 | 5768 | 9.507 | 559.3844 | [M+H-2O] ⁺ | C30H56O10 | | 576.387348 | -0.0003402 |
| 397 | 6641 | 9.726 | 673.39301 | [M+Na] ⁺ | C36H58O10 | | 650.4029981 | -0.0007812 |
| 398 | 6797 | 11.857 | 716.55249 | [M+NH4] ⁺ | C42H70N2O6 | | 698.523388 | 0.0047135 |
| 399 | 6811 | 10.362 | 722.5058 | [2M+NH4] ⁺ | C24H32O2 | | 352.2402303 | 0.0039687 |
| 400 | 6825 | 11.745 | 727.47375 | [M+H] ⁺ | C46H66N2O52 | | 726.4616567 | -0.0048668 |
| 401 | 6826 | 11.473 | 727.47595 | [M+Na] ⁺ | C41H68O9 | | 704.4863338 | -0.0004455 |
| 402 | 6831 | 8.628 | 728.52075 | [M+NH4] ⁺ | C42H66N2O7 | | 710.4870025 | 2.80E-05 |

| | | | | | | | |
|-----|------|--------|------------|-------------------------|--------------------------------|-------------|------------|
| 403 | 6837 | 12.351 | 730.56769 | [M+NH4] ⁺ | C37H76O12 | 712.5336779 | -0.0001966 |
| 404 | 6849 | 10.772 | 732.54779 | [2M+NH4] ⁺ | C20H31N5O | 357.2528606 | -0.0044009 |
| 405 | 6880 | 11.106 | 743.47247 | [M+Na] ⁺ | C41H68O10 | 720.4812484 | -0.0020309 |
| 406 | 691 | 3.292 | 174.11212 | [M+H] ⁺ | C8H15NO3 | 173.1051933 | 0.0003698 |
| 407 | 692 | 4.502 | 174.11235 | [M+H] ⁺ | C8H15NO3 | 173.1051933 | 6.98E-05 |
| 408 | 6929 | 11.432 | 757.48816 | [M+H] ⁺ | C45H64N4O6 | 756.4825858 | 0.0016622 |
| 409 | 694 | 4.337 | 174.11304 | [M+H-2H2O] ⁺ | C9H15N5O | 209.1276601 | 0.0008072 |
| 410 | 6942 | 11.827 | 760.58075 | [M+NH4] ⁺ | | | |
| 411 | 6946 | 9.518 | 761.50525 | [M+H] ⁺ | C43H73N2O5P5 | 760.4977811 | -0.0001425 |
| 412 | 695 | 3.696 | 174.11308 | [M+H-2H2O] ⁺ | C9H15N5O | 209.1276601 | 0.0007072 |
| 413 | 6954 | 10.336 | 766.53149 | [M+NH4] ⁺ | C45H68N2O7 | 748.5026525 | 0.0049781 |
| 414 | 6962 | 11.263 | 767.49323 | [M+Na] ⁺ | C46H68N2O6 | 744.5077379 | 0.0003786 |
| 415 | 698 | 10.003 | 174.12245 | [M+H] ⁺ | C7H15N3O2 | 173.1164267 | 0.0012032 |
| 416 | 7001 | 10.679 | 780.54779 | [M+NH4] ⁺ | C40H74O13 | 762.5129424 | -0.001032 |
| 417 | 7010 | 10.202 | 783.48877 | [M+Na] ⁺ | C43H73N2O5P5 | 760.4977811 | -0.0017982 |
| 418 | 675 | 10.453 | 172.13318 | [M+NH4] ⁺ | C9H14O2 | 154.0993797 | 5.20E-06 |
| 419 | 7016 | 10.693 | 785.5033 | [M+Na] ⁺ | C40H74O13 | 762.5129424 | -0.0011369 |
| 420 | 7050 | 7.135 | 795.3009 | [M+Na] ⁺ | | | |
| 421 | 7100 | 10.432 | 810.41052 | [M+NH4] ⁺ | C45H52N4O9 | 792.3734292 | -0.0032452 |
| 422 | 7102 | 10.308 | 810.55902 | [M+Na] ⁺ | C43H82NO9P | 787.5727198 | 0.0029405 |
| 423 | 7147 | 10.729 | 829.38354 | [M+Na] ⁺ | C44H58N2O12 | 806.3989753 | 0.004696 |
| 424 | 7151 | 10.533 | 829.53046 | [M+H-2O] ⁺ | | | |
| 425 | 7226 | 9.082 | 868.61835 | [2M+H] ⁺ | | | |
| 426 | 7234 | 11.28 | 871.39429 | [M+Na] ⁺ | C49H52N8O6 | 848.4009814 | -0.0040979 |
| 427 | 7266 | 7.69 | 883.50903 | [M+H] ⁺ | C52H70N2O10 | 882.5030464 | 0.0013229 |
| 428 | 7279 | 9.378 | 884.64563 | [M+H] ⁺ | | | |
| 429 | 7280 | 9.526 | 884.64648 | [M+Na] ⁺ | | | |
| 430 | 7287 | 9.11 | 886.62878 | [M+H] ⁺ | C52H88NO8P | 885.6247554 | 0.00032319 |
| 431 | 7289 | 9.938 | 886.66052 | [M+H] ⁺ | | | |
| 432 | 729 | 7.775 | 177.0546 | [M+H] ⁺ | C10H8O3 | 176.0473441 | 2.06E-05 |
| 433 | 7317 | 10.254 | 898.61163 | [2M+Na] ⁺ | Hemiarin | | |
| 434 | 1530 | 7.397 | 225.18933 | [M+H] ⁺ | C14H24O2 | 224.17763 | -0.0043935 |
| 435 | 7388 | 7.537 | 919.14746 | [2M+H] ⁺ | C20H17N3O8S | 459.0736355 | 0.00032494 |
| 436 | 745 | 4.774 | 179.06444 | [M+NH4] ⁺ | C5H7NO5 | 161.0324223 | 0.0018479 |
| 437 | 7453 | 9.409 | 944.66229 | [M+Na] ⁺ | | | |
| 438 | 747 | 11.265 | 179.07018 | [M+H] ⁺ | C10H10O3 | 178.0629942 | 7.06E-05 |
| 439 | 4846 | 11.193 | 449.30286 | [M+H-2H2O] ⁺ | 4-hydroxy-3-methoxyphenyl]prol | 484.3188745 | 0.0021216 |
| 440 | 7514 | 11.557 | 971.62823 | [M+Na] ⁺ | C30H44O5 | | |
| 441 | 7539 | 10.559 | 987.62299 | [M+Na] ⁺ | C63H84N2O6 | 964.6329384 | -0.0008409 |
| 442 | 7540 | 10.745 | 987.62366 | [M+Na] ⁺ | C63H84N2O6 | 964.6329384 | -0.0015409 |
| 443 | 755 | 4.357 | 179.11789 | [M+H-2O] ⁺ | C10H16N2O2 | 196.1211778 | -1.05E-05 |
| 444 | 7553 | 10.022 | 1003.61829 | [M+Na] ⁺ | cyclo(L-Pro-L-Val) | | |
| 445 | 762 | 3.758 | 180.10234 | [M+H] ⁺ | C10H13NO2 | 179.0946287 | -0.0003949 |
| 446 | 763 | 4.056 | 180.10242 | [M+H] ⁺ | C10H13NO2 | 179.0946287 | -0.0004949 |
| 447 | 764 | 4.498 | 180.10249 | [M+H] ⁺ | C10H13NO2 | 179.0946287 | -0.0005949 |

| | | | | | | | | |
|-----|------|-------|-----------|------------------------|------------|----------------------------------|-------------|------------|
| 448 | 1806 | 2.013 | 245.09537 | [M+FA+H] ⁺ | C12H10N2O | | 198.0793129 | -0.0033316 |
| 449 | 3056 | 7.007 | 313.22824 | [M+H-H2O] ⁺ | C20H30N2O2 | | 330.2307282 | -0.00076 |
| 450 | 1849 | 5.257 | 247.1443 | [M+H] ⁺ | C14H18N2O2 | | 246.1368278 | -0.0001957 |
| 451 | 874 | 6.192 | 187.13298 | [M+H] ⁺ | C10H18O3 | | 186.1255944 | -0.0001291 |
| 452 | 892 | 4.397 | 188.12881 | [M+H] ⁺ | C9H17NO3 | Polyethylene glycol | 187.1208434 | -0.0006801 |
| 453 | 926 | 5.459 | 191.06464 | [M+H] ⁺ | C6H10N2O5 | | 190.0589714 | 0.0016479 |
| 454 | 2045 | 3.55 | 261.12344 | [M+H] ⁺ | C14H16N2O3 | ine-L-tyrosine anhydride:Cyclol- | 260.1160924 | -3.12E-05 |
| 455 | 928 | 5.691 | 191.08453 | [M+Na] ⁺ | C13H12 | | 168.0939004 | -0.0013789 |
| 456 | 1378 | 5.629 | 215.06871 | [M+Na] ⁺ | C11H12O3 | | 192.0786442 | -0.0008351 |

ANNEXE 3 - EPIGENETIC MODIFIERS

| ID | Alignment | Average-Rt.min | Average-Mz | Adduct type | Formula | Structure | Total score | Theoretical,mas | Mass error |
|----|-----------|----------------|------------|------------------------|------------|---|-------------|-----------------|------------|
| 1 | 101 | 3.671 | 180.10167 | [M+H] ⁺ | C10H13NO2 | | 8.1144 | 179.0946287 | 0.0002051 |
| 2 | 103 | 4.535 | 182.02747 | [M+H] ⁺ | C5H11NO2S2 | | 7.3486 | 181.0231206 | 0.002897 |
| 3 | 105 | 4.326 | 183.09195 | [M+H] ⁺ | C7H10N4O2 | | 6.1565 | 182.0803756 | -0.004248 |
| 4 | 107 | 3.916 | 185.12848 | [M+H] ⁺ | C9H16N2O2 | | 6.8801 | 184.1211778 | -4.58E-05 |
| 5 | 110 | 4.289 | 188.12839 | [M+H] ⁺ | C9H17NO3 | Polyethylene glycol | 5.2244 | 187.1208434 | -0.0002801 |
| 6 | 112 | 5.313 | 191.07054 | [M+H] ⁺ | C11H10O3 | | 8.1144 | 190.0629942 | -0.0002294 |
| 7 | 113 | 5.494 | 193.08578 | [M+H] ⁺ | C11H12O3 | Terricolyne | 7.6517 | 192.0786442 | 0.0001207 |
| 8 | 139 | 6.877 | 200.28011 | [M+H] ⁺ | | | 6.9737 | 201.1364935 | -0.0002301 |
| 9 | 142 | 5.504 | 202.14395 | [M+H] ⁺ | C10H19NO3 | | 4.9472 | 203.115758 | 0.0001345 |
| 10 | 143 | 3.361 | 204.12291 | [M+H] ⁺ | C9H17NO4 | acine B1:(1S,2R,3R,5R,7R)-1,2-Dihydroxy-3,5 | 7.3486 | 204.0898776 | 5.41E-05 |
| 11 | 144 | 3.524 | 205.09708 | [M+H] ⁺ | C11H12N2O2 | L-Tryptophan | 6.1565 | 207.0531578 | -0.0034658 |
| 12 | 146 | 0.933 | 208.06393 | [M+H] ⁺ | C10H9NO4 | | 7.6517 | 207.0895433 | 0.0004197 |
| 13 | 147 | 4.624 | 208.09639 | [M+H] ⁺ | C11H13NO3 | | 6.9737 | 207.0895433 | 1.97E-05 |
| 14 | 148 | 5.535 | 208.09685 | [M+H] ⁺ | C11H13NO3 | (+)-Streptazolin | 6.1565 | 207.0895433 | -0.0001803 |
| 15 | 149 | 4.255 | 208.09695 | [M+H] ⁺ | C11H15NO4 | | 6.1578 | 225.100108 | -0.0001803 |
| 16 | 150 | 6.156 | 208.09697 | [M+H] ⁺ | C11H13NO3 | Thalifoline | 6.1578 | 207.0895433 | -0.0001803 |
| 17 | 151 | 6.366 | 208.13306 | [M+H] ⁺ | C12H17NO2 | | 4.9472 | 207.1259288 | 0.0001052 |
| 18 | 152 | 6.923 | 209.08092 | [M+H] ⁺ | C11H12O4 | 6-Methoxytellin | 6.1565 | 208.0735589 | -6.47E-05 |
| 19 | 154 | 4.092 | 209.08105 | [M+H] ⁺ | C11H12O4 | | 6.1565 | 208.0735589 | -0.0002647 |
| 20 | 160 | 7.13 | 211.08679 | [M+H] ⁺ | C13H10N2O | | 6.1565 | 210.0793129 | -0.0002106 |
| 21 | 114 | 4.267 | 193.08595 | [M+H+H2O] ⁺ | C11H14O4 | | 6.1578 | 210.0892089 | -7.93E-05 |
| 22 | 164 | 4.431 | 211.1445 | [M+H] ⁺ | C11H18N2O2 | L,L-Cyclo(leucylprolyl) | 6.1578 | 210.1368278 | -0.0003957 |
| 23 | 171 | 4.899 | 213.1597 | [M+H] ⁺ | C11H20N2O2 | | 6.1565 | 212.1524779 | 5.43E-05 |
| 24 | 183 | 6.884 | 222.11258 | [M+H] ⁺ | C12H15NO3 | | 6.1565 | 221.1051933 | -0.0001302 |
| 25 | 187 | 6.266 | 225.08711 | [M+H] ⁺ | C10H12N2O4 | Stavudine | 6.8801 | 224.0797069 | -0.0001167 |
| 26 | 188 | 5.556 | 225.11209 | [M+H] ⁺ | C12H16O4 | | 6.1565 | 224.104859 | 3.54E-05 |
| 27 | 189 | 7.284 | 225.19597 | [M+H] ⁺ | C13H24N2O | | 6.1565 | 224.1888634 | 0.0001398 |
| 28 | 193 | 4.331 | 227.09178 | [M+H] ⁺ | C11H14O5 | | 6.1565 | 226.0841235 | -4.00E-04 |
| 29 | 195 | 7.677 | 227.12784 | [M+H] ⁺ | C12H18O4 | | 6.1565 | 226.1205091 | -1.45E-05 |
| 30 | 196 | 3.776 | 227.13875 | [M+H] ⁺ | C11H18N2O3 | | 6.1565 | 226.1317424 | 0.0003189 |
| 31 | 197 | 5.156 | 227.13885 | [M+H] ⁺ | C11H18N2O3 | Amobarbital | 6.1565 | 226.1317424 | 0.0001189 |
| 32 | 198 | 5.464 | 227.17537 | [M+H] ⁺ | C12H22N2O2 | | 6.1565 | 226.1681279 | 4.40E-06 |
| 33 | 203 | 2.814 | 228.13431 | [M+H] ⁺ | C10H17N3O3 | | 6.1565 | 227.1269914 | -3.21E-05 |
| 34 | 207 | 7.764 | 229.14339 | [M+H] ⁺ | C12H20O4 | | 6.1565 | 228.1361591 | 3.56E-05 |
| 35 | 208 | 6.842 | 229.14355 | [M+H] ⁺ | C12H20O4 | | 6.1565 | 228.1361591 | -0.0001644 |
| 36 | 21 | 4.196 | 122.05992 | [M+H] ⁺ | C7H7NO | | 6.1565 | 121.0527638 | 0.0001403 |
| 37 | 211 | 4.469 | 231.12236 | [M+H] ⁺ | C12H14N4O | | 6.1565 | 230.1167611 | 0.0016375 |
| 38 | 224 | 4.328 | 240.1234 | [M+H] ⁺ | C12H17NO4 | | 6.1565 | 239.115758 | -0.0003655 |
| 39 | 225 | 6.468 | 240.15939 | [M+H] ⁺ | C13H21NO3 | | 6.1565 | 239.1521435 | 2.00E-05 |
| 40 | 226 | 0.873 | 242.10225 | [M+H] ⁺ | C11H15NO5 | | 6.1565 | 241.0950226 | 9.90E-05 |
| 41 | 228 | 3.285 | 242.14995 | [M+H] ⁺ | C11H19N3O3 | | 6.1565 | 241.1426415 | 1.79E-05 |
| 42 | 229 | 3.712 | 243.13336 | [M+H] ⁺ | C16H18O2 | | 6.1565 | 242.1306798 | 0.0045563 |

| | | | | | | | | | |
|----|-----|-------|-----------|-----------|-------------|---|--------|-------------|------------|
| 43 | 23 | 1.209 | 123.05505 | [M++]+ | G6H6N2O | Niacinamide | 5.8124 | 122.0480128 | 0.0001893 |
| 44 | 230 | 4.092 | 243.13396 | [M++]+ | C11H18N2O4 | | 5.8124 | 196.1211778 | -6.68E-05 |
| 45 | 232 | 6.576 | 244.09666 | [M++]+ | G9H13N3O5 | Verticillin A methyl ester | 5.2244 | 243.0855205 | -0.003903 |
| 46 | 234 | 4.887 | 244.15408 | [M++]+ | C12H21NO4 | | 5.2244 | 243.1470582 | 0.0002346 |
| 47 | 235 | 4.597 | 245.05583 | [M++]+ | G9H12N2O4S | | 7.5918 | 244.0517779 | 0.0032543 |
| 48 | 236 | 1.873 | 245.09558 | [M++]+ | C10H16N2O3S | Biotin | 7.5918 | 244.0881634 | -0.0001602 |
| 49 | 238 | 4.507 | 245.12859 | [M++]+ | C14H16N2O2 | Limazepine F(+)-Limazepine F | 6.5852 | 244.1211778 | -0.0001458 |
| 50 | 239 | 4.708 | 245.12877 | [M++]+ | C14H16N2O2 | Limazepine F(+)-Limazepine F | 6.9662 | 244.1211778 | -0.0003458 |
| 51 | 244 | 5.679 | 247.05338 | [M++H2O]+ | C12H12N2O3S | | 6.9662 | 264.0568632 | 0.000275 |
| 52 | 245 | 6.771 | 247.05367 | [M++H+] | C12H10N2O2S | | 6.9662 | 200.0408193 | -0.0001253 |
| 53 | 248 | 5.147 | 247.14398 | [M++H+] | C14H18N2O2 | | 6.9662 | 246.1368278 | 0.0001043 |
| 54 | 250 | 6.53 | 249.06929 | [M++H+] | C12H12N2O2S | | 6.9662 | 248.0619486 | -7.49E-05 |
| 55 | 252 | 4.868 | 251.05513 | [M++H+] | C12H10O6 | | 6.9662 | 250.047738 | -8.55E-05 |
| 56 | 254 | 3.849 | 252.08658 | [M++H+] | C12H13NO5 | | 6.9662 | 251.0793725 | 4.90E-05 |
| 57 | 255 | 4.67 | 252.09789 | [M++H+] | C11H13N3O4 | | 6.9662 | 251.0906059 | -1.77E-05 |
| 58 | 256 | 3.81 | 253.07056 | [M++H+] | C12H12O6 | | 6.9662 | 252.0633881 | 6.46E-05 |
| 59 | 261 | 5.437 | 255.06551 | [M++H+] | C15H10O4 | Chrysophanol | 6.4368 | 254.0579088 | -0.0003147 |
| 60 | 263 | 5.305 | 255.08643 | [M++H+] | C12H14O6 | Fumiquinone A | 6.3439 | 254.0790382 | -8.54E-05 |
| 61 | 264 | 5.276 | 255.13429 | [M+NH4]+ | C12H15NO4 | | 6.3439 | 237.100108 | -0.0003665 |
| 62 | 268 | 0.913 | 256.11823 | [M+NH4]+ | C12H14O5 | | 6.3439 | 238.0841235 | -0.0002509 |
| 63 | 271 | 5.514 | 257.08063 | [M++H+] | C15H12O4 | (2S)-Liquiritigenin | 7.6136 | 256.0735589 | 0.0002353 |
| 64 | 278 | 4.351 | 261.12323 | [M++H+] | C14H16N2O3 | | 7.6136 | 260.1160924 | 0.0001688 |
| 65 | 182 | 5.608 | 222.11255 | [M++H+] | C12H15NO3 | | 7.6136 | 221.1051933 | -3.02E-05 |
| 66 | 286 | 3.886 | 263.13876 | [M++H+] | C14H18N2O3 | | 7.6136 | 262.1317424 | 0.0002189 |
| 67 | 287 | 3.606 | 264.12308 | [M++H+] | C14H17NO4 | | 7.6136 | 263.115758 | -6.55E-05 |
| 68 | 288 | 6.955 | 264.15927 | [M++H+] | C15H21NO3 | | 7.6136 | 263.1521435 | 0.00012 |
| 69 | 290 | 4.815 | 265.0976 | [M++H+] | C13H16N2O2S | | 7.6136 | 264.0932488 | 0.0029252 |
| 70 | 291 | 5.104 | 265.11813 | [M++H+] | C13H16N2O4 | | 7.6136 | 264.111007 | 0.0001834 |
| 71 | 292 | 4.177 | 265.11832 | [M++H+] | C13H16N2O4 | | 7.6136 | 264.111007 | -1.66E-05 |
| 72 | 297 | 0.919 | 268.10425 | [2M+NH4]+ | | | 7.6136 | | |
| 73 | 298 | 1.232 | 268.10431 | [M++H+] | C10H13N5O4 | Adenosine | 9.2014 | 267.0967539 | -0.0002697 |
| 74 | 301 | 3.746 | 269.06564 | [M++H+] | C12H12O7 | | 9.2014 | 268.0583027 | -2.08E-05 |
| 75 | 302 | 6.43 | 270.11267 | [M+Na]+ | C14H17NO3 | | 9.2014 | 247.1208434 | -0.0026359 |
| 76 | 304 | 6.11 | 271.06003 | [M++H+] | C15H10O5 | Emodin | 6.5017 | 270.0528234 | 9.99E-05 |
| 77 | 306 | 5.912 | 271.11795 | [M++H+] | C13H18O6 | | 6.5017 | 270.1103383 | -0.0003853 |
| 78 | 308 | 0.927 | 272.11301 | [M++H2O]+ | C12H19NO7 | | 6.5017 | 289.1161519 | -0.0001363 |
| 79 | 314 | 4.763 | 276.12094 | [M+Na]+ | C13H19NO4 | | 6.5017 | 253.1314081 | -0.0002712 |
| 80 | 317 | 4.34 | 277.11844 | [M++H+] | G9H16N4O6 | | 6.5017 | 276.1069842 | -0.0041393 |
| 81 | 163 | 4.264 | 211.14444 | [M++H+] | C11H18N2O2 | L-L-Cycl(Leucylproyl) | 6.1323 | 210.1368278 | -0.0002957 |
| 82 | 319 | 4.03 | 277.155 | [M++H+] | C15H20N2O3 | | 6.1323 | 276.1473925 | -0.000331 |
| 83 | 320 | 8.906 | 277.21597 | [M++H2O]+ | C18H30O3 | ;:1,2Z,15Z)-9-Hydroxy-10,12,15-octadecatrieni | 6.6235 | 294.2194948 | 0.0002066 |
| 84 | 323 | 4.837 | 279.13385 | [M++H+] | C14H18N2O4 | 1-(alpha-D-riboseyl)-5,6-dimethyl-benzimidazo | 7.3687 | 278.1266571 | 3.35E-05 |
| 85 | 326 | 1.499 | 282.06436 | [M++H+] | C12H11NO7 | | 7.3687 | 281.0535517 | -0.0035719 |
| 86 | 329 | 4.366 | 283.10764 | [M++H+] | C16H14N2O3 | | 7.3687 | 282.1004423 | 0.0001188 |
| 87 | 33 | 1.605 | 131.07024 | [M++H+] | G6H10O3 | Ketoleucine | 5.4301 | 130.0629942 | 7.06E-05 |

| | | | | | | | | |
|-----|-----|--------|-----------|-----------|-------------|--|-------------|------------|
| 88 | 332 | 4.469 | 283.10828 | [M++]+ | C13H18N2O3S | | 282.1038134 | 0.0027899 |
| 89 | 336 | 5.563 | 285.07632 | [M++]+ | C16H12O5 | | 284.0684735 | -0.0005501 |
| 90 | 337 | 0.87 | 285.10825 | [M++]+ | C12H16N2O6 | | 284.1008362 | -8.73E-05 |
| 91 | 100 | 6.869 | 179.07039 | [M++]+ | C10H10O3 | Cinnamaldehyde | 132.0575149 | -0.0001297 |
| 92 | 341 | 5.931 | 287.19659 | [M++]+ | C19H26O2 | | 286.1932801 | 0.0039565 |
| 93 | 347 | 4.68 | 290.13901 | [M++]+ | C16H19NO4 | | 289.1314081 | -0.0003155 |
| 94 | 352 | 5.032 | 292.1546 | [M++]+ | C16H21NO4 | | 291.1470582 | -0.0002654 |
| 95 | 353 | 5.245 | 292.15475 | [M++]+ | C16H21NO4 | | 291.1470582 | -0.0004654 |
| 96 | 355 | 8.058 | 292.19116 | [M++]+ | C17H25NO3 | | 291.1834437 | -0.0004799 |
| 97 | 357 | 5.841 | 293.0593 | [M++]+ | C9H8N8O2S | | 292.0490925 | -0.0029311 |
| 98 | 361 | 6.211 | 293.12866 | [M++]+ | C18H16N2O2 | | 292.1211778 | -0.0002458 |
| 99 | 363 | 5.817 | 295.14465 | [M++]+ | C18H18N2O2 | | 294.1368278 | -0.0005957 |
| 100 | 364 | 9.819 | 295.22684 | [M++]+ | C18H30O3 | o-2-(2-enteny)(cyclopentaneoctanoic acid;OP1 | 294.2194948 | -2.87E-05 |
| 101 | 366 | 5.019 | 297.18549 | [M++]+ | C20H24O2 | 17a-Ethynyl/estradiol | 296.17763 | -0.0005935 |
| 102 | 368 | 4.463 | 298.12869 | [M++]+ | C14H19NO6 | UNPD147549 | 297.1212373 | -0.0001862 |
| 103 | 369 | 5.454 | 298.16476 | [M++]+ | C15H23NO5 | | 297.1576228 | 9.93E-05 |
| 104 | 370 | 6.634 | 298.16476 | [M++]+ | C15H23NO5 | | 297.1576228 | 9.93E-05 |
| 105 | 371 | 5.62 | 298.16498 | [M++]+ | C15H23NO5 | | 297.1576228 | -0.0001007 |
| 106 | 372 | 0.894 | 299.12433 | [M++]+ | C18H18O4 | | 298.1205091 | 0.0034855 |
| 107 | 374 | 6.412 | 300.14392 | [M++]+ | C14H21NO6 | | 299.134202 | -0.0024215 |
| 108 | 376 | 5.348 | 300.17035 | [M++]+ | C17H21N3O2 | | 299.1633769 | 0.0003534 |
| 109 | 377 | 4.161 | 301.11859 | [M++]+ | C16H16N2O4 | | 300.111007 | -0.0003166 |
| 110 | 386 | 5.986 | 306.17023 | [M++]+ | C17H23NO4 | | 305.1627082 | -0.0002153 |
| 111 | 388 | 0.919 | 308.14969 | [M++]+ | C16H21NO5 | | 307.1419728 | -0.0004508 |
| 112 | 389 | 5.73 | 309.08728 | [M++]+ | C17H12N2O4 | | 308.0797069 | -0.0003167 |
| 113 | 390 | 5.082 | 309.12366 | [M++]+ | C18H16N2O3 | | 308.1160924 | -0.0003312 |
| 114 | 391 | 5.613 | 309.12378 | [M++]+ | C18H16N2O3 | | 308.1160924 | -0.0004312 |
| 115 | 392 | 3.835 | 310.16467 | [M++]+ | C16H23NO5 | | 309.1576228 | 0.0001993 |
| 116 | 394 | 5.499 | 311.13892 | [M++]+ | C18H18N2O3 | | 310.1317424 | 0.0001189 |
| 117 | 395 | 5.898 | 311.1391 | [M++]+ | C18H18N2O3 | | 310.1317424 | -8.11E-05 |
| 118 | 396 | 4.658 | 311.13953 | [M++]+ | C18H18N2O3 | | 310.1317424 | -0.0004811 |
| 119 | 397 | 5.701 | 311.15985 | [M++]+ | C20H22O3 | | 310.1568946 | 0.004271 |
| 120 | 399 | 11.016 | 311.29407 | [M++]+ | C20H38O2 | Eicosenoic acid | 310.2871805 | 0.0003569 |
| 121 | 403 | 2.801 | 313.18091 | [M++]+ | C20H24O3 | | 312.1725446 | -0.0010789 |
| 122 | 404 | 4.375 | 315.13422 | [M++]+ | C17H18N2O4 | | 314.1266571 | -0.0002665 |
| 123 | 405 | 4.46 | 315.13467 | [M++]+ | C17H18N2O4 | | 314.1266571 | -0.0007665 |
| 124 | 409 | 9.121 | 320.22214 | [M++]+ | C19H29NO3 | | 319.2147438 | -7.98E-05 |
| 125 | 414 | 6.906 | 323.13937 | [M++]+ | C19H18N2O3 | Diphenylalazine B(+)-Diphenylalazine B | 322.1317424 | -0.0003811 |
| 126 | 416 | 4.552 | 324.18127 | [M++]+ | C17H25NO5 | | 323.1732729 | -0.0007506 |
| 127 | 418 | 6.791 | 326.23215 | [M++]+ | C18H31NO4 | | 325.2253085 | 0.0004849 |
| 128 | 419 | 11.091 | 326.37772 | [M++]+ | C22H47N | | 325.3708505 | 0.000427 |
| 129 | 421 | 7.133 | 327.13397 | [M++]+ | C18H18N2O4 | | 280.117155 | -0.0040896 |
| 130 | 426 | 9.049 | 331.24826 | [M++H2O]+ | C18H36O6 | | 348.2511889 | -0.0003994 |
| 131 | 427 | 8.46 | 332.27954 | [M+NH4]+ | C18H34O4 | 9,10-Epoxy-18-hydroxy-octadecanoic acid | 314.2457096 | 3.51E-05 |
| 132 | 430 | 6.031 | 335.16028 | [M++]+ | C17H22N2O5 | | 334.1528718 | -0.0001517 |

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|-----|-----|--------|-----------|-----------|--------------|--|--------|-------------|------------|
| 133 | 431 | 4.623 | 336.16736 | [M++]+ | C15H21NSO4 | | | 335.1593542 | -0.0007694 |
| 134 | 432 | 8.585 | 337.10455 | [M++]+ | C20H16O5 | | | 336.0997736 | 0.0024501 |
| 135 | 439 | 11.914 | 339.32611 | [M++]+ | C22H42O2 | Fructose acid | 6.2462 | 338.3184806 | -0.000343 |
| 136 | 44 | 3.09 | 141.0545 | [M++]+ | C7H8O3 | | | 140.0473441 | 0.0001206 |
| 137 | 443 | 7.015 | 341.15942 | [M++]+ | C17H24O7 | | | 340.1522031 | 7.96E-05 |
| 138 | 446 | 5.894 | 343.12894 | [M++]+ | C18H18N2O5 | | | 342.1215717 | -5.19E-05 |
| 139 | 447 | 3.967 | 345.14447 | [M++]+ | C18H20N2O5 | | | 344.1372217 | -1.80E-06 |
| 140 | 262 | 6.499 | 255.07651 | [M++]+ | C14H10N2O3 | | | 254.0691422 | -8.14E-05 |
| 141 | 453 | 4.506 | 350.16034 | [M++]+ | C18H23NO6 | | | 349.1525375 | -0.0004861 |
| 142 | 454 | 7.843 | 351.0972 | [M++]+ | C16H18N2O5S | | | 350.0936427 | 0.0037191 |
| 143 | 455 | 5.599 | 351.11118 | [M++]+ | C15H18N4O4S | | | 350.1048761 | 0.0009525 |
| 144 | 456 | 5.375 | 351.11139 | [M++]+ | C17H18O8 | | | 350.1001675 | -0.003956 |
| 145 | 428 | 6.211 | 335.11606 | [M++]+ | C17H18O7 | | | 334.1052529 | -0.0035706 |
| 146 | 429 | 6.018 | 335.11636 | [M++]+ | C17H18O7 | | | 334.1052529 | -0.0038706 |
| 147 | 460 | 7.762 | 353.14981 | [M++]+ | C20H20N2O4 | | | 352.1423071 | -0.0002164 |
| 148 | 462 | 10.806 | 356.38834 | [M++]+ | C23H49NO | | | | |
| 149 | 464 | 4.399 | 357.09042 | [M++]+ | C15H20N2O4S2 | Bisdethiobis (methylthio)-gliotoxin | 6.8552 | 356.0864491 | 0.0033256 |
| 150 | 466 | 6.021 | 357.10794 | [M++]+ | C18H16N2O6 | | | 356.1008362 | 0.0002127 |
| 151 | 467 | 5.735 | 359.07312 | [M++]+ | C18H14O8 | | | 358.0688674 | 0.0030439 |
| 152 | 471 | 9.611 | 365.13712 | [M++]+ | C22H20O5 | | | 364.1310737 | 0.0012502 |
| 153 | 472 | 6.374 | 367.09293 | [M++]+ | C16H18N2O6S | | | 366.0885573 | 0.0029337 |
| 154 | 474 | 5.377 | 367.1062 | [M++]+ | C15H18N4O5S | | | 366.0997907 | 0.0008671 |
| 155 | 477 | 7.446 | 371.05176 | [M++]+ | C18H14N2O3S2 | | | 370.0445843 | 6.08E-05 |
| 156 | 482 | 4.134 | 375.06784 | [M++]+ | C15H14N6O2S2 | | | 374.063782 | 0.0032585 |
| 157 | 483 | 5.598 | 375.10089 | [M++]+ | C18H18N2O5S | | | 374.0936427 | 1.91E-05 |
| 158 | 484 | 4.095 | 375.1012 | [M++]+ | C18H18N2O5S | | | 374.0936427 | -0.0002809 |
| 159 | 485 | 5.732 | 375.10135 | [M++]+ | C18H18N2O5S | | | 374.0936427 | -0.0003809 |
| 160 | 486 | 8.53 | 376.30505 | [M++]+ | C20H41NO5 | | | 375.2984734 | 0.0006499 |
| 161 | 488 | 5.774 | 378.11893 | [M++]+ | C18H19NO8 | | | 377.1110666 | -0.000557 |
| 162 | 489 | 6.676 | 379.14188 | [M++]+ | C19H22O8 | | | 378.1314677 | -0.0031559 |
| 163 | 493 | 5.893 | 384.21317 | [M++]+ | C23H29NO4 | | | 383.2096584 | 0.0037349 |
| 164 | 502 | 5.391 | 390.20255 | [M++]+ | C20H27N3O5 | | | 389.195071 | -0.0001526 |
| 165 | 504 | 5.058 | 391.26895 | [M++]+ | C20H38O7 | | | 390.2617536 | 3.00E-05 |
| 166 | 505 | 7.673 | 396.21707 | [M++]+ | C24H29NO4 | 1-(1-(2,4-dimethoxyphenyl)-decahydroisouquin | 7.8266 | 395.2096584 | -0.0001651 |
| 167 | 509 | 7.706 | 399.1011 | [M++]+ | C17H22N2O5S2 | | | 398.0970138 | 0.0031903 |
| 168 | 510 | 7.833 | 399.1012 | [M++]+ | C20H18N2O5S | | | 398.0936427 | -0.0002809 |
| 169 | 512 | 6.577 | 400.28067 | [M++]+ | C25H37NO3 | | | 399.277344 | 0.0039205 |
| 170 | 520 | 6.182 | 408.27411 | [M++]+ | C23H37NO5 | | | 407.2671733 | 0.0003497 |
| 171 | 524 | 6.615 | 412.21146 | [2M+NH4]+ | C7H11NSO2 | 3-Methylglutaraconic acid | 5.9764 | 197.0912746 | 0.0021631 |
| 172 | 529 | 7.394 | 417.05701 | [M++]+ | C23H12O8 | | | 416.0532173 | 0.0034938 |
| 173 | 53 | 1.764 | 145.04936 | [M++]+ | C6H8O4 | | | 144.0422587 | 0.0001352 |
| 174 | 530 | 5.974 | 417.11136 | [M++]+ | C15H20N4O8S | | | 416.1001846 | -0.0039389 |
| 175 | 533 | 4.059 | 417.11856 | [2M+H]+ | C11H12O2S | | | 208.0583027 | 0.0023667 |
| 176 | 536 | 6.374 | 419.12741 | [M++]+ | C20H22N2O6S | | | 418.1198574 | -0.0002661 |
| 177 | 540 | 8.345 | 423.20093 | [M++]+ | C22H30O8 | | | 422.1940679 | 0.0004444 |

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|-----|-----|--------|-----------|-----------------------|--------------------------|---|--------|-------------|------------|
| 178 | 541 | 10.536 | 425.21414 | [M+Na] ⁺ | C20H34O8 | UNPDI73832 | 6.9284 | 402.225368 | 0.0004887 |
| 179 | 526 | 5.661 | 415.04208 | [M++H2O] ⁺ | C23H12O9 | | | 432.048132 | 0.0027437 |
| 180 | 55 | 3.233 | 146.05971 | [M+H] ⁺ | C9H7NO | | | 145.0527638 | 0.0003403 |
| 181 | 550 | 6.625 | 433.05283 | [M+H] ⁺ | C23H12O9 | | | 432.048132 | 0.0026084 |
| 182 | 552 | 6.272 | 433.10684 | [M+H] ⁺ | C20H20N2O7S | | | 432.099122 | -0.0004016 |
| 183 | 554 | 7.366 | 434.08426 | [M+H] ⁺ | C19H19N3O5S2 | | | 433.0766127 | -0.0004108 |
| 184 | 531 | 6.13 | 417.11142 | [M+H] ⁺ | C15H20N4O8S | | | 416.1001846 | -0.0039389 |
| 185 | 556 | 8.599 | 435.06665 | [M+H] ⁺ | C22H14N2O6S | | | 434.0572572 | -0.0021664 |
| 186 | 557 | 6.93 | 435.06802 | [M+H] ⁺ | C23H14O9 | | | 434.063782 | 0.0030585 |
| 187 | 194 | 6.578 | 227.1026 | [M+H] ⁺ | C10H14N2O4 | Porphobilinogen | 6.3809 | 226.0953569 | 3.34E-05 |
| 188 | 56 | 5.027 | 146.05972 | [M+H] ⁺ | C9H7NO | | | 145.0527638 | 0.0003403 |
| 189 | 566 | 4.79 | 442.03427 | [M+H] ⁺ | ¹⁴ H16N7O2PS3 | | | 445.188923 | -0.0006006 |
| 190 | 567 | 4.397 | 446.19684 | [M+H] ⁺ | C20H27N7O3S | | | 446.0603807 | -0.0001428 |
| 191 | 569 | 7.076 | 447.06781 | [M+H] ⁺ | ¹⁴ H20N4O11P. | CDP-Ethanolamine | 6.176 | 446.0603807 | 0.0026195 |
| 192 | 545 | 6.188 | 431.09149 | [M+H] ⁺ | C17H22N2O7S2 | | | 430.086843 | 0.0026195 |
| 193 | 574 | 6.659 | 449.04733 | [M+H] ⁺ | C20H23N3O7S | | | 449.1256711 | 0.0005475 |
| 194 | 580 | 4.383 | 450.13239 | [M+H] ⁺ | C23H14O10 | | | 450.0586966 | 0.0029731 |
| 195 | 583 | 6.837 | 451.06299 | [M+H] ⁺ | C23H14O10 | | | 454.0300139 | 0.0034904 |
| 196 | 593 | 7.509 | 455.03375 | [M+H] ⁺ | C14H14N8O4S3 | | | 228.0997736 | -0.0005125 |
| 197 | 594 | 4.265 | 457.20728 | [2M+H] ⁺ | C11H16O5 | | | 458.2569429 | 0.0003194 |
| 198 | 597 | 6.439 | 459.04837 | [M+H] ⁺ | C29H34N2O3 | | | 458.2569429 | -8.06E-05 |
| 199 | 598 | 7.757 | 459.26392 | [M+H] ⁺ | C29H34N2O3 | | | 462.0900274 | -0.0017961 |
| 200 | 599 | 8.363 | 459.26431 | [M+H] ⁺ | C14H19N6O10F | Botrysosulfuranol A isomer | | 462.1162115 | 0.000188 |
| 201 | 602 | 6.272 | 463.09909 | [M+H] ⁺ | C22H22O11 | 6-Methoxyulteriorin 7-riamnoside | 7.6539 | 464.0743467 | 0.0030232 |
| 202 | 604 | 5.942 | 463.12332 | [M+H] ⁺ | C24H16O10 | | | 465.1180836 | -0.00274 |
| 203 | 609 | 8.04 | 465.07861 | [M+H] ⁺ | C24H23N3O3S2 | | | 432.1056468 | -0.0006767 |
| 204 | 614 | 3.719 | 466.12805 | [M+H] ⁺ | C21H20O10 | henyl)-6-[3,4,5-trihydroxy-6-(hydroxymethyl)c | 8.2188 | 466.1263823 | 0.002903 |
| 205 | 553 | 4.558 | 433.11356 | [M+H] ⁺ | C21H20O10 | | | 450.0586966 | 0.0028731 |
| 206 | 616 | 6.007 | 467.05765 | [M+H] ⁺ | C25H22O9 | | | 467.1337337 | -0.0025546 |
| 207 | 648 | 7.917 | 489.11267 | [M+Na] ⁺ | C25H22O9 | | | 467.1328646 | -0.0032589 |
| 208 | 584 | 6.45 | 451.06308 | [M+H] ⁺ | C23H14O10 | | | 472.35526 | -0.0005635 |
| 209 | 581 | 4.815 | 450.13297 | [M++H2O] ⁺ | C24H25N3O3S2 | | | 477.1028275 | 0.0003039 |
| 210 | 620 | 4.021 | 468.14337 | [M+H] ⁺ | C23H21N3O8 | | | 478.1111261 | -9.74E-05 |
| 211 | 621 | 9.578 | 473.36307 | [M+H] ⁺ | C30H48O4 | Ganodermanotriol | 5.9807 | 456.2723182 | 0.0007947 |
| 212 | 624 | 7.575 | 478.10983 | [M+H] ⁺ | C21H23N3O6S2 | | | 462.0950822 | 0.0030586 |
| 213 | 628 | 5.516 | 479.11847 | [M+H] ⁺ | C22H22O12 | yp(henyl)-3-[(3,4,5-trihydroxy-6-(hydroxymethyl | 8.7521 | 480.0661076 | -0.0001159 |
| 214 | 595 | 9.583 | 457.27881 | [M+H] ⁺ | C24H40O8 | | | | |
| 215 | 603 | 7.049 | 463.09927 | [M+H] ⁺ | C25H18O9 | Botrysosulfuranol A | | | |
| 216 | 632 | 6.518 | 481.07349 | [M+H] ⁺ | C20H20N2O8S2 | Gliovirin | 5.8412 | | |
| 217 | 635 | 7.205 | 482.05057 | [M+NH4] ⁺ | | | | | |
| 218 | 641 | 6.648 | 483.03506 | [M+H] ⁺ | | | | | |
| 219 | 642 | 5.915 | 484.0849 | [M+H] ⁺ | C17H18N5O10P | | | 483.0791284 | 0.0015049 |
| 220 | 649 | 4.847 | 489.23529 | [M+H] ⁺ | C29H32N2O5 | | | 488.2311221 | 0.0030986 |
| 221 | 650 | 9.76 | 491.16754 | [M+H] ⁺ | C28H26O8 | Homodimericin A | 5.6618 | 490.1627678 | 0.0025442 |
| 222 | 653 | 5.761 | 496.11722 | [M+H] ⁺ | C21H25N3O7S2 | | | 495.1133921 | 0.0034686 |

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|-----|-----|--------|-----------|------------------------------------|---|--|--|--|--|-------------|------------|
| 223 | 473 | 4.967 | 367.09338 | [M++H ₂ O] ⁺ | C19H16N ₂ O ₇ | | | | | 384.0957509 | -0.0009374 |
| 224 | 658 | 6.068 | 498.13278 | [M++H] ⁺ | C ₂₄ H ₂₃ N ₃ O ₇ S | | | | | 497.1256711 | 0.0001475 |
| 225 | 659 | 5.295 | 498.13614 | [M+NH ₄] ⁺ | C ₂₄ H ₂₀ N ₂ O ₇ S | | | | | 480.099122 | -0.0031525 |
| 226 | 634 | 6.476 | 481.11002 | [M++H] ⁺ | C ₂ 5H ₂ O ₁₀ | | | | | 480.1056468 | 0.0029233 |
| 227 | 662 | 9.886 | 498.37875 | [M++H] ⁺ | C ₂ 8H ₅ NO ₆ | | | | | 497.3716384 | 0.0001148 |
| 228 | 663 | 6.52 | 500.06149 | [M+NH ₄] ⁺ | | | | | | | |
| 229 | 664 | 4.979 | 501.0755 | [M++H] ⁺ | ¹⁴ H ₂ N ₄ O ₁₂ P ₂ | | | | | 500.0709454 | 0.0027219 |
| 230 | 667 | 10.042 | 505.1832 | [M++H] ⁺ | C ₂₄ H ₂₈ N ₂ O ₁₀ | | | | | 504.1743951 | -0.0015285 |
| 231 | 668 | 7.181 | 507.08881 | [M++H] ⁺ | C ₂ 6H ₁₈ O ₁₁ | | | | | 506.0849114 | 0.0033878 |
| 232 | 67 | 0.802 | 151.03505 | [M++H] ⁺ | C ₈ H ₆ O ₃ | | | | | 150.0316941 | 0.0039705 |
| 233 | 519 | 6.095 | 407.18143 | [M++H] ⁺ | C ₂₀ H ₂₆ N ₂ O ₇ | | | | | 406.1740012 | -0.0001224 |
| 234 | 677 | 6.794 | 513.04437 | [M++H] ⁺ | | | | | | | |
| 235 | 678 | 5.673 | 514.11017 | [M++H] ⁺ | C ₂ 8H ₁₉ NO ₉ | | | | | 513.1059812 | 0.0030576 |
| 236 | 656 | 7.802 | 497.08688 | [M++H] ⁺ | C ₂ 8H ₁₆ O ₉ | | | | | 496.0794321 | -0.0001915 |
| 237 | 682 | 10.012 | 519.19891 | [M++H] ⁺ | C ₃₀ H ₃ O ₈ | | | | | 518.1940679 | 0.0024444 |
| 238 | 280 | 3.428 | 261.12369 | [M++H] ⁺ | C ₁₄ H ₁₆ N ₂ O ₃ | | | | | 260.1160924 | -0.0003312 |
| 239 | 689 | 5.218 | 524.11145 | [M++H] ⁺ | | | | | | | |
| 240 | 691 | 6.049 | 526.11005 | [M++H] ⁺ | C ₁₈ H ₂₃ N ₉ O ₄ S | | | | | 525.1035132 | 0.0007897 |
| 241 | 693 | 6.012 | 528.12579 | [M++H] ⁺ | C ₂ 9H ₂₁ NO ₉ | | | | | 527.1216313 | 0.0031077 |
| 242 | 696 | 5.734 | 530.10486 | [M++H] ⁺ | ¹⁸ H ₂₄ N ⁷ O ₆ P ₅ S ₂ | | | | | 529.1008958 | 0.0032723 |
| 243 | 698 | 5.944 | 538.13153 | [M+NH ₄] ⁺ | C ₂₀ H ₂₀ N ₆ O ₉ S | | | | | 520.1012472 | 0.0035728 |
| 244 | 684 | 6.328 | 521.10516 | [M++H] ⁺ | C ₂₀ H ₂₀ N ₆ O ₉ S | | | | | 520.1012472 | 0.0033237 |
| 245 | 1 | 2.987 | 102.0547 | [M++H] ⁺ | C ₄ H ⁷ N ₂ O ₂ | | | | | 520.1012472 | 0.0002549 |
| 246 | 417 | 7.181 | 324.21756 | [M++H] ⁺ | C ₁₈ H ₂₉ NO ₄ | | | | | 101.0476785 | 0.0006551 |
| 247 | 704 | 6.5 | 540.14697 | [M+NH ₄] ⁺ | C ₁₉ H ₂₂ N ₈ O ₆ S ₂ | | | | | 323.2096584 | -0.0006651 |
| 248 | 705 | 6.055 | 542.14154 | [M++H] ⁺ | C ₃₀ H ₂ NO ₉ | | | | | 522.1103724 | -0.002802 |
| 249 | 708 | 6.172 | 544.12091 | [M++H] ⁺ | C ₁₇ H ₂₆ N ₃ O ₁₅ P | | | | | 541.1372813 | 0.0030578 |
| 250 | 711 | 6.163 | 549.15088 | [M++H] ⁺ | C ₂₈ H ₂₄ N ₂ O ₁₀ | | | | | 543.1101538 | -0.0034698 |
| 251 | 714 | 4.78 | 551.13977 | [2M+Na] ⁺ | C ₁₇ H ₁₂ O ₃ | | | | | 548.143095 | -0.0005286 |
| 252 | 701 | 8.707 | 538.39587 | [M++H] ⁺ | C ₃₃ H ₅ IN ₃ O ₃ | | | | | 264.0786442 | 0.0030803 |
| 253 | 718 | 5.84 | 560.15167 | [M++H] ⁺ | C ₃₀ H ₂ NO ₁₀ | | | | | 537.3930425 | 0.0044189 |
| 254 | 72 | 1.432 | 155.0815 | [M++H] ⁺ | C ₇ H ₁₀ N ₂ O ₂ | | | | | 559.147846 | 0.0034225 |
| 255 | 734 | 6.007 | 569.07159 | [M++H] ⁺ | C ₃₀ H ₁₆ O ₁₂ | | | | | 154.0742276 | 4.00E-06 |
| 256 | 735 | 6.292 | 569.35461 | [M++H] ⁺ | C ₃₃ H ₄₈ N ₂ O ₆ | | | | | 568.064176 | -0.0001476 |
| 257 | 716 | 9.037 | 552.4126 | [M++H] ⁺ | C ₂₉ H ₅ IN ₃ O ₅ | | | | | 568.3512373 | 0.0039137 |
| 258 | 738 | 6.323 | 571.08716 | [M++H] ⁺ | C ₃₀ H ₁₈ O ₁₂ | | | | | 551.4046698 | -0.0006538 |
| 259 | 742 | 6.386 | 574.16791 | [M++H] ⁺ | C ₃₁ H ₂ N ₂ O ₁₀ | | | | | 570.079826 | -9.75E-05 |
| 260 | 745 | 4.748 | 581.15155 | [M++H] ⁺ | C ₂₆ H ₂₈ O ₁₅ | | | | | 573.1634961 | 0.0028725 |
| 261 | 746 | 6.964 | 582.10327 | [M+NH ₄] ⁺ | C ₂₇ H ₂₀ N ₂ O ₈ S ₂ | | | | | 580.1428202 | -0.0015034 |
| 262 | 730 | 9.366 | 566.42798 | [M++H] ⁺ | C ₃₀ H ₅ IN ₃ O ₅ | | | | | 564.0661076 | -0.0033668 |
| 263 | 761 | 9.252 | 600.41089 | [M++H] ⁺ | C ₃₃ H ₅ IN ₃ O ₅ | | | | | 565.4203199 | -0.0004037 |
| 264 | 762 | 6.255 | 601.09735 | [M++H] ⁺ | ²⁴ H ₂₈ N ₂ O ₁₀ S ₃ | | | | | 599.4046698 | 0.0010462 |
| 265 | 763 | 6.693 | 602.1297 | [M++H] ⁺ | | | | | | 600.0906081 | 0.0004845 |
| 266 | 750 | 6.794 | 585.10376 | [M++H] ⁺ | C ₂₄ H ₂₈ N ₂ O ₉ S ₃ | | | | | 584.0956935 | -0.0008301 |
| 267 | 770 | 5.213 | 625.1767 | [M++H] ⁺ | C ₂₈ H ₃ Z ₂ O ₁₆ | | | | | 624.1690349 | -0.0003886 |

Tamarixetin 3-robino bioside

| | | | | | | | | |
|-----|-----|--------|------------|-----------|--------------|---|-------------|------------|
| 268 | 771 | 5.105 | 631.12927 | [M++]+ | C29H26O16 | | 630.1220848 | 6.12E-05 |
| 269 | 330 | 6.254 | 283.10782 | [M++]+ | C16H14N2O3 | | 282.1004423 | -8.12E-05 |
| 270 | 775 | 6.293 | 649.35559 | [M++]+ | C35H52O11 | | 648.3509625 | 0.0026389 |
| 271 | 781 | 9.523 | 681.44171 | [M++]+ | C34H64O13 | | 680.4346921 | 0.0002686 |
| 272 | 783 | 12.131 | 685.43445 | [M++]+ | C34H60N4O10 | | 684.4309441 | 0.0038206 |
| 273 | 791 | 5.714 | 747.37244 | [M++]+ | C44H50N4O7 | Pandicine | 746.3679499 | 0.0028264 |
| 274 | 793 | 11.516 | 758.59125 | [M++]+ | | | | |
| 275 | 293 | 2.194 | 266.15485 | [M++]+ | C18H19NO | | 265.1466642 | -0.0008593 |
| 276 | 812 | 6.161 | 869.17889 | [M++]+ | | | | |
| 277 | 813 | 12.11 | 871.57373 | [M++]+ | C55H74N4O5 | Pheophytin-a;Pheophytin a5 | 870.5659215 | -0.0005021 |
| 278 | 548 | 7.496 | 433.05215 | [M++]+ | C23H12O9 | Botrysulfuranol C | 432.048132 | 0.0032084 |
| 279 | 83 | 5.566 | 166.12236 | [M++]+ | C10H15NO | Pseudoephedrine | 165.1153641 | 0.0002406 |
| 280 | 834 | 7.47 | 919.14563 | [M+Na]+ | | | | |
| 281 | 839 | 7.095 | 931.14307 | [M++]+ | | | | |
| 282 | 84 | 0.802 | 167.01257 | [M++]+ | C4H7O5P | | 166.00311 | -0.0022136 |
| 283 | 610 | 7.317 | 465.11496 | [M++]+ | C25H20O9 | Botrysulfuranol B | 464.1107322 | 0.0030087 |
| 284 | 858 | 7.526 | 1014.14862 | [M++]+ | | | | |
| 285 | 109 | 5.025 | 187.13281 | [M++]+ | C10H18O3 | | 186.1255944 | 7.09E-05 |
| 286 | 860 | 8.63 | 1049.6969 | [M++]+ | C68H92N2O7 | | 1048.690453 | 0.0007297 |
| 287 | 861 | 9.188 | 1053.72791 | [M++]+ | | | | |
| 288 | 863 | 9.378 | 1079.74487 | [M++]+ | | | | |
| 289 | 867 | 9.784 | 1093.75769 | [M++]+ | 55H100N10O12 | | 1092.752219 | 0.0014951 |
| 290 | 90 | 1.344 | 174.05482 | [M++]+ | C10H7NO2 | | 173.0476785 | 0.0001549 |
| 291 | 91 | 4.352 | 174.11247 | [M++]+ | C8H15NO3 | | 173.1051933 | -3.02E-05 |
| 292 | 96 | 4.826 | 177.05453 | [M++]+ | C10H8O3 | 10-Hydroxy-2,8-decadiene-4,6-dinyric acid | 176.0473441 | 0.0001206 |
| 293 | 98 | 5.301 | 177.05461 | [M++]+ | C10H8O3 | Hemitarin | 176.0473441 | 2.06E-05 |
| 294 | 99 | 4.557 | 179.07024 | [M++H2O]+ | C10H12O4 | | 196.0735589 | 7.06E-05 |

ANNEXE 4 - ADDITION OF AMINO ACIDS

| ID | Alignment | Average-Rt.min. | Average.Mz | Adducttype | Formula | Structure | Theoreticalmass | Mass.error |
|----|-----------|-----------------|------------|-------------------------|--------------|----------------------|-----------------|------------|
| 1 | 100 | 3.856 | 154.04971 | [M+H] ⁺ | C7H7NO3 | hydroxyanthranilic a | 153.0425931 | 0.0001695 |
| 2 | 1019 | 6.25 | 313.08124 | [M+H] ⁺ | C19H20O4 | | 312.1361591 | |
| 3 | 1025 | 9.9 | 313.14404 | [M+H] ⁺ | C8H11NO2 | | 153.0789786 | |
| 4 | 103 | 3.209 | 154.08612 | [M+H] ⁺ | C3H8O2S2 | | 139.9965715 | |
| 5 | 106 | 0.581 | 158.02655 | [M+NH4] ⁺ | | | | |
| 6 | 1238 | 5.232 | 345.10742 | [M+FA+H] ⁺ | C16H14N2O4 | | 298.0953569 | |
| 7 | 1271 | 5.674 | 351.09702 | [M+H] ⁺ | C16H18N2O5S | | 350.0936427 | |
| 8 | 1296 | 9.054 | 354.33679 | [M+H] ⁺ | C22H43NO2 | | 353.3293796 | |
| 9 | 1305 | 8.187 | 356.10223 | [M+FA+H] ⁺ | C14H15NO7 | | 309.0848518 | |
| 10 | 1306 | 5.454 | 356.13873 | [M+H-2H2O] ⁺ | C19H25N3O4S | | 391.1565773 | |
| 11 | 1339 | 5.893 | 360.17987 | [M+H] ⁺ | C20H25NO5 | | 359.1732729 | |
| 12 | 1364 | 9.742 | 365.13605 | [M+H] ⁺ | C22H20O5 | | 364.1310737 | |
| 13 | 1449 | 5.889 | 382.16165 | [M+FA+H] ⁺ | C16H21N3O5 | | 335.1481208 | |
| 14 | 151 | 1.382 | 174.05469 | [M+H] ⁺ | C10H7NO2 | | 173.0476785 | |
| 15 | 1542 | 6.857 | 401.09784 | [2M+H] ⁺ | C12H8O3 | | 200.0473441 | |
| 16 | 1549 | 10.659 | 403.23297 | [M+H] ⁺ | C20H34O8 | | 402.225368 | |
| 17 | 155 | 3.073 | 176.07042 | [M+H] ⁺ | C10H9NO2 | Indoleacetic acid | 175.0633285 | 0.000205 |
| 18 | 157 | 1.852 | 176.07047 | [M+H] ⁺ | C10H9NO2 | nino-4-methylcourr | 175.0633285 | 0.000105 |
| 19 | 1592 | 7.51 | 417.05621 | [M+H] ⁺ | | | | |
| 20 | 162 | 4.315 | 178.08632 | [M+H] ⁺ | C10H11NO2 | 3a-Hydroxyfuroindo | 177.0789786 | -4.50E-05 |
| 21 | 1628 | 11.006 | 424.30621 | [M+NH4] ⁺ | C24H38O5 | | 406.2719243 | |
| 22 | 1650 | 11.228 | 429.26089 | [M+H] ⁺ | C26H36O5 | | 428.2562743 | |
| 23 | 1651 | 10.998 | 429.26105 | [M+Na] ⁺ | C24H38O5 | 3-Oxochoic acid | 406.2719243 | 0.000145 |
| 24 | 1652 | 11.629 | 429.26196 | [M+H] ⁺ | C26H36O5 | | 428.2562743 | |
| 25 | 167 | 3.752 | 180.10172 | [M+H] ⁺ | C10H13NO2 | | 179.0946287 | |
| 26 | 168 | 4.079 | 180.10191 | [M+H] ⁺ | C10H13NO2 | | 179.0946287 | |
| 27 | 1692 | 8.311 | 438.24847 | [M+H] ⁺ | C23H35NO7 | | 437.2413525 | |
| 28 | 1702 | 6.756 | 440.21707 | [M+H] ⁺ | C21H33N3O5S | | 439.2140922 | |
| 29 | 1734 | 6.275 | 448.11594 | [M+NH4] ⁺ | C17H22N2O7S2 | | 430.086843 | |
| 30 | 1742 | 7.604 | 450.07709 | [M+NH4] ⁺ | C19H16N2O6S2 | Botrysulfuranol C | 432.04 | |
| 31 | 1745 | 6.593 | 450.13159 | [M+H] ⁺ | C20H23N3O7S | | 449.1256711 | |
| 32 | 1757 | 6.277 | 453.07156 | [M+Na] ⁺ | C17H22N2O7S2 | | 430.086843 | |
| 33 | 1806 | 5.565 | 464.11197 | [M+NH4] ⁺ | | | | |
| 34 | 1123 | 7.228 | 327.13315 | [M+H] ⁺ | C13H18N4O6 | | 326.1226343 | |
| 35 | 1884 | 7.144 | 480.05118 | [M+H] ⁺ | C22H13N3O8S | | 479.0423354 | |
| 36 | 1784 | 7.14 | 463.09753 | [M+H] ⁺ | C21H22N2O6S2 | Botrysulfuranol A | 462.0900274 | |
| 37 | 1891 | 7.14 | 480.21042 | [M+H] ⁺ | C26H29N3O6 | amide H(-)-Notoarr | 479.2056356 | 0.0025121 |
| 38 | 1908 | 7.415 | 482.06784 | [M+H] ⁺ | | | | |
| 39 | 1910 | 7.408 | 482.13934 | [M+NH4] ⁺ | C21H24N2O6S2 | Botrysulfuranol B | 464.11 | |
| 40 | 1911 | 8.292 | 482.14224 | [M+NH4] ⁺ | C25H20O9 | | 464.1107322 | |
| 41 | 1871 | 6.858 | 478.14502 | [M+NH4] ⁺ | C22H24N2O5S2 | | 460.1126639 | |
| 42 | 1370 | 5.055 | 367.09192 | [M+H-2O] ⁺ | C19H16N2O7 | | 384.0957509 | |

| | | | | | | | | |
|----|------|--------|-----------|------------------------|--------------|------------------------|-------------|-----------|
| 43 | 1970 | 6.552 | 498.13516 | [M+NH4] ⁺ | C24H20N2O7S | | 480.099122 | |
| 44 | 1980 | 5.061 | 501.0752 | [M+Na] ⁺ | C25H18O10 | | 478.089968 | |
| 45 | 199 | 1.583 | 192.12306 | [M+H] ⁺ | C8H17NO4 | | 191.115758 | |
| 46 | 2005 | 7.425 | 510.17102 | [M+FA+H] ⁺ | C25H25N3O4S | | 463.1565773 | |
| 47 | 203 | 5.594 | 193.08574 | [M+H-H2O] ⁺ | C11H14O4 | | 210.0892089 | |
| 48 | 1718 | 8.384 | 445.23178 | [M+H] ⁺ | C24H32N2O6 | | 444.2260367 | |
| 49 | 2097 | 11.828 | 554.17615 | [M+NH4] ⁺ | C33H51N3O3 | | 537.3930425 | |
| 50 | 2099 | 8.794 | 555.42218 | [M+NH4] ⁺ | C30H55N5O5 | -Ile-L-Leu-L-Leu-L-Ile | 565.4203199 | 0.0005963 |
| 51 | 2131 | 9.448 | 566.427 | [M+H] ⁺ | C19H24N7O12P | | 573.1220558 | |
| 52 | 2166 | 5.367 | 574.12628 | [M+H] ⁺ | | | | |
| 53 | 2180 | 7.061 | 582.10187 | [M+H] ⁺ | | | | |
| 54 | 2208 | 9.451 | 588.40869 | [M+Na] ⁺ | C30H55N5O5 | | 565.4203199 | |
| 55 | 2209 | 11.101 | 588.4101 | [M+H] ⁺ | C32H53N5O5 | | 587.4046698 | |
| 56 | 2170 | 9.125 | 574.39386 | [M+Na] ⁺ | C29H53N5O5 | Leu-L-Leu-D-Leu-L-I | 551.4046698 | -9.50E-06 |
| 57 | 2230 | 7.744 | 600.37756 | [M+H] ⁺ | C31H53NO10 | | 599.3669469 | |
| 58 | 2240 | 9.189 | 603.42151 | [M+NH4] ⁺ | C31H55NO9 | | 585.3876823 | |
| 59 | 2256 | 9.475 | 611.48566 | [M+H] ⁺ | | | | |
| 60 | 2231 | 9.511 | 600.41119 | [M+H] ⁺ | C33H53N5O5 | -Phe-L-Leu-L-Leu-L- | 599.4046698 | 0.0007462 |
| 61 | 238 | 4.371 | 202.14395 | [M+H] ⁺ | C10H19NO3 | | 201.1364935 | |
| 62 | 2397 | 7.607 | 903.0509 | [M+H] ⁺ | | | | |
| 63 | 2398 | 7.461 | 903.14423 | [M+H] ⁺ | | | | |
| 64 | 2409 | 7.591 | 919.14124 | [M+Na] ⁺ | | | | |
| 65 | 2421 | 7.174 | 931.14014 | [2M+Na] ⁺ | C13H19N4O12P | | 454.0737087 | |
| 66 | 2438 | 7.411 | 951.20111 | [2M+Na] ⁺ | C21H24N2O6S2 | | 464.11 | |
| 67 | 245 | 10.066 | 205.086 | [M+H] ⁺ | C12H12O3 | | 204.0786442 | |
| 68 | 273 | 3.837 | 210.07599 | [M+H] ⁺ | C10H11NO4 | Ile-Ile-Ile | 209.0688078 | 8.43E-05 |
| 69 | 274 | 7.009 | 210.11195 | [M+H] ⁺ | C11H15NO3 | | 209.1051933 | |
| 70 | 283 | 4.548 | 211.08899 | [M+H] ⁺ | C10H14N2O5 | | 210.0826841 | |
| 71 | 284 | 6.421 | 211.09618 | [M+H] ⁺ | C11H14O4 | | 210.0892089 | |
| 72 | 293 | 0.583 | 212.07339 | [M+H] ⁺ | C10H13NO2S | | 211.0666997 | |
| 73 | 311 | 0.597 | 214.08955 | [M+H] ⁺ | C10H15NO2S | | 213.0823497 | |
| 74 | 312 | 1.184 | 214.08961 | [M+H] ⁺ | C10H15NO2S | | 213.0823497 | |
| 75 | 340 | 2.573 | 220.118 | [M+H] ⁺ | C9H17NO5 | | 219.1106726 | |
| 76 | 347 | 5.055 | 221.07068 | [M+H] ⁺ | C11H12N2O5 | | 220.067034 | |
| 77 | 364 | 4.643 | 224.1281 | [M+NH4] ⁺ | C12H14O3 | | 206.0942943 | |
| 78 | 1367 | 5.333 | 366.15405 | [M+H] ⁺ | C18H23NO7 | | 365.1474521 | |
| 79 | 381 | 5.348 | 227.08084 | [M+H] ⁺ | C8H10N4O4 | | 226.0702048 | |
| 80 | 393 | 5.304 | 228.159 | [M+H] ⁺ | C12H21NO3 | | 227.1521435 | |
| 81 | 394 | 5.476 | 228.15903 | [M+H] ⁺ | C12H21NO3 | | 227.1521435 | |
| 82 | 395 | 5.163 | 228.15909 | [M+H] ⁺ | C12H21NO3 | | 227.1521435 | |
| 83 | 405 | 3.259 | 229.11809 | [M+H] ⁺ | C10H16N2O4 | | 228.111007 | |
| 84 | 41 | 1.675 | 131.06998 | [2M+H] ⁺ | | | | |
| 85 | 413 | 4.295 | 230.13885 | [M+H] ⁺ | C11H19NO4 | | 229.1314081 | |
| 86 | 455 | 6.405 | 238.10693 | [M+H] ⁺ | C12H15NO4 | (+)-Flavipucine | 237.100108 | 0.0004844 |
| 87 | 456 | 5.958 | 238.10707 | [M+H] ⁺ | C12H15NO4 | (+)-Flavipucine | 237.100108 | 0.0002844 |

| | | | | | | | | |
|-----|-----|--------|-----------|------------------------|-------------|----------------------|-------------|-----------|
| 88 | 458 | 4.405 | 238.10724 | [M+H] ⁺ | C12H15NO4 | | 237.100108 | |
| 89 | 477 | 6.175 | 241.09668 | [M+H] ⁺ | C14H12N2O2 | b-tetrahydroquinol | 240.0898776 | |
| 90 | 490 | 4.124 | 243.11374 | [M+FA+H] ⁺ | C8H12N4O2 | | 196.0960256 | 0.0004541 |
| 91 | 499 | 4.98 | 244.1541 | [M+H] ⁺ | C12H21NO4 | | 243.1470582 | |
| 92 | 514 | 4.52 | 246.07339 | [M+Na] ⁺ | C11H13NO4 | | 223.0844579 | |
| 93 | 516 | 5.27 | 246.16963 | [M+H] ⁺ | C12H23NO4 | | 245.1627082 | |
| 94 | 517 | 3.562 | 246.17004 | [M+H] ⁺ | C12H23NO4 | | 245.1627082 | |
| 95 | 520 | 4.209 | 246.17024 | [M+H] ⁺ | C12H23NO4 | | 245.1627082 | |
| 96 | 521 | 4.22 | 246.22508 | [M+H] ⁺ | C17H27N | | 245.2143499 | |
| 97 | 538 | 4.102 | 248.18547 | [M+NH4] ⁺ | C12H22O4 | | 230.1518092 | |
| 98 | 569 | 9.648 | 253.13345 | [M+H] ⁺ | C16H16N2O | | 252.1262631 | |
| 99 | 57 | 2.178 | 140.07048 | [M+H] ⁺ | C7H9NO2 | | 139.0633285 | |
| 100 | 570 | 10.149 | 253.13368 | [M+H] ⁺ | C16H16N2O | | 252.1262631 | |
| 101 | 578 | 4.83 | 254.13855 | [M+H] ⁺ | C13H19NO4 | | 253.1314081 | |
| 102 | 579 | 4.721 | 254.13873 | [M+H] ⁺ | C13H19NO4 | | 253.1314081 | |
| 103 | 457 | 5.379 | 238.10713 | [M+H] ⁺ | C12H15NO4 | (+)-Flavipucine | 237.100108 | 0.0002844 |
| 104 | 599 | 3.945 | 256.11783 | [M+H] ⁺ | C12H17NO5 | | 255.1106726 | |
| 105 | 626 | 5.638 | 259.12277 | [M+H] ⁺ | C13H14N4O2 | | 258.1116757 | |
| 106 | 629 | 5.96 | 260.08865 | [M+Na] ⁺ | C12H15NO4 | | 237.100108 | |
| 107 | 644 | 4.384 | 262.10495 | [M+Na] ⁺ | C12H17NO4 | | 239.115758 | |
| 108 | 649 | 5.026 | 262.14352 | [M+H] ⁺ | C15H19NO3 | -methyl(ethyl)-3-(ph | 261.1364935 | 0.0002699 |
| 109 | 454 | 5.751 | 238.10692 | [M+H] ⁺ | C12H15NO4 | (+)-Flavipucine | 237.100108 | 0.0004844 |
| 110 | 703 | 6.633 | 269.09183 | [M+H-H2O] ⁺ | C15H14N2O4 | | 286.0953569 | |
| 111 | 729 | 6.084 | 273.07507 | [M+H] ⁺ | C15H12O5 | | 272.0684735 | |
| 112 | 752 | 4.826 | 276.12036 | [M+FA+H] ⁺ | C9H15N3O4 | | 229.106256 | |
| 113 | 758 | 3.866 | 277.11813 | [M+H] ⁺ | C9H16N4O6 | | 276.1069842 | |
| 114 | 764 | 4.384 | 278.07883 | [M+H] ⁺ | C9H15N3O5S | | 277.0732416 | |
| 115 | 779 | 10.05 | 279.15918 | [M+H] ⁺ | C16H22O4 | | 278.1518092 | |
| 116 | 810 | 5.831 | 284.11801 | [M+H] ⁺ | C14H13N5O2 | | 283.1069247 | |
| 117 | 824 | 4.697 | 285.12283 | [M+H] ⁺ | C11H16N4O5 | | 284.1120696 | |
| 118 | 825 | 4.804 | 285.12283 | [M+H] ⁺ | C16H16N2O3 | | 284.1160924 | |
| 119 | 835 | 5.726 | 287.09076 | [M+H] ⁺ | C16H14O5 | | 286.0841235 | |
| 120 | 869 | 4.828 | 292.09439 | [2M+NH4] ⁺ | C7H7NS | | 137.0299202 | |
| 121 | 871 | 4.862 | 292.1539 | [M+H] ⁺ | C16H21NO4 | | 291.1470582 | |
| 122 | 874 | 5.941 | 293.05847 | [M+H] ⁺ | C9H8N8O2S | | 292.0490925 | |
| 123 | 879 | 3.274 | 293.11301 | [M+H] ⁺ | C14H16N2O5 | | 292.1059216 | |
| 124 | 909 | 8.384 | 297.12256 | [M+H] ⁺ | C17H16N2O3 | | 296.1160924 | |
| 125 | 929 | 4.637 | 299.13858 | [M+H] ⁺ | C17H18N2O3 | Luteoride A | 298.1317424 | 0.0004189 |
| 126 | 932 | 5.484 | 299.15918 | [M+H] ⁺ | C19H22O3 | | 298.1568946 | |
| 127 | 939 | 4.173 | 301.11761 | [M+H] ⁺ | C14H21O5P | | 300.1126604 | |
| 128 | 941 | 4.735 | 301.11856 | [M+Na] ⁺ | C11H22N2O4S | | 278.1300282 | |
| 129 | 95 | 1.549 | 153.06566 | [M+H] ⁺ | C7H8N2O2 | | 152.0585775 | |
| 130 | 975 | 5.202 | 307.1286 | [M+H] ⁺ | C15H18N2O5 | | 306.1215717 | |
| 131 | 98 | 5.963 | 154.04935 | [M+H] ⁺ | C7H7NO3 | hydroxyanthranilic e | 153.0425931 | 0.0005695 |
| 132 | 99 | 4.647 | 154.0497 | [M+H] ⁺ | C7H7NO3 | hydroxyanthranilic e | 153.0425931 | 0.0001695 |

ANNEXE 5 - HEAT-KILLED LEISHMANIA

| ID | Alignment | Average-Rt.min | Average.Mz | Adduct.type | Formula | Structure | Theoretical.mas: | Mass.error |
|----|-----------|----------------|------------|-----------------------|--------------|--|------------------|------------|
| 1 | 1009 | 5.168 | 366.15494 | [M+H] ⁺ | C18H23NO7 | | 365.1474521 | |
| 2 | 1097 | 7.42 | 385.10333 | [M+H] ⁺ | C19H16N2O7 | | 384.0957509 | |
| 3 | 1108 | 6.946 | 387.1004 | [M+H] ⁺ | C19H18N2O5S | | 386.0936427 | |
| 4 | 1119 | 6.067 | 392.2272 | [M+H] ⁺ | C22H33NO3S | | 391.2181149 | |
| 5 | 1129 | 6.574 | 396.22192 | [M+NH4] ⁺ | C24H26O4 | | 378.1831093 | |
| 6 | 1134 | 10.857 | 398.23294 | [M+H] ⁺ | C24H31NO4 | | 397.2253085 | |
| 7 | 1332 | 11.468 | 461.32388 | [M+Na] ⁺ | C26H46O5 | | 438.3345246 | |
| 8 | 122 | 3.726 | 174.11235 | [M+H] ⁺ | C8H15NO3 | Swainsonine(-)-Swainsonine | 173.1051933 | 6.98E-05 |
| 9 | 1240 | 10.762 | 429.26041 | [M+Na] ⁺ | C24H38O5 | 3-Oxochohic acid | 406.2719243 | 0.000745 |
| 10 | 1295 | 6.09 | 448.11673 | [M+NH4] ⁺ | C17H22N2O7S2 | | 430.086843 | |
| 11 | 133 | 4.165 | 179.11769 | [M+H] ⁺ | C10H14N2O | Nicotine-1'-N-oxide | 178.1106131 | 0.0001895 |
| 12 | 136 | 3.908 | 180.10149 | [M+H] ⁺ | C10H13NO2 | | 179.0946287 | |
| 13 | 1367 | 6.981 | 469.04996 | [M+H] ⁺ | | | | |
| 14 | 1292 | 7.603 | 447.10309 | [M+H] ⁺ | C21H22N2O5S2 | | 446.0970138 | |
| 15 | 138 | 3.59 | 180.10202 | [M+H] ⁺ | C10H13NO2 | (R)-Salisolinol | 179.0946287 | -9.49E-05 |
| 16 | 1315 | 7.396 | 455.03323 | [M+Na] ⁺ | C19H16N2O6S2 | Botryosulfuranol C | 432.0455 | |
| 17 | 1390 | 6.67 | 478.14548 | [M+NH4] ⁺ | C22H24N2O5S2 | | 460.1126639 | |
| 18 | 1439 | 7.212 | 487.09592 | [M+Na] ⁺ | C21H24N2O6S2 | Botryosulfuranol B | 464.1077 | |
| 19 | 1461 | 4.882 | 496.12042 | [M+NH4] ⁺ | C25H18O10 | | 478.0899968 | |
| 20 | 1467 | 5.208 | 498.13568 | [M+NH4] ⁺ | C24H20N2O7S | | 480.099122 | |
| 21 | 1402 | 6.946 | 480.12448 | [M+NH4] ⁺ | C21H22N2O6S2 | Botryosulfuranol A | 462.092 | |
| 22 | 150 | 1.647 | 184.09685 | [M+H] ⁺ | C9H13NO3 | | 183.0895433 | |
| 23 | 1528 | 5.857 | 538.13086 | [M+NH4] ⁺ | C27H20O11 | | 520.1005615 | |
| 24 | 1529 | 8.592 | 538.39722 | [M+H] ⁺ | C33H51N3O3 | | 537.3930425 | |
| 25 | 161 | 4.219 | 188.12782 | [M+H] ⁺ | C9H11NO3 | Polyethylene glycol | 187.1208434 | 0.0003199 |
| 26 | 1637 | 9.244 | 588.40851 | [M+Na] ⁺ | C30H55N5O5 | Cyclo-(L-Ile-L-Leu-L-Leu-L-Leu) | 565.4203199 | 0.0010406 |
| 27 | 1582 | 9.072 | 566.42725 | [M+H] ⁺ | C30H55N5O5 | Cyclo-(L-Ile-L-Leu-L-Leu-L-Leu) | 565.4203199 | 0.0003963 |
| 28 | 1628 | 11.514 | 584.47296 | [M+H] ⁺ | | | | |
| 29 | 163 | 2.215 | 190.10732 | [M+H] ⁺ | C8H15NO4 | | 189.100108 | |
| 30 | 165 | 5.229 | 191.07011 | [M+H] ⁺ | C11H10O3 | | 190.0629942 | |
| 31 | 1662 | 6.595 | 602.12854 | [M+NH4] ⁺ | C31H20O12 | | 584.0954761 | |
| 32 | 1664 | 9.245 | 604.38171 | [M+FA+H] ⁺ | C33H51NO6 | | 557.3716384 | |
| 33 | 167 | 2.141 | 191.17557 | [M+H] ⁺ | C14H22 | | 190.1721507 | |
| 34 | 1670 | 12.394 | 610.18408 | [M+H] ⁺ | | | 191.115758 | |
| 35 | 168 | 1.461 | 192.12288 | [M+H] ⁺ | C8H17NO4 | | 191.115758 | |
| 36 | 1657 | 9.307 | 600.41162 | [M+H] ⁺ | C33H53N5O5 | yclo-(L-Phe-L-Leu1-L-Leu2-L-Leu3-L-Ile | 599.4046698 | 0.0003462 |
| 37 | 169 | 1.618 | 192.1232 | [M+H] ⁺ | C8H17NO4 | | 191.115758 | |
| 38 | 171 | 5.401 | 193.08588 | [M+H] ⁺ | C11H12O3 | | 192.0786442 | |
| 39 | 1745 | 7.216 | 729.2132 | [M+H] ⁺ | C34H36N2O16 | | 728.2064831 | |
| 40 | 1776 | 12.015 | 823.51117 | [M+H] ⁺ | C48H66N6O6 | | 822.5043838 | |
| 41 | 1429 | 6.947 | 485.07956 | [M+Na] ⁺ | | | 462.092 | |
| 42 | 1829 | 7.209 | 949.19055 | [M+H] ⁺ | C14H19N6O10P | 3'-L-aspartyl-AMP | | -0.0003519 |

| | | | | | | | | |
|----|------|-------|-----------|-----------------------|-------------|--|-------------|------------|
| 43 | 1601 | 8.925 | 574.39343 | [M+Na] ⁺ | C29H53N5O5 | Cyclo(-L-Leu-L-Leu-D-Leu-L-Leu-L-Val) | 551.4046698 | 0.0004905 |
| 44 | 228 | 4.589 | 209.11719 | [M+H] ⁺ | C12H16O3 | | 208.1099444 | |
| 45 | 233 | 6.814 | 210.11198 | [M+H] ⁺ | C11H15NO3 | | 209.1051933 | |
| 46 | 240 | 9.12 | 211.10956 | [M+H] ⁺ | C10H14N2O3 | | 210.1004423 | |
| 47 | 287 | 2.326 | 220.11769 | [M+H] ⁺ | C9H17NO5 | Pantothenic acid | 219.1106726 | 0.0002491 |
| 48 | 29 | 1.541 | 131.07004 | [M+H] ⁺ | C6H10O3 | Ketoleucine | 130.0629942 | 0.0002706 |
| 49 | 372 | 6.22 | 238.10693 | [M+H] ⁺ | C12H15NO4 | (+)-Flavipucine | 237.100108 | 0.0004844 |
| 50 | 375 | 5.681 | 238.10715 | [M+H] ⁺ | C12H15NO4 | (+)-Flavipucine | 237.100108 | 0.0002844 |
| 51 | 384 | 4.235 | 240.12276 | [M+FA+H] ⁺ | C11H15NO2 | | 193.1102787 | |
| 52 | 430 | 5.088 | 246.1702 | [M+H] ⁺ | C12H23NO4 | | 245.1627082 | |
| 53 | 440 | 6.43 | 249.06882 | [M+H] ⁺ | C12H12N2O25 | | 248.0619486 | |
| 54 | 459 | 3.757 | 253.07077 | [M+H] ⁺ | C12H12O6 | | 252.0633881 | |
| 55 | 463 | 4.572 | 254.1384 | [M+H] ⁺ | C13H19NO4 | | 253.1314081 | |
| 56 | 481 | 3.797 | 256.11804 | [M+H] ⁺ | C12H17NO5 | | 255.1106726 | |
| 57 | 482 | 0.884 | 256.11816 | [M+H] ⁺ | C12H17NO5 | | 255.1106726 | |
| 58 | 490 | 5.437 | 257.08087 | [M+H] ⁺ | C15H12O4 | (2S)-Liquiritigenin | 256.0735589 | -6.47E-05 |
| 59 | 493 | 7.503 | 257.17432 | [M+H] ⁺ | C14H24O4 | | 256.1674592 | |
| 60 | 505 | 6.764 | 259.1647 | [M+H] ⁺ | C12H22N2O4 | | 258.1579572 | |
| 61 | 376 | 5.192 | 238.10722 | [M+H] ⁺ | C12H15NO4 | | 237.100108 | 0.0001844 |
| 62 | 522 | 4.229 | 262.10474 | [M+FA+H] ⁺ | C8H13N3O4 | (+)-Flavipucine | 215.0906059 | |
| 63 | 526 | 4.872 | 262.14374 | [M+H] ⁺ | C15H19NO3 | γ-Hyl-6-(1-methylethyl)-3-(phenylmethyl)-6-(1-methylethyl)-3-(phenylmethyl)-4-oxo-1-cyclohexa-2,5-dienylidene)me | 261.1364935 | 6.99E-05 |
| 64 | 538 | 5.596 | 265.06384 | [M+H] ⁺ | C15H8N2O3 | | 264.0534921 | -0.0030314 |
| 65 | 573 | 2.93 | 272.14917 | [M+H] ⁺ | C13H21NO5 | | 271.1419728 | |
| 66 | 574 | 4.669 | 272.14951 | [M+H] ⁺ | C13H21NO5 | | 271.1419728 | |
| 67 | 590 | 8.198 | 275.20053 | [M+FA+H] ⁺ | C17H24 | | 228.1878008 | |
| 68 | 60 | 2.143 | 146.11751 | [M+H] ⁺ | C7H15NO2 | | 145.1102787 | |
| 69 | 613 | 9.371 | 279.23151 | [M+H] ⁺ | C18H30O2 | Crepenynic acid | 278.2245802 | 0.0003567 |
| 70 | 627 | 5.196 | 283.16492 | [2M+NH4] ⁺ | | | | |
| 71 | 641 | 7.36 | 285.16977 | [M+H] ⁺ | C15H24O5 | | 284.1623739 | |
| 72 | 648 | 5.555 | 287.09113 | [M+H] ⁺ | C16H14O5 | Sakuranetin | 286.0841235 | 3.00E-04 |
| 73 | 651 | 6.466 | 288.17978 | [M+H] ⁺ | C14H25NO5 | | 287.1732729 | |
| 74 | 664 | 4.159 | 290.15958 | [2M+NH4] ⁺ | C7H8N2O | | 136.0636629 | |
| 75 | 676 | 5.752 | 293.05927 | [M+H] ⁺ | C13H12N2O45 | | 292.0517779 | |
| 76 | 679 | 6.117 | 293.12842 | [M+H] ⁺ | C18H16N2O2 | | 292.1211778 | 5.42E-05 |
| 77 | 688 | 6.671 | 295.05655 | [M+H] ⁺ | C17H10O5 | UNPD92998 | 294.0528234 | |
| 78 | 692 | 7.313 | 295.22626 | [M+H] ⁺ | C18H30O3 | (2-entenyl)cyclopentaneoctanoic acid | 294.2194948 | 0.0004713 |
| 79 | 693 | 7.719 | 295.22632 | [M+H] ⁺ | C18H30O3 | (2-entenyl)cyclopentaneoctanoic acid | 294.2194948 | 0.0004713 |
| 80 | 72 | 3.682 | 154.04979 | [M+Na] ⁺ | C5H9NO3 | | 131.0582431 | |
| 81 | 73 | 4.491 | 154.04991 | [M+Na] ⁺ | C5H9NO3 | | 131.0582431 | |
| 82 | 748 | 8.745 | 309.24274 | [M+Na] ⁺ | C17H34O3 | | 286.2507949 | |
| 83 | 752 | 8.199 | 310.23773 | [M+H] ⁺ | C18H31NO3 | | 309.2303939 | |
| 84 | 604 | 8.787 | 277.21631 | [M+H] ⁺ | C18H28O2 | | 276.2089301 | |
| 85 | 805 | 6.533 | 320.20627 | [M+H] ⁺ | C16H25N5O2 | | 319.200825 | |
| 86 | 806 | 5.588 | 320.20676 | [M+H] ⁺ | C16H25N5O2 | | 319.200825 | |
| 87 | 826 | 7.037 | 327.13339 | [M+H] ⁺ | C13H18N4O6 | cyclo(tyrosyl-tyrosyl) | 326.1226343 | -0.0034893 |

| | | | | | | |
|----|-----|--------|-----------|----------------------|------------|-------------|
| 88 | 867 | 10.447 | 336.27484 | [M+NH4] ⁺ | C17H34O5 | 318.2406242 |
| 89 | 878 | 4.239 | 340.18695 | [M+H] ⁺ | C16H25N3O5 | 339.1794209 |
| 90 | 939 | 4.722 | 352.19669 | [M+H] ⁺ | C19H29NO3S | 351.1868148 |
| 91 | 982 | 5.746 | 360.1803 | [M+H] ⁺ | C20H25NO5 | 359.1732729 |
| 92 | 992 | 8.545 | 362.21741 | [M+H] ⁺ | C18H27N5O3 | 361.2113897 |
| 93 | 994 | 8.213 | 362.21793 | [M+H] ⁺ | C18H27N5O3 | 361.2113897 |

Fungal endophytes: a source of anti-parasitic and antibacterial compounds

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Abstract

The increase and emergence of resistant microbes (bacteria, fungi, and parasites) have led researchers to search for new anti-infective drugs and to study new sources of bioactive compounds. Among them, endophytic fungi are attracting more and more attention because this source is still largely under-exploited and relatively little studied from a chemical point of view. Because of their specific environment, and especially their constant interactions with their host plant and its entire microbiome, they represent a huge reservoir of bioactive compounds. This review lists the most active antibacterial and antiparasitic compounds isolated from endophytic fungi over the past two decades (2000-2019). A discussion of the strains producing these compounds as well as their host plant is followed by a review of new methods used to induce or modify fungal metabolism to discover novel and bioactive compounds.

1. Introduction

1.1. Current situation of microbial infections

The golden era of the antibiotic occurred between the early 1930s and late 1960s, a period where most of the new classes of drugs were discovered. This period was followed by an important decline in drug discovery rate along with the increase and emergence of resistant microbes, including bacteria, viruses, fungi and parasites (Aminov, 2010). In 2019, the WHO listed 32 antibiotics in clinical development that were addressed to the list of priority pathogens, of which only 6 were considered innovative. Antimicrobial resistance affects health care systems from all countries found in any level of development, generating a great economic burden while other medical procedures become more risky.

Protozoal diseases such as Malaria (*Plasmodium* spp.), Leishmaniasis (*Leishmania* spp.), and Trypanosomiasis (*Trypanosoma* spp.) affect mainly tropical and subtropical regions with great health and economic impacts worldwide. These unicellular eukaryotic organisms are free-living organisms and their developmental stages include intracellular stages within the host cell or extracellular stages in body fluids, hollow organs or between

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cells in the interstitial space. Additionally, they can possess dormant forms that help them to survive under extreme conditions over a long period of time. Their transmission is mainly vector-borne but also fecal-oral or predator-prey transmission.

According to the WHO, there are currently 20 diseases caused by bacteria, helminthes, protozoa or viruses considered as part of the neglected tropical diseases (NTDs), including Chagas disease, human African trypanosomiasis, leishmaniasis and many others. Individually, none of them represents a global priority however, counted together, they affect approximately 2 billion people with a collective number of people affected equivalent to HIV/AIDS, tuberculosis or malaria (Engels & Zhou, 2020).

In general, the arsenal of antimicrobial drugs are becoming very limited while the resistance development is rising against many of the main treatments. The problem of antibiotic resistance also involves natural environmental changes and proliferation of infections due to human activities. The absence of effective vaccines and the development of resistance to the current drugs situates these infections as major problems in endemic countries and highlight the need of novel molecules acting on new targets.

1.2. Microbial natural products as sources of new drugs

A significant number of compounds, including drugs and drug leads, are produced by microorganisms during the interaction with other microorganisms and with their environment. Almost 50% of the total small molecules approved as drugs between 1981 and 2019 are derived from natural products or from their synthetic variations (Newman & Cragg, 2020). In general, microorganisms respond to environmental signals which provide them better survival mechanisms and in this process, many secondary metabolites with different biological activities are produced. Most of the relevant classes that are currently clinically important are derived from fungi, actinomycetes and other bacteria, besides the compounds obtained from medicinal plants (Hutchings et al., 2019). Environments with high biodiversity will potentially harbor new species or strains of microorganisms capable of producing novel compounds. These environments are considered as hot spots, such as the tropical forests, where plants represent excellent niches for interactions between microorganisms, including fungi and bacteria (Knight et al., 2003).

Species from fungal kingdom, including basidiomycetes and filamentous fungi, are responsible for 45% of the microbial metabolites production, mainly from species corresponding to *Penicillium*, *Aspergillus* and *Trichoderma* genera (Berdy et al., 2012). Massive whole-genome sequencing has shown the excellent capacity of fungi to produce a variety of secondary metabolites, however, the current challenge is to access these biosynthetic pathways that are usually silenced under standard culture conditions (Challinor & Bode, 2015). Secondary metabolites produced by fungi are chemically diverse including polyketides, aromatic compounds, alkaloids or terpenoids, which are mainly synthesized by polyketide synthases and non-ribosomal peptide synthetases. Additionally, there are other biosynthetic systems, including hybrid pathways that give rise to a greater chemical diversity (Keller, 2019). They exhibit a broad spectrum of biological activities such as antifungal, antibacterial, antiviral, anti-parasitic, anticancer, immunosuppressant properties, and also possess high chemical originality, especially from fungal species obtained as endophytes or from marine ecosystems (Schueffler & Ankle, 2014).

1.3. Endophytic fungi

Endophytes are microorganisms, mainly bacteria and fungi that colonize internal plant tissues, including branches, leaves, seeds, roots, without generating visible harmful effects to the host plant. This successful endophyte colonization involves compatible interactions between the plant and its microbiome and the recognition of the endophyte during the invasion to the plant through the initiation of a cross-talk of signal molecules (Khare et al., 2018). It is important to consider that these interactions can be facultative or obligate which also depend on environmental conditions and on the physiological state of the plant, ranging from mutualism to commensalism and parasitism. Besides this, endophytic fungal communities can exhibit host plant species specificity and within the host plant, organ and tissue specificity (Aly et al., 2011).

It is considered that almost all plants on earth are in constant association and interaction with microorganisms, especially fungal endophytes (Arnold, 2007). This special relationship has evolved over long periods of time and approximately 18% of known plant metabolites can be produced by their associated fungi. The intrinsic relationships occur at different metabolic levels and have also shown to improve plant growth and tolerance to biotic and abiotic stress factors including resistance to environment, herbivores attacks, via the production of antimicrobials, insecticides and growth regulators while plants may provide them their nutrients and sugars. Specifically, a total of 46 families of medicinal plants distributed in tropical and subtropical or extreme environments, have shown to exhibit a mutualistic relationship with their endophytes (Jia et al., 2016).

Taken together, the rationale for studying endophytic microbes as potential sources of new drugs is related to the fact that they represent a relatively unexplored area of biochemical diversity. In particular, the protection provided by endophytes to the host plant by various bioactive compounds increases the attractiveness of these compounds in medical applications. Furthermore, since toxicity to higher organisms is a concern of any potential drug, natural products isolated from endophytes may be an attractive alternative, since these compounds are produced in an eukaryotic system without apparent damage. Therefore, the host plant itself has naturally served as a selection system for microbes producing bioactive compounds with reduced toxicity to higher organisms.

Given the number of existing plant species on the planet, what are the justifications for selecting a plant to be studied for its endophytes? It is noteworthy that each of the approximately 300,000 plant species that exist on our planet is a host for one or more endophytes (Strobel and Daisy 2003) and it is widely accepted that there are few plants without endophytes. It is estimated that there may be as many as 1 million different fungal species, only a few of which have been studied in detail (Ganley et al. 2004). The presence of plant-associated microorganisms detected in fossilized stem and leaf tissues indicates that endophyte-host associations may have evolved since the appearance of higher plants on earth (Strobel 2003, Andrzej 2002). Endophytes are detected in a wide variety of plant tissue types, such as seeds and ovules (Siegel et al. 1987), fruits (Sчена et al. 2003), stems (Gutierrez-Zamora and Martínez-Romero, 2001), roots (Germida et al. 1998), leaves (Smith et al. 1996), tubers (Sturz et al. 1998), buds (Ragazzi et al. 1999), xylem (Hoff et al. 2004), rachis (Rodriguez and Samuels 1999) and bark (Raviraja 2005).

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There are different ways to select plants to study their endophytes. The selection of a plant will be guided by different criteria ranging from random selection of a number of different plants in an ecosystem to the search for a useful plant in the hope of finding a useful molecule (e.g. medicinal plants). This selection rationale may be based on a particular morphology of the plant, its age, its ecological niche, its environment, and/or its ethnobotanical history (medicinal uses) (Strobel and Daisy 2003). For example, the research that led to the discovery of the Paclitaxel-producing endophyte followed an ethnomedical rationale (isolated from a tree known to have medicinal uses, due in part to the presence of Paclitaxel in its bark).

A total of 449 new secondary metabolites were isolated from endophytic fungi between 2017 and 2019, corresponding to terpenoids and polyketones, alkaloids, steroids and other compounds (Zheng et al., 2021). Many of these compounds exhibited biological activities (Manganyi et al., 2020) (Figure 1) important for public health, agriculture and pharmaceutical industries (Gupta et al., 2020). For instance, the *in vivo* and *in vitro* screening of griseofulvin isolated from different endophytic fungi from the genus *Xylaria* have revealed its microbicidal activity against plant pathogens allowing its potential use in the control of crop diseases. Also, the compound lectin produced by endophytic fungus *Alternaria* sp. has shown its antidiabetic activity in rats and *in vitro* with no harmful effects compared to the side effects observed with some antidiabetic drugs (Saanu Adeleke et al., 2021). In addition to this, 224 patents between 2001 and 2019 are related to secondary metabolite production and biotechnological processes involving endophytic fungi from *Aspergillus*, *Fusarium*, *Penicillium*, *Trichoderma* and *Phomopsis* genera. For example, one submitted patent of *Trichoderma* acid is involved with the preparation of antifungal agents, another patent is about obtaining three isopiramide diterpenoids compounds from *Xylaria* sp. with antifungal activity and with potential application in agriculture and medicine. Moreover, another patent is related to the production of antifungal and immunosuppressive compounds by endophytic fungus *Colletotrichum* sp. (Torres-Mendoza et al., 2020).

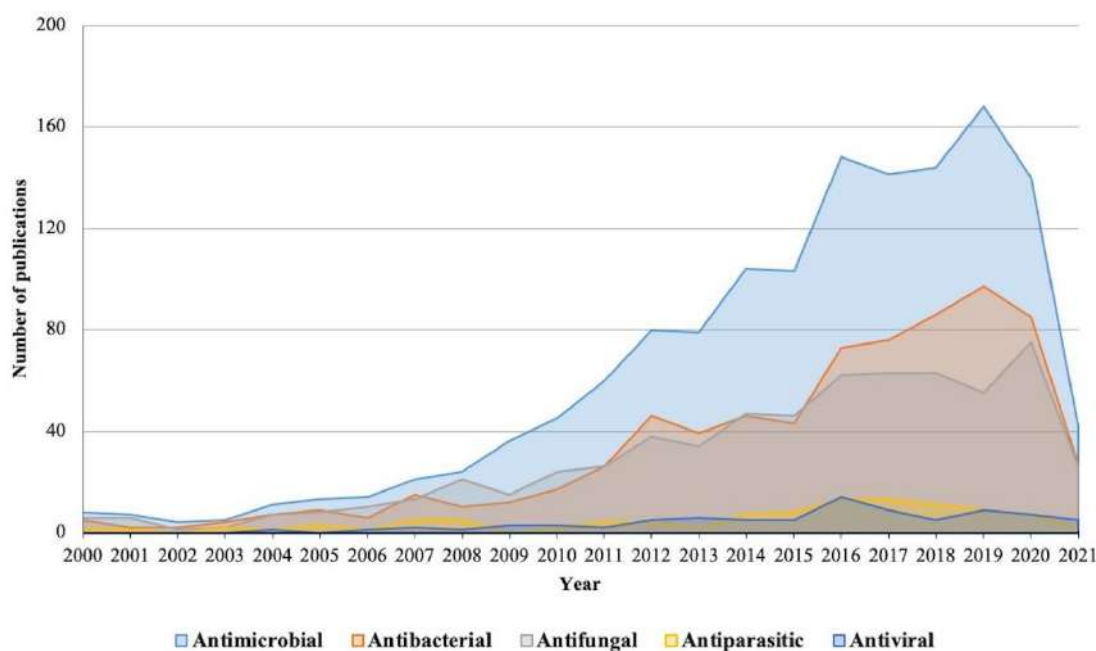


Figure 1. Number of publications using the Search query: (“bioactivity”) AND (“endophytic fungi”) in PubMed.

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The endophytic community present on the host plant is affected by different environmental factors such as the growth state and physiological conditions of the plant or the sampling season, while the culture conditions play an important role for their recovery (Gouda et al., 2016). For the isolation of endophytes, the selection of a healthy plant is mandatory to avoid the isolation of pathogenic fungi as well as the preservation of the tissue to be used within 24 hours of harvesting to prevent death of the endophytic community. This process is followed by a series of sterilization steps to ensure the isolation of endophytes instead of epiphytes or root actinomycetes. Before surface sterilization, washing plant tissues under tap water can help to reduce the soil particles. Among the most used solutions for the surface sterilization are 2-10% of hypochlorite solution, 3% of hydrogen peroxide or 2% of potassium permanganate combined with 70-96% of ethanol washing steps and rinses with sterile distilled water (Martínez-Klimova et al., 2017). Time in each bath depends on the plant material (leaves, roots, stems) especially their thickness. Sterilized tissues are cut in small fragments (3-5 mm) and dried under aseptically conditions to be later placed on solid agar media and incubated during 2 – 4 weeks. Usually, plates are discarded after this period of time to avoid the growth of contaminants. However, this can be corroborated with the control plates containing culture media with the sterile distilled water used for the surface sterilization. Some of the endophytes are not readily culturable or they are found to be under-represented in the selected tissue, therefore some culture-independent techniques are used for studying or identifying the unculturable endophytes. For the identification of pure isolates, sequencing of 18S rDNA, internal transcribed spacer (ITS1 and ITS2), 5.8S rDNA or 28S subunit of rDNA are usually done (Zhang et al., 2006).

2. Antimicrobial compounds from endophytic fungi

Endophytes are in close interaction with their host-plant but also with the entire microbiome of the plant. The microbiome can also change over time, becoming a dynamic interaction and a constant microbial competition which at the same time stimulates metabolite production. This trend of life is a huge opportunity for researchers looking for new anti-infectives compounds such as antibacterial or antifungal metabolites. Endophytes are not naturally in interaction with pathogens of animals, such as *Plasmodium*, *Leishmania* or other *Trypanosomatidae* parasites, however, their capacity to produce bioactive metabolites in answer to the presence of other microorganisms led the scientific community to screen fungal endophytes extracts for this kind of activity. Nowadays, there is an increasing interest for the research of anti-parasitic compounds from fungal endophytes although these organisms do not share the same biotopes.

This work aims at reviewing the most active antibacterial and antiparasitic compounds isolated from endophytic fungi, between 2000 and 2019.

2.1. Antibacterial compounds

As briefly introduced in point 1.1, drug and multi drug resistance in bacteria is a global health and development threat and is caused mainly by the large amounts of antibiotics used for human therapy, as well as for fish in aquaculture and for farm animals. The two principal mechanisms in the generation of drug resistance are the accumulation of resistance plasmids and the increase in the expression of the genes that code for drug efflux

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pumps. These mechanisms have produced bacterial strains resistant to methicillin, aminoglycosides, macrolides, tetracyclines, chloramphenicol, lincosamides and/or penicilline agents. An important group of these drug/multidrug resistant bacteria are the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) which are the leading cause of nosocomial infections throughout the world and one of the greatest challenges in clinical practice.

The urgency of finding new agents to fight these pathogens or new antibacterial mechanisms of actions has been one of the most important focuses of science in the last decades. A special focus on natural products has been applied in the research for new antibacterial compounds with marine, plant or fungi origins. In the following section (Table 1), compounds with potent antibacterial activity isolated from endophytic fungi, obtained from mainly terrestrial but also some marine plants, have been listed. They exhibited activities with MIC values less than 20 µg/mL and/or with important inhibition zones (> 8 mm with at least 50 µg per disk).

In particular, the derivatives with MIC values lower than 10 µg/mL are described and discussed. Compounds are organized according to families of metabolites and the possible mechanisms of action proposed in the literature will be mentioned. Finally, we describe some cases of antivirulence compounds against different pathogens of human concern in order to evaluate new strategies to fight bacterial infections.

- Alkaloids

Pyrazin-2-one

Neoaspergilliac acid (**6**), an alkaloid pyrazinoic derivative, was isolated from a co-culture of two *Aspergillus* strains (A. FSY-01 and A. FSW-02), which were isolated from the mangrove tree *Avicennia marina* (Acanthaceae) and showed antibacterial activity in a serial dilution assay with MIC values in 0.98 and 0.49 µg/mL against *Staphylococcus aureus* and *S. epidermidis* bacteria species (Zhu et al., 2011). In another study, neoaspergilliac acid significantly inhibited the cell proliferation of SGC-7901 and K562 cancer cell lines with IC₅₀ values of 8.24 and 7.99 µg/mL respectively, and moderate cytotoxicity to SPA-A-1 and BEL-7402 cell lines with IC₅₀ values of 22.19 and 24.19 µg/mL, respectively.

Piperine

Piperine (**10**), a well-known plant-produced alkaloid, was found to be produced by an endophytic strain of *Periconia* sp. isolated from *Piper longum* L (Piperaceae). Additionally, piperine was reported to possess a MIC value of 1.74 µg/mL against a multi-drug resistance strain of *M. tuberculosis* (Verma et al., 2011). There are several reports in the literature that confirm the activity of piperine against *Mycobacterium* spp. and its role in the inhibition of Rv1258c, a putative multidrug efflux pump of *M. tuberculosis* (Sharma et al., 2010).

Pyrocidines

The 13-member macrocycle alkaloids, pyrocidine A (**12**) and B (**13**) were isolated for the first time from the endophytic fungi *Acremonium zeae* NRRL 13540, which was isolated from maize, and they showed MICs values of 0.25-2 and 4-8 µg/mL, respectively, against four strains of *Staphylococcus aureus* (including two piperacillin-

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resistant strains); 0.25 and 8 µg/mL, respectively, against *S. haemolyticus* GC4546; 0.5 and 4-8 µg/mL against three strains of *Enterococcus faecalis* and 0.5-1 and 4-8 µg/mL, respectively, against three strains (including two vancomycin-resistant strains) of *Enterococcus faecium* (He et al., 2002; Wicklow and Poling, 2009). In addition, pyrrocidine A also exerted moderate antifungal activity against a strain of *Candida albicans* with a MIC value of 8 µg/mL. Another member of this family, pyrrocidine C (**14**) was isolated from the endophytic fungi *Lewia infectoria* SNB-GTC2402, which was obtained from the amazonian tree *Besleria insolita* (Gesneriaceae), and showed antibacterial activity against *S. aureus* ATCC 29213 with a MIC value of 2 µg/mL (Casella et al., 2013). In addition, pyrrocidine C showed cytotoxicity activity against the cervical uterine cell line KB with an IC₅₀ value of 10 µM, however it was inactive against MDA-MB-435 and MRC-5 cell lines.

Bisindoles

From the cultures of the same strain of *L. infectoria* described above, two bisindole alkaloids identified as semicochliodinol A (**15**) and cochliodinol (**3**) were also isolated and showed MICs values of 2 and 4 µg/mL, respectively, against *Staphylococcus aureus* ATCC 29213 strain. In addition, both derivatives showed cytotoxic activity against the KB cancer cell line with IC₅₀ values of 0.31 and 0.53 µM respectively. Cochliodinol also showed antifungal activity against *Candida albicans* ATCC 10213 strain with a MIC value of 2 µg/mL. Semicochliodinol A has also been reported with anti-HIV activity by inhibiting the HIV-1 protease (IC₅₀ of 0.37 µM) and as anticancer agent by inhibiting the epidermal growth factor receptor protein tyrosine kinase (IC₅₀ of 20 µM). (Casella et al., 2013; Fredenhagen et al., 1997).

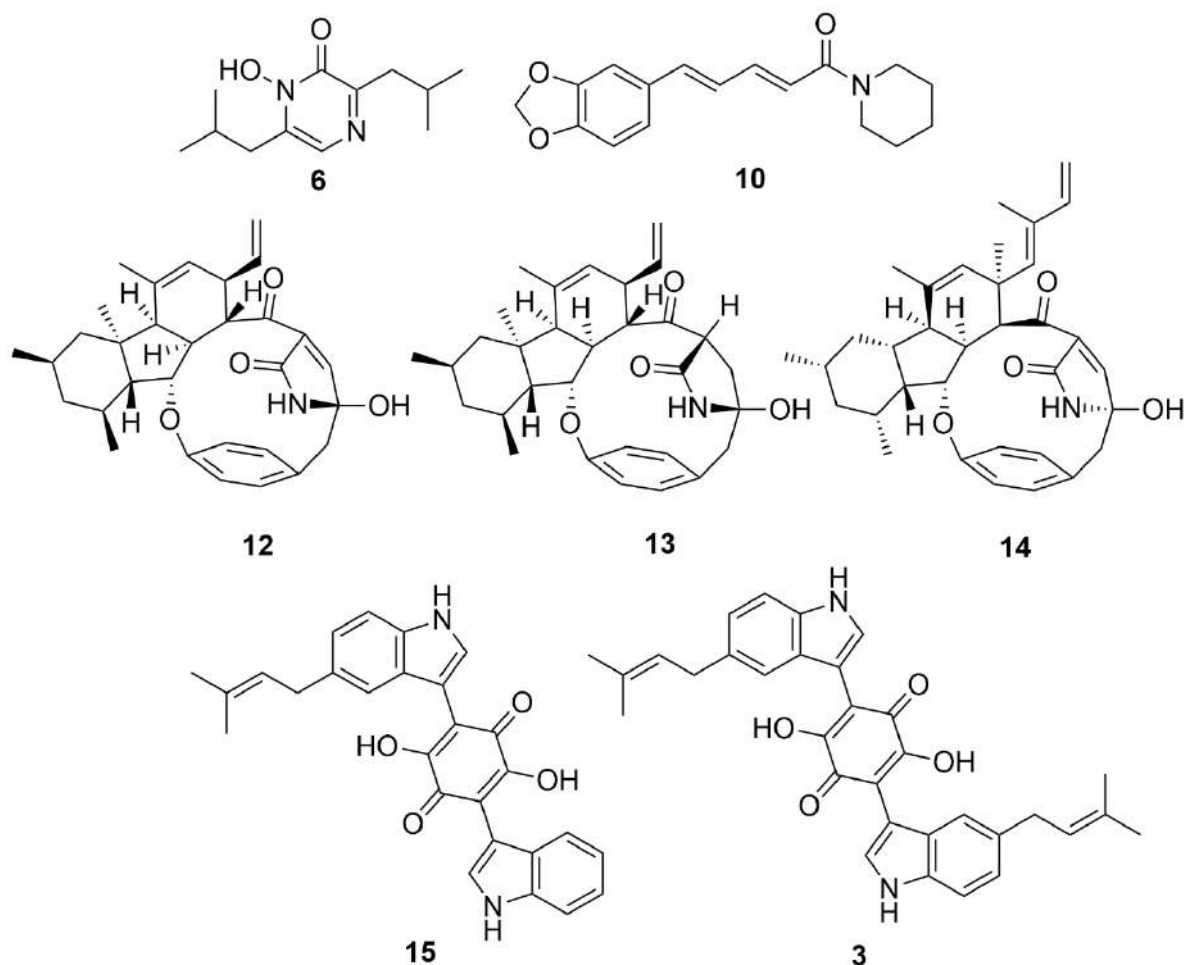


Figure 2. Chemical structures of neoaspergilliac acid (6), piperine (10), pyrrocidine A (12), pyrrocidine B (13), semiochliodinol A (15) and cochliodinol (3).

- Peptides

Dipeptides

The 2,5-diketopiperazine derivative, tardioxopiperazine A (**92**), was isolated from culture extract of *Eurotium cristatum* EN-220, an endophytic fungus isolated from the marine alga *Sargassum thunbergii* (Du et al. 2012). This compound exerted antibacterial activity in a well-diffusion method against a strain of *Staphylococcus aureus*, with a MIC value of 8 $\mu\text{g/mL}$. In addition, tardioxopiperazine A showed no cytotoxic activity against P388, HL-60, BEL-7402 and A-549 cell lines *in vitro* ($\text{IC}_{50} > 50 \mu\text{M}$) (Wang et al. 2007).

Polypeptides

Two cyclopeptides PF1022F (**91**) and halobacillin (**90**) have been isolated from the endophytic fungus *Trichoderma asperellum*, residing in the traditional chinese medicinal plant *Panax notoginseng*. Both compounds showed antibacterial activity *in vitro* in a serial dilution model against *Enterococcus faecium* CGMCC 1.2025 strain with MICs values of 7.3 and 5.4 $\mu\text{g/mL}$, respectively (Ding et al. 2012). Halobacillin, previously isolated from cultures of *Bacillus* sp. (CND-914) obtained from a deep-sea sediment core, showed also strong cytotoxic

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activity, inhibiting the growth of human colon tumor cells (HCT-116) with an IC_{50} of 0.98 $\mu\text{g/mL}$ (Trischman et al. 1994).

In 2013, the cyclic depsipeptide enniatin A1 (**87**), a cyclic hexadepsipeptide of alternating D- α -hydroxyisovaleric acids and N-methyl-L-amino acids, was isolated from the fungal endophyte *Fusarium tricinctum* with antibacterial activity showing MIC value of 2 $\mu\text{g/mL}$ against *Staphylococcus pneumoniae* ATCC 49619. Remarkably, the co-culturing of *F. tricinctum* with the bacterium *Bacillus subtilis* 168 trpC2 resulted in an up to 78-fold increase in the production of enniatin A1 (Ola et al., 2013). Enniatin A1 also possesses anticarcinogenic properties by induction of apoptosis of HepG2 liver cancer cell line and disruption of extracellular signal-regulated kinases signalling pathway. In addition, this compound has been probed as an enzyme inhibitor of acyl-CoA:cholesterol acyltransferase with an IC_{50} of 49 μM in rat liver microsomes (García et al. 2015; Tomoda et al. 1992).

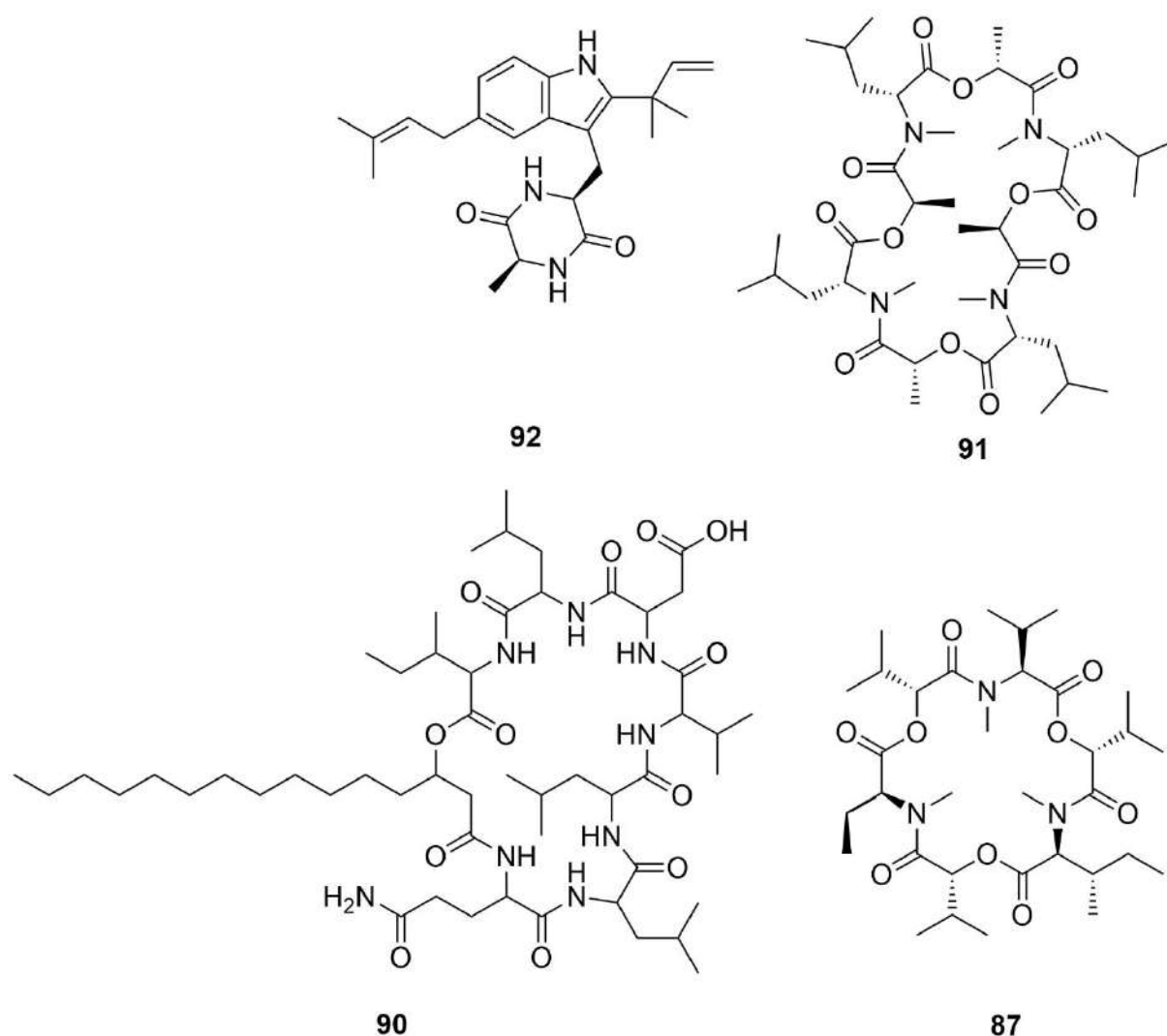


Figure 3. Chemical structures of tardioxopiperazine A (**92**), PF1022F (**91**), halobacillin (**90**) and enniatin A1 (**87**).

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- Polyketides

Chromones

The chromone derivative altechromone A (**37**), firstly isolated from a strain of *Alternaria* sp. in 1992, which was obtained from an ear of oat, was reported as a plant growth promoter. Also isolated from several fungi including *Hypoxylon truncatum*, Ascomycota spp. and from *Alternaria brassicicola*, altechromone A has showed antibacterial activity with a MIC value of 3.9, 3.9, 1.8 and 7.8 µg/mL against *Bacillus subtilis* CGMCC1.1162, *Escherichia coli* CGMCC1.1571, *Pseudomonas fluorescens* CGMCC1.1828 and *Staphylococcus aureus* CGMCC1.1361 strains, respectively (Gu, 2009; Kimura et al., 1992).

Alternariol (**39**), a chromone derivative isolated from the crude extract of *Alternaria alternata* ZHJG5 (an endophytic fungus isolated from healthy leaves of *Cercis chinensis*), showed antibacterial activity against the phytopathogen *Xanthomonas oryzae* pv. *oryzae* KACC10331 with a MIC value of 4 µg/mL. This activity was further evaluated in a protective rice-infected model, showing protection efficacy of 52.8 and 66.2 % at 100 and 200 µg/mL, respectively, against the same pathogen. Alternariol was found to inhibit the β-ketoacyl-acyl carrier protein synthase III, an essential protein for bacterial fatty acid biosynthesis (Zhao et al. 2020).

Quinones

A new quinone derivative, the 2,3-didehydro-19a-hydroxy-14-epicochlioquinone B (**29**) was isolated from the endophytic fungus *Nigrospora* sp. MA75 obtained from the marine semi-mangrove plant *Pongamia pinnata* (Fabaceae), and showed antibacterial activity in a serial dilution assay with MICs values of 8.0, 4.0, 4.0, 0.5 and 0.5 µg/mL against a methicillin-resistant *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*; *P. fluorescens* and *S. epidermidis*, respectively. In addition, this compound showed cytotoxic effect *in vitro* against several tumor cell lines with IC₅₀ values of 4, 5 and 7 µg/mL for MCF-7, SW1990 and SMMC7721 strains respectively (Shang et al., 2012).

Xanthones

From the same endophytic fungus of *Nigrospora* sp. MA75 described previously, a xanthone derivative, norlichexanthone (**62**) was isolated and showed antibacterial activity against *Staphylococcus epidermidis* with a MIC value of 0.5 µg/mL. This compound showed cytotoxic effects against HepG2 cell line with a IC₅₀ of 15 µg/mL (Shang et al. 2012). In addition, norlichexanthone has been identified as a selective ligand of estrogen receptor-alpha (ERα) by activating the Gal4/DBD-ERα/LDB fusion protein in a dose-dependently with an EC₅₀ of 38.8 nM. The potential efficacy to prevent osteoporosis of norlichexanthone was evaluated in an ovariectomized mouse model, preventing bone loss at the dosage of 1 mg/kg (Wang et al. 2021).

Two novel xanthone dimers derivatives, phomoxanthone A (**69**) and B (**70**), were isolated from culture of the endophytic fungi *Phomopsis* sp. BSS 1323, an endophytic fungus isolated from *Tectona grandis* (Lamiaceae) and showed antitubercular activity against the strain *Mycobacterium tuberculosis* H37Ra with a MIC values of 0.5 and 6.25 µg/mL, respectively (Isaka et al., 2001). In addition, these two xanthone dimers have been shown to inhibit the proliferation of cancer cell lines (IC₅₀ of 0.9 and 4.1 µg/mL against KB cells, 0.51 and 0.70 µg/mL against

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BC-1 cells and 1.4 and 1.8 $\mu\text{g/mL}$ against vero cells, respectively) and inhibit the tyrosine phosphatase SHP1 in MCF7 cells leading to the upregulation of inflammatory factors (Yang et al. 2020).

Quinones

From the endophyte fungus *Nigrospora* sp. MA75, described previously, two anthraquinones derivatives, tetrahydrobostrycin (**76**) and 4-deoxytetrahydrobostrycin (**30**), were isolated with antibacterial activity with MICs values of 0.5 and 4.0 $\mu\text{g/mL}$ against a strain of *Escherichia coli*, respectively. Tetrahydrobostrycin was active also against a methicillin-resistant *Staphylococcus aureus* with a MIC value of 2.0 $\mu\text{g/mL}$ (Shang et al. 2012). In the same study, only deoxytetrahydrobostrycin showed moderate cytotoxic activity against HeLa cell line with a IC_{50} of 22.0 $\mu\text{g/mL}$.

Javanicin (**56**), a highly functionalized naphthoquinone, was isolated from an endophytic fungus, *Chloridium* sp. (J. F. H. Beyma), which was obtained from the roots of *Azadirachta indica* (Meliaceae). This compound showed antibacterial activity in a standard plate bioassay test method against *Pseudomonas* spp., *P. aeruginosa* and *P. fluorescens* with a MIC value of 2.0 $\mu\text{g/mL}$ against both species. In addition, javanicin has shown antifungal activity against the fungal plant pathogen species *Cercospora arachidicola*, with a MIC value of 5.0 $\mu\text{g/mL}$ (Kharwar et al. 2009).

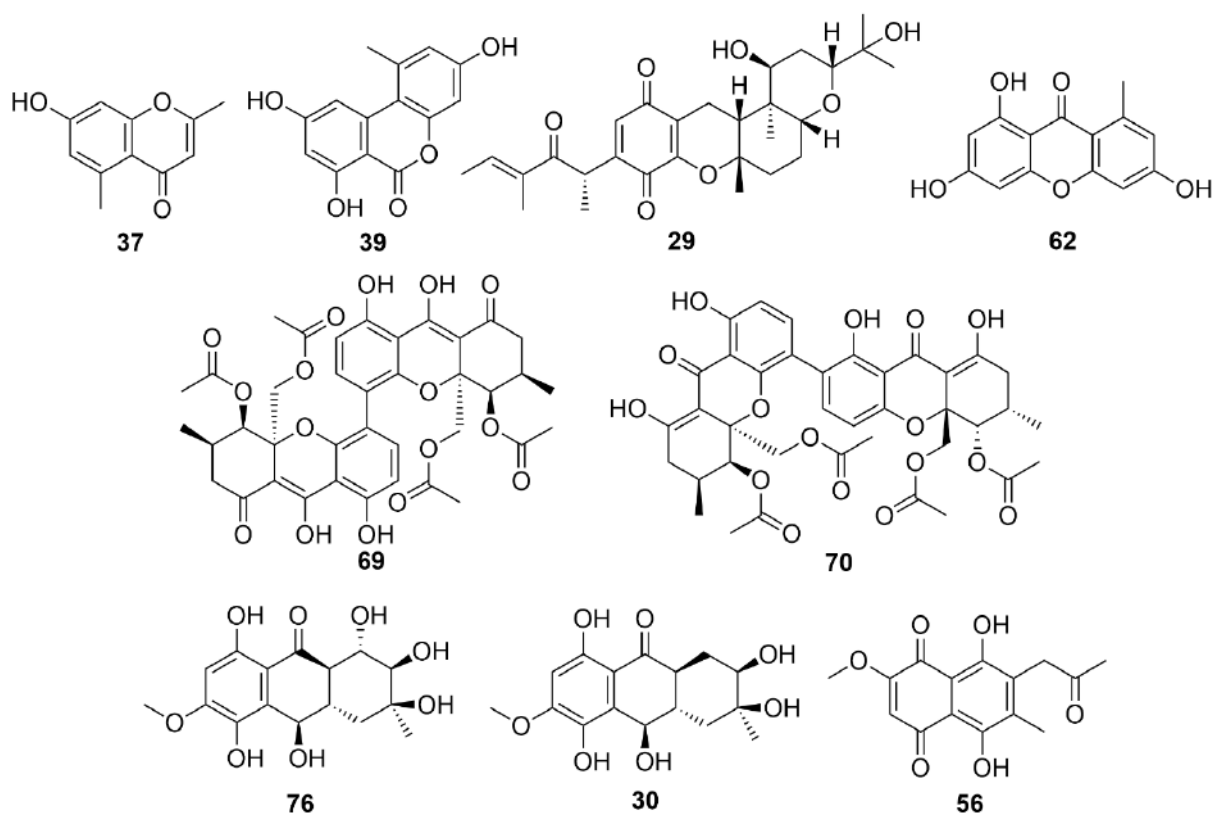


Figure 4. Chemical structures of altechromone A (**37**), alternariol (**39**), 2,3-didehydro-19a-hydroxy-14-epicochlioquinone B (**29**), norlichexanthone (**62**), phomoxanthone A (**69**), phomoxanthone B (**70**), tetrahydrobostrycin (**76**), 4-deoxytetrahydrobostrycin (**30**) and javanicin (**56**).

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Benzofurans

Usnic acid (**78**) and two of its derivatives, cercosporamide (**42**) and phomodione (**65**), were isolated from the endophytic fungus *Phoma* sp. NG-25, which was isolated from *Saurauia scaberrima* (Actinidiaceae) and they showed antibacterial activity with MIC values of 2.0 µg per disc with an halo of inhibition of 0.5 mm against the strain *Staphylococcus aureus* ATCC 25923 (Hoffman et al., 2008). Previously, isolated from a lichen, usnic acid showed antibacterial activity against *Clostridium perfringens* ATCC 13124, *Propionibacterium acnes*, and 29 clinical isolated of *Enterococcus faecalis* and 8 clinically isolated strains of methicillin-resistant *S. aureus* with MICs values of 4.0, 2.0, 8.0 and 8.0 µg/mL, respectively (Lauterwein et al., 1995). Usnic acid showed also antibiofilm activity, inhibiting the formation of biofilm by loaded *S. aureus* cells and altering the biofilm morphology formed by *Pseudomonas aeruginosa* cells, indicating the possibility of interference in signaling pathways (Francolini et al; 2004). Recently, the strong inhibition of RNA and DNA synthesis in *Bacillus subtilis* and *S. aureus*, by usnic acid has been demonstrated as a possible antibacterial mechanism of action. Interestingly, DNA synthesis was halted rapidly, suggesting interference of usnic acid with the elongation of DNA replication. It was also observed a slight inhibition of RNA synthesis in a Gram-negative bacterium, *Vibrio harveyi* (Maciag-Dorszynska et al. 2014).

Octaketides

Two new octaketides, cytosporone D (**44**) and E (**45**), were isolated from extracts obtained from the endophytic fungus *Cytospora* sp. CR200, isolated from the tissue of the mangrove shrub *Conocarpus erecta* (Combretaceae). These compounds showed antibacterial activity against representative strains of *Staphylococcus aureus* and *Enterococcus faecalis* with MICs values of 8 µg/mL. In addition, cytosporone D also showed antifungal activity with a MIC value of 4 µg/mL against the fungus *Candida albicans*.

Benzophenones

From the endophyte fungus *Nigrospora* sp. MA75, described previously, a benzophenone identified as griseophenone C (**49**) was also isolated. This compound showed MIC value of 0.5 µg/mL against *Pseudomonas aeruginosa*, *P. fluorescens* and a methicillin-resistant *Staphylococcus aureus* strain.

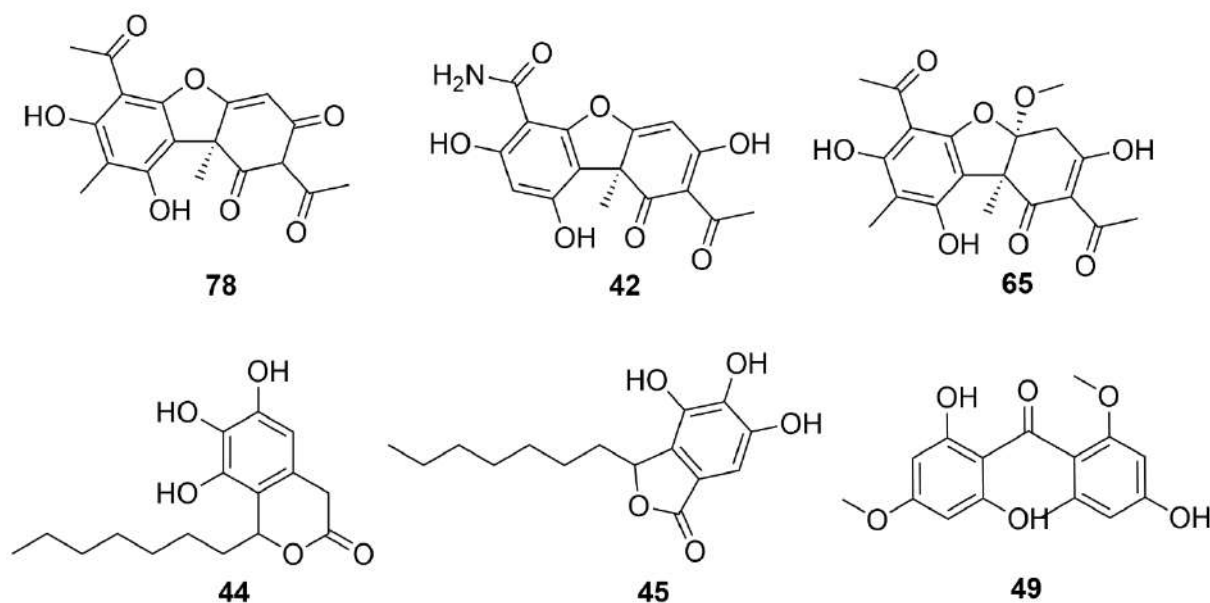


Figure 5. Chemical structures of usnic acid (78), cercosporamide (42), phomodione (65), cytosporone D (44), cytosporone E (45) and griseophenone C (49).

- Terpenoids

A meroterpene with a cyclohexane acid moiety, named fusariumin C (**98**), was isolated from a crude extract of an endophytic fungus *Fusarium oxysporum* ZYP-R1, obtained from the roots of coastal plant *Rumex madaio* (Polygonaceae). This terpenoid possesses significant antibacterial activity against *Staphylococcus aureus* ATCC 25923 strain with a MIC value of 6.25 µg/mL (Chen et al. 2019).

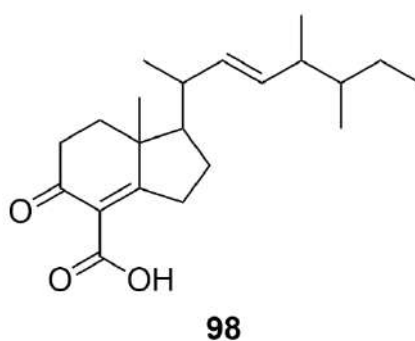


Figure 6. Chemical structure of fusariumin C (98).

Antivirulence compounds

In the last years, and in order to find new strategies to fight some persistent and multidrug pathogens, the expression, production, secretion or activity of different virulence factors have been targeted. Some of these factors are essential in the invasion, colonization and pathogenicity of bacterial infections, such as the production of gelatinase, which is inhibited by ambuic acid (**41**) isolated from different strains of *Pestalotiopsis* sp. and *Monochaetia* sp. (Li et al., 2001 ; Nakayama et al, 2009). In the exploration of new bioactive compounds, scientifics have been screening isolated compounds from endophytic fungi.

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During a screening of 100 extracts from 50 *Penicillium* species, 33 were found to produce QS inhibitory (QSI) compounds. From the extracts of the endophytic fungus of *Penicillium radicolica* (IBT 10696) and *Penicillium coprobium* (IBT 6895) bioguided isolation led to the identification of penicillic acid (**64**) and patulin (**63**) respectively, as potent quorum system inhibitors of a *Pseudomonas aeruginosa* PA01 strain. These compounds were found to target the transcription of different genes encoding the expression of RhlR and LasR proteins at 80-40 μM (Rasmussen et al. 2005).

From the endophytic fungi *Penicillium restrictum* G85ITS, obtained from healthy parts of *Silybum marianum* (Asteraceae), a series of anti quorum sensing polyhydroxyanthraquinones were isolated. The most active compounds were identified as ω -hydroxyemodin (**82**) and isorhodoptilometrin (**55**) with IC_{50} values of 8.1 and 8.4 respectively against the expression of a functional accessory gene regulator (*agr*), which is required for the infection of clinical strains of methicillin-resistant *Staphylococcus aureus* (Figuerola et al. 2014).

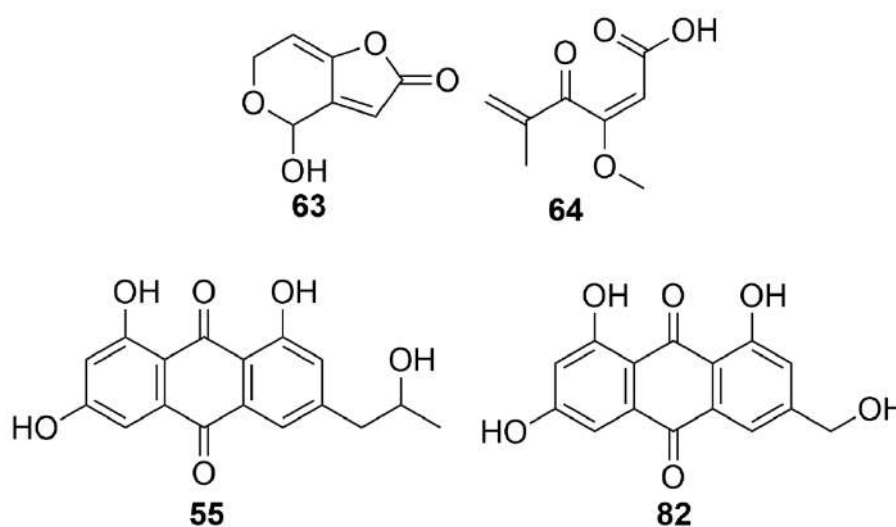


Figure 7. Chemical structures of patulin (63), penicillic acid (64), ω -hydroxyemodin (82) and isorhodoptilometrin (55).

2.2. Antiparasitic compounds

Among all the works carried out on the search for bioactive compounds from endophytic fungi, many studies have focused on their activity against parasitic infections (Ibrahim et al., 2018; Toghueo et al., 2019; Hzounda Fokou et al., 2021). The reported antiparasitic activities in the literature can vary up to $\text{IC}_{50} > 290 \mu\text{M}$ against *P. falciparum* such as the lactones isolated from *Xylaria* sp. isolated from the leaves of *Siparuna* sp. (Siparunaceae) (Jimenez Romero et al., 2008) or up to $\text{IC}_{50} > 169 \mu\text{M}$ against *L. donovani* such as the polyketides isolated from *Mycocleptodiscus indicus* isolated from the plant *Borreria verticillata* (Rubiaceae) (Andrioli et al., 2014). Here, we focused on the compounds with IC_{50} values lower than $10 \mu\text{M}$ active against *Plasmodium* spp., *Leishmania* spp. and *Trypanosoma* spp. and we describe the culture methods and their potential cytotoxicity against different cancer cell lines. Interestingly, these compounds belong to several chemical classes, such as polyketides, terpenoids, alkaloids and polypeptides.

2.2.1. Antileishmanial

Among the most active compounds isolated from endophytic fungi active against *Leishmania donovani* with an IC_{50} below 1 μM , we can mention preussomerin EG1 (IC_{50} 0.12 μM) followed by FD-838 (IC_{50} 0.2 μM), fumiquinone B and pseurotin D (IC_{50} 0.5 μM), palmarumycin CP18 (IC_{50} 0.62 μM) and purpureone (IC_{50} 0.87 μM). The compounds exhibiting a moderate activity against *L. donovani* with an IC_{50} between 1 and 5 μM are palmarumycin CP17 (IC_{50} 1.34 μM) followed by integracide J (IC_{50} 3.29 μM), palmarumycin CP2 (IC_{50} 3.93 μM), 14-norpseurotin A (IC_{50} 4.4 μM) and integracide H (IC_{50} 4.75 μM). Similarly, cochlioquinone A and isocochlioquinone A exhibited moderate activities with EC_{50} values of 1.7 and 4.1 μM , respectively and were tested against *Leishmania amazonensis* promastigotes (Campos et al., 2008). Finally, the compounds presenting a weaker activity against *L. donovani* with an IC_{50} between 5 and less than 10 μM are pseurotin A (IC_{50} 5.8 μM) and CJ-12,371 (IC_{50} 8.4 μM). Additionally, amphotericin B is one of the reference drugs to be used in biological assays against *Leishmania donovani* exhibiting an IC_{50} value of 0.07 μM , besides being part of one of the most important antileishmanial current treatments.

- **Polyketides**

Isocochlioquinone A, cochlioquinone A

Phytotoxic compounds, isocochlioquinone A (**126**) and cochlioquinone A (**123**), were isolated for the first time from the fungal pathogen *Bipolaris bicolor* (*Cochliobolus* sp.) from a lesioned leaf of *Eleusine coracana* (Poaceae) (Miyagawa et al., 1994). Isocochlioquinone A and cochlioquinone A were later isolated from a different strain of *Cochliobolus* sp. from the plant *Piptadenia adiantoides* (Fabaceae). These compounds were tested against *L. amazonensis* amastigotes, exhibiting an EC_{50} value (effective concentration to kill 50% of the parasites) of 4.1 μM and 1.7 μM , respectively. Additionally, they are considered to possess some degree of selectivity since no cytotoxic activity was detected against 3 human cancer cell lines [UACC-62 (melanoma), MCF-7 (breast) and TK-10 (renal)] (Campos et al., 2008). Amphotericin B was used at 0.2 $\mu g/mL$ as a control drug. In another study, the same compounds were isolated but not separated and therefore their activity was tested as a mixture against *L. amazonensis* promastigotes, exhibiting an IC_{50} value of 10.16 μM (do Nascimento et al., 2015).

CJ-12,371

This compound with DNA-gyrase activity was previously isolated from a non-identified fungus (N983-46) (Sakemi et al., 1995) and recently isolated from *Edenia* sp. (Martinez-Luis et al., 2008). In this last study, CJ-12,371 (**122**) exhibited a weak activity with an IC_{50} value of 8.40 μM against the amastigotes of *Leishmania donovani*. Amphotericin B was used as the positive control with an IC_{50} value between 0.07 – 0.12 μM .

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Palmarumycin CP17, palmarumycin CP18 (news), preussomerin EG1, palmarumycin CP2

Two new members of the palmarumycin family with potent antileishmanial activity were isolated from an endophytic strain of *Edenia sp.* from a mature leaf of *Petrea volubilis* (Verbenaceae). This fungus was cultivated in malt extract media for 15 days before the mycelium extraction. Palmarumycin CP17 (**133**) and CP18 (**134**) exhibited an IC₅₀ of 1.34 μM and 0.62 μM, respectively, against the amastigotes of *Leishmania donovani* (Martinez-Luis et al., 2008). In addition, two already known compounds isolated from *Edenia sp.* exhibited potent antileishmanial activity, being preussomerin EG1 (**139**) the most active (IC₅₀=0.12 μM), followed by palmarumycin CP2 (**135**, IC₅₀=3.93 μM). Amphotericin B was used as the positive control with an IC₅₀ value between 0.07 – 0.12 μM. Preussomerin EG1 was first described in *Edenia gomezpompae*, an endophytic fungus isolated from *Callicarpa acuminata* (Verbenaceae) (Macias-Rubacalva et al., 2008) while palmarumycin CP2 was first obtained from *Coniothyrium sp.* isolated from the forest soil in West Borneo (Indonesia), being also the first described member of the palmarumycin family (Krohn et al., 1994).

Purpureone

The new ergochrome compound was isolated for the first time from the endophytic fungus *Purpureocillium lilacinum* from the roots of the medicinal plant *Rauvolfia macrophylla* (Apocynaceae). This endophyte was cultivated in a solid rice medium for 3 weeks at room temperature. Purpureone (**140**) possesses a potent activity against the amastigotes of *Leishmania donovani* with an IC₅₀ value of 0.87 μM, showing also a good selectivity index (SI=49.5) while control drug miltefosine showed an IC₅₀ value of 0.35 μM. Additionally, it exhibited good antimicrobial activity, signalling the promising future investigations for drug development (Lenta et al., 2016).

Fumiquinone B

This compound was first isolated from the culture of a soil-strain of *Aspergillus fumigatus*, and showed a nematicidal activity against *Bursaphelenchus xylophilus* (pine wood nematode) (Hayashi et al., 2007). Later, fumiquinone B (**125**) was also isolated from *Aspergillus sp.* F1544, a fungal endophyte isolated from a mature leaf of *Guapira standleyana* (Nyctaginaceae) in Panama. *Aspergillus sp.* F1544 was cultured in Potato dextrose agar and in Czapek-Dox medium at 30°C during 15 days at 150 rpm for whole culture extraction. Fumiquinone B exhibited a strong activity against the amastigotes of *Leishmania donovani* with an IC₅₀ value of 0.5 μM and a moderate activity against *Plasmodium falciparum* with an IC₅₀ value of 5.4 μM. Amphotericin B was used as the positive control with an IC₅₀ value between 0.07 – 0.12 μM. Additionally, it presented a moderate cytotoxicity against the human breast cancer cell MCF-7 with an IC₅₀ value of 23.9 μM (Martinez-Luis et al., 2012).

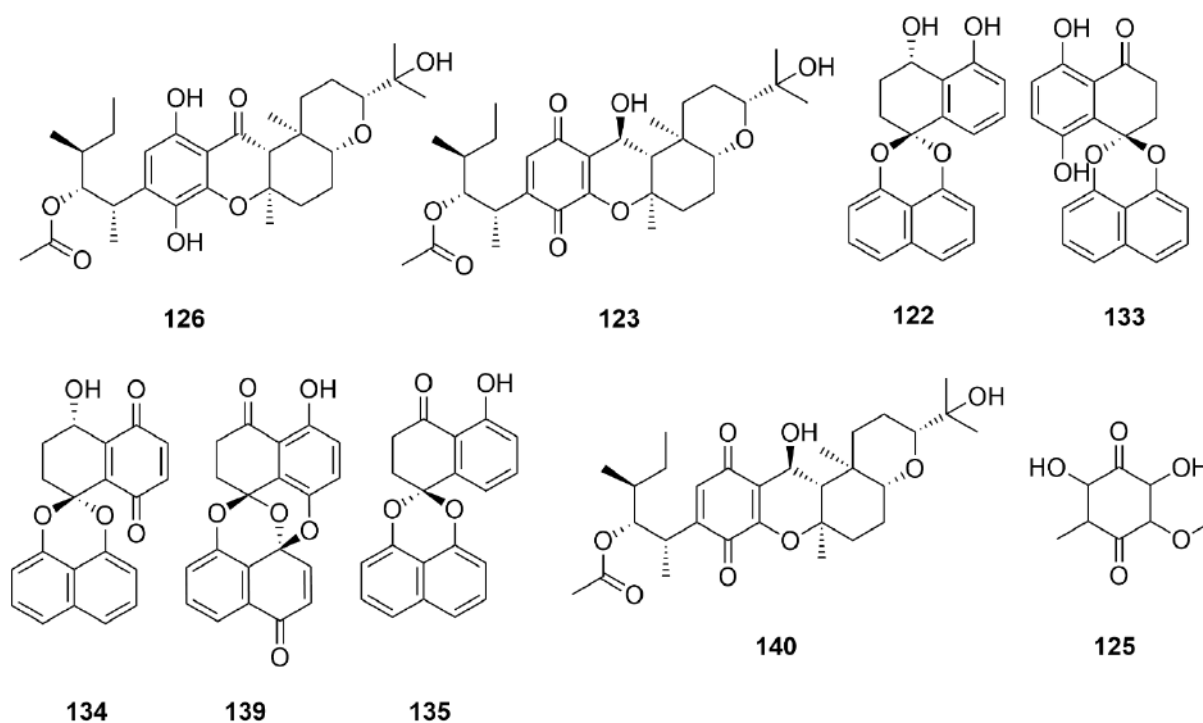


Figure 8. Chemical structures of isocochlioquinone A (126), cochlioquinone A (123), CJ-12,371 (122), palmarumycin CP17 (133), palmarumycin CP18 (134), preussomerin EG1 (139), palmarumycin CP2 (135), purpureone (140) and fumiquinone B (125).

- **Polyketide – Alkaloids**

14-Norpseurotin A, FD-838, pseurotin A, pseurotin D

Along with the isolation of fumiquinone B from endophytic fungus *Aspergillus* sp. F1544, 4 other known compounds were also isolated, 14-norpseurotin A (**142**), FD-838 (**144**), pseurotin A (**145**) and pseurotin D (**146**) (Martinez-Luis et al., 2012). 14-Norpseurotin was first isolated from a marine-derived strain of *Aspergillus sydowii* as a new oxaspirol [4,4]lactam exhibiting a significant antimicrobial activity (Zhang et al., 2008). Pseurotin A was first isolated from *Pseudeurotium ovalis* as a minor metabolite from the culture of this fungus (Bloch & Tamm, 1976) while pseurotin D was isolated 5 years later from the same strain along with other compounds from the same family (pseurotin B, C, D and E) which possess a rare substituted skeleton found in natural product chemistry (Breitenstein et al., 1981). When 14-norpseurotin A, pseurotin A and pseurotin D were tested against the amastigotes of *Leishmania donovani*, they exhibited an IC₅₀ value of 4.4, 5.8 and 0.5 μM, respectively. However, compound FD-838 (patent application EP0216607) exhibited the highest antileishmanial activity with an IC₅₀ value of 0.2 μM (Martinez-Luis et al., 2012). Amphotericin B was the positive control of the assay exhibiting IC₅₀ values between 0.07 – 0.12 μM.

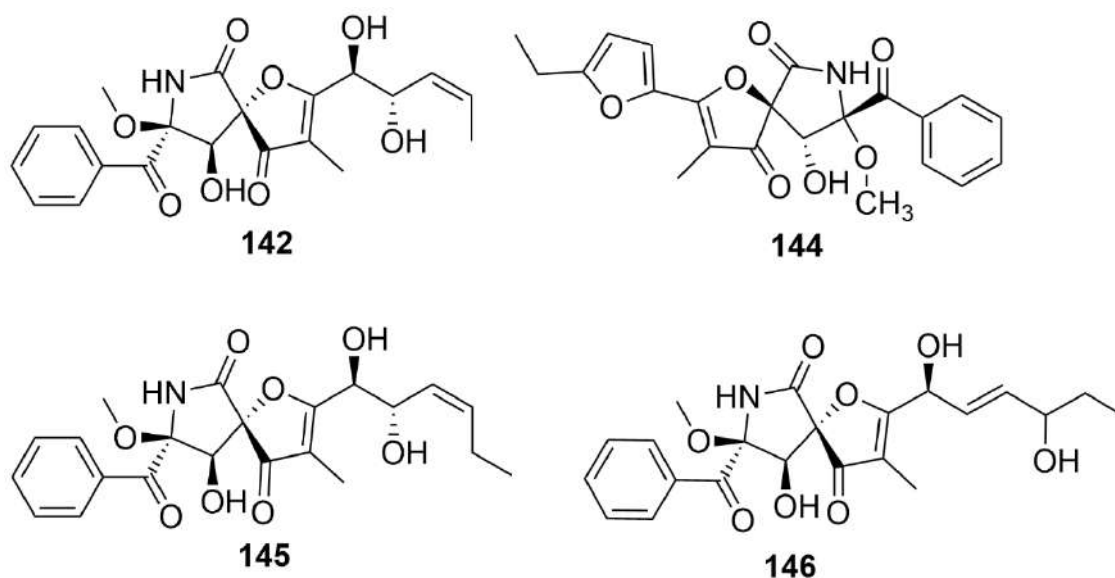


Figure 9. Chemical structures of 14-norpseurotin A (142), FD-838 (144), pseurotin A (145) and pseurotin D (146).

- **Terpenoids**

Integracide H, integracide J

Two new tetracyclic triterpenoids with antileishmanial activity were isolated from *Fusarium* sp. isolated from the roots of *Mentha longifolia* (Lamiaceae). The fungal endophyte was cultivated in a rice solid medium for 30 days at 27°C before extraction. Integracides H (**156**) and J (**157**) exhibited moderate activity against the amastigotes of *Leishmania donovani* with an IC_{50} value of 4.75 and 3.29 μ M, respectively (Ibrahim et al., 2016). Pentamidine was used as the positive control with an IC_{50} value of 6.35 μ M. Moreover, integracide H presented a high cytotoxicity against the human cancer cell lines BT-549, SKOV and KB with IC_{50} values of 1.82, 1.32 and 0.18 μ M, respectively; while Integracide H exhibited IC_{50} values of 2.46, 3.01 and 2.54 μ M, respectively (Ibrahim et al., 2016).

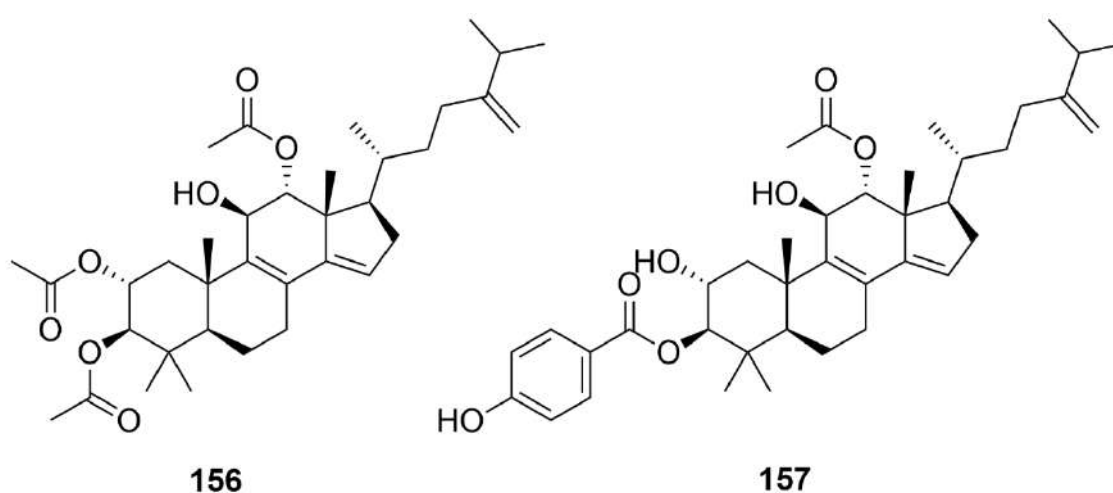


Figure 10. Chemical structures of integracide H (156) and integracide J (157).

2.2.2. Antiplasmodial

The following compounds isolated from endophytic fungi have been tested against different strains of *Plasmodium falciparum*: 3D7 (drug sensitive), D6 (chloroquine sensitive), W2 (chloroquine resistant), NF54 (chloroquine resistant) and K1 (multidrug resistant).

Among the most active compounds against *P. falciparum* 3D7 are cytochalasins H, J and O, 12,13-deoxyroridin E and roridin E with IC_{50} values $<0.02 \mu\text{M}$, followed by cytochalasin D (IC_{50} $0.0258 \mu\text{M}$), 2,5-dihydroxy-1-(hydroxymethyl)pyridin-4-one (IC_{50} $0.127 \mu\text{M}$), 7-hydroxy-3,4,5-trimethyl-6-on2,3,4,6- tetrahydroisoquinoline-8-carboxylic acid (IC_{50} $0.129 \mu\text{M}$), 19,20-epoxycytochalasin D (IC_{50} $0.136 \mu\text{M}$) and dicerandrol D (IC_{50} $0.6 \mu\text{M}$). Compounds with potent activity against *P. falciparum* D6 and W2 are 19,20-epoxycytochalasin D with an IC_{50} value of $0.04 \mu\text{M}$ followed by 19,20-epoxycytochalasin C (IC_{50} $0.05\text{-}0.07 \mu\text{M}$), epoxycytochalasin H (IC_{50} $0.07\text{-}0.1 \mu\text{M}$) and 18-deoxy-19,20-epoxycytochalasin C (IC_{50} $0.19\text{-}0.56 \mu\text{M}$) while fusaripeptide A was tested only against *P. falciparum* D6 and it exhibited an IC_{50} value of $0.34 \mu\text{M}$. Three other compounds presented potent activity against *P. falciparum* K1 being phomoxanthone A with an IC_{50} value of $0.15 \mu\text{M}$ followed by phomoxanthone B (IC_{50} $0.44 \mu\text{M}$) and monocerin (IC_{50} $0.68 \mu\text{M}$). Additionally, apicidins B and C were tested against *P. falciparum* (strain not specified) exhibiting IC_{50} values of 0.189 and $0.069 \mu\text{M}$, respectively.

Compounds presenting a moderate activity in terms of IC_{50} were tested against *P. falciparum* K1, a multidrug resistant strain. Chaetoxanthone B was the most active, with an IC_{50} value of $1.41 \mu\text{M}$ followed by 2-chloro-5-methoxy-3-methylcyclohexa-2,5- diene-1,4-dione (IC_{50} $1.84 \mu\text{M}$), phomoarcherin B (IC_{50} $2.05 \mu\text{M}$), 12,12a-dihydro antibiotic PI 016 (IC_{50} $2.95 \mu\text{M}$), mollicellin K (IC_{50} $1.84 \mu\text{M}$), pullularin B (IC_{50} $4.18 \mu\text{M}$) and pullularin A (IC_{50} $4.64 \mu\text{M}$). Codinaeopsin was tested against *P. falciparum* 3D7 and exhibited a moderate activity with an IC_{50} value of $4.7 \mu\text{M}$.

Finally, those exhibiting a weaker activity in terms of IC_{50} , were also mainly tested against *P. falciparum* K1. Xylariaquinone A presented an IC_{50} value of $6.68 \mu\text{M}$ followed by mollicellin M (IC_{50} $6.95 \mu\text{M}$), mollicellin E (IC_{50} $7.16 \mu\text{M}$), 11-hydroxymonocerin (IC_{50} $7.7 \mu\text{M}$), $7\alpha,10\alpha$ -dihydroxy-1 β -methoxyeremophil-11(13)-en-12,8 β olide (IC_{50} $8.1 \mu\text{M}$), 7-butyl-6,8-dihydroxy-3(R)-pentylochroman-1-one (IC_{50} $8.48 \mu\text{M}$), mollicellin L (IC_{50} $8.6 \mu\text{M}$) and KS-501a (IC_{50} $9.9 \mu\text{M}$). Asterric acid, preussiafuran A and cissetin were tested against *P. falciparum* NF54 exhibiting weak activities with IC_{50} values of 8.67 , 8.76 and $10.3 \mu\text{M}$, respectively.

- **Alkaloids**

18-Deoxy-19,20-epoxycytochalasin C; 19,20-epoxycytochalasin C; 19,20-epoxycytochalasin D

Three known cytochalasins were obtained from the endophytic fungus *Nemania* sp. isolated from the diseased leaves of *Torreya taxifolia* Arnott (Taxaceae) (Kumarihamy et al., 2019). Compounds 19,20-epoxycytochalasin C (**103**) and D (**104**) were first isolated from the fungus *Xylaria hypoxylon* and by this time, there were many biological activities, such as antibiotic and antitumor activities, that were already reported for this family of compounds (Espada et al., 1997). Moreover, 18-deoxy-19,20-epoxycytochalasin C was first isolated from a non-

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identified fungus (KL-1.1) isolated from the leaves of the medicinal plant *Psidium guajava* (Myrtaceae) (Okoye et al., 2015).

For the isolation of secondary metabolites, *Nemania* sp. was cultivated in Potato dextrose broth at 27 °C during 30 days at 100 rpm. These three known cytochalasins exhibited potent *in vitro* activity against two strains of *Plasmodium falciparum* (D6 and W2). Compound 19,20-epoxycytochalasin C (**103**) was isolated as the major compound and it exhibited IC₅₀ values of 0.07 and 0.05 μM against the D6 and W2 strains, respectively. Compound 18-deoxy-19,20-epoxycytochalasin C (**102**) exhibited IC₅₀ values of 0.56 and 0.19 μM against the D6 and W2 strains, respectively while compound 19,20-epoxycytochalasin D (**104**) exhibited an IC₅₀ value of 0.04 μM for both *P. falciparum* strains (Kumarihamy et al., 2019). Positive standard controls used were chloroquine (IC₅₀ 0.03 μM) and artemisinin (IC₅₀ 0.02 μM). Similarly, when the compound 19,20-epoxycytochalasin D was isolated from endophytic fungus *Diaporthe* sp., it exhibited an IC₅₀ value of 0.136 μM against *P. falciparum* 3D7 (Calcul et al., 2013).

Additionally, all three compounds presented moderate toxicity against different tumor cell lines, 19,20-epoxycytochalasin C exhibited an IC₅₀ value of 8.02 μM against SK-MEL (malignant melanoma); 19,20-epoxycytochalasin D exhibited IC₅₀ values of 7.84 and 8.4 μM against BT-549 (breast ductal carcinoma) and LLC-PK₁₁ (kidney epithelial cells), respectively. While 18-Deoxy-19,20-epoxycytochalasin C exhibited an IC₅₀ value of 6.89 μM against BT-549. None of these compounds were toxic against Vero Cells (kidney fibroblast), therefore showing their selectivity to *Plasmodium falciparum* strains (Kumarihamy et al., 2019).

Cissetin

Cissetin (**107**) was first isolated from a non-identified fungus (OSI 50185) isolated from a decomposed plant in Peru, showing antibacterial activity against penicillin-resistant *Streptococcus pneumoniae* (MIC 2 μg/mL) (Boros et al., 2003). This compound was later isolated from endophytic fungus *Preussia* sp. isolated from the leaves and barks of the plant *Enantia chlorantha* (Annonaceae) (Talonsi et al., 2014). For the isolation of compounds, *Preussia* sp. was cultivated in a rice medium at 25°C for 30 days under static conditions. Cissetin exhibited a weak activity against *Plasmodium falciparum* (NF54) with an IC₅₀ value of 10.3 μM and a weak cytotoxicity against L6 cells (rat skeletal myoblast) with an IC₅₀ value of 42.1 μM.

2,5-Dihydroxy-1-(hydroxymethyl)pyridin-4-one; 7-hydroxy-3,4,5-trimethyl-6-on 2,3,4,6-tetrahydroisoquinoline-8-carboxylic acid

Two new alkaloids were isolated from a non-identified endophytic fungus (strain BB4) isolated from the stem and leaves of *Tinospora crispa* (Menispermaceae), a plant traditionally used for malaria treatment. This endophytic fungal strain was cultivated in Potato dextrose broth for 3 weeks for further extraction. Compounds 2,5-dihydroxy-1-(hydroxymethyl)pyridin-4-one (**105**) and 7-hydroxy-3,4,5-trimethyl-6-on-2,3,4,6-tetrahydroisoquinoline-8-carboxylic acid (**106**) exhibited potent activity against *Plasmodium falciparum* (3D7) with IC₅₀ values of 0.127 and 0.129 μM, respectively (Elfita et al., 2011). Positive standard chloroquine presented an IC₅₀ value of 0.02 μM.

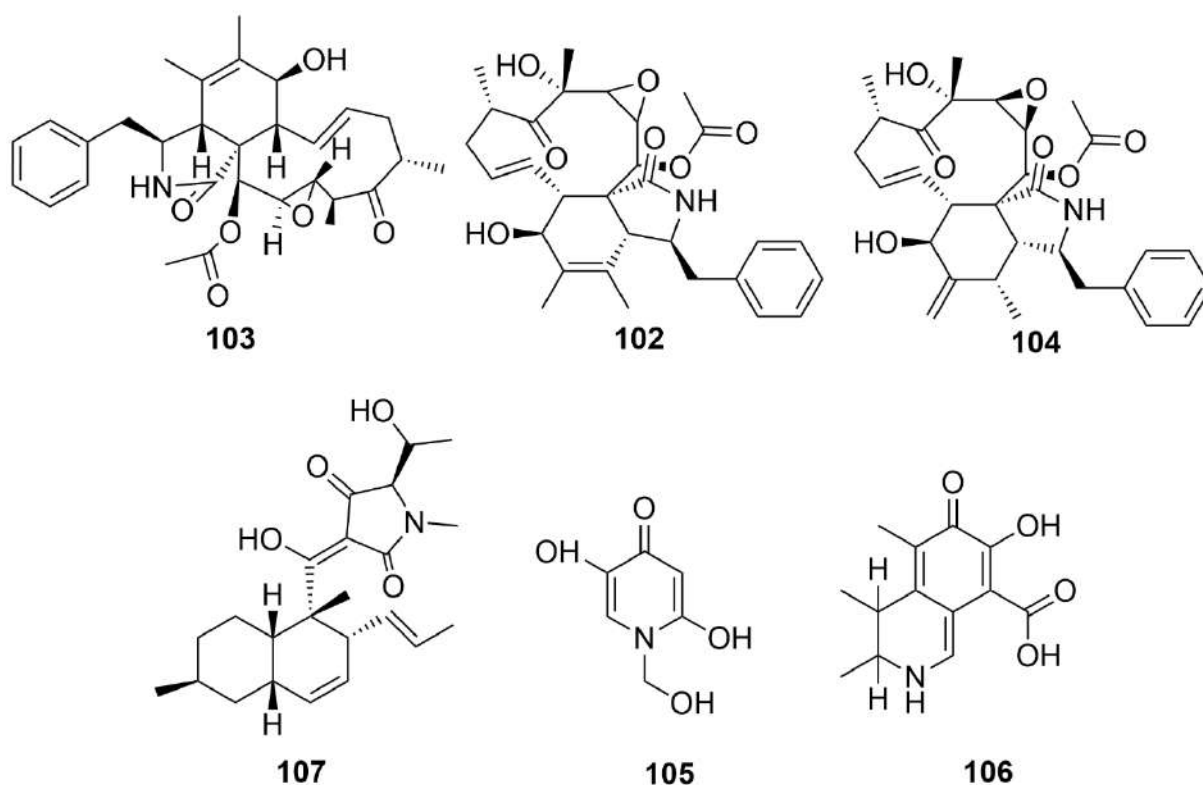


Figure 11. Chemical structures of 18-deoxy-19,20-epoxycytochalasin C (102), 19,20-epoxycytochalasin C (103), 19,20-epoxycytochalasin D (104), cissetin (107), 2,5-dihydroxy-1-(hydroxymethyl)pyridin-4-one (105) and 7-hydroxy-3,4,5-trimethyl-6-on-2,3,4,6-tetrahydroisoquinoline-8-carboxylic acid (106)

Cytochalasin D, cytochalasin H, cytochalasin J, cytochalasin O

Cytochalasin D (**108**) was first isolated from endophytic fungus, *Xylaria* sp. from the plant *Palicourea marcgravii* (Rubiaceae), showing antifungal activity (Cafeu et al., 2005). This compound was able to reduce the attachment of the promastigotes of leishmania by the destabilization of the actin cytoskeleton of macrophages, accompanied by a reduction of the intracellular amastigote load (Roy et al., 2014). Cytochalasin H (**109**) and J (**110**) were previously isolated from *Phomopsis* sp. (Wells et al., 1976; Izawa et al., 1989) while cytochalasin O (**111**) was isolated from the fungus *Hypoxylon terricola* (Edwards et al., 1989). As part of a bigger study, endophytic fungi *Diaporthe* sp., *Xylaria* sp. and *Verticillium* sp. were isolated from the barks and leaves of 3 different plants; *Kandelia obovata* (Rhizophoraceae), *Avicennia marina* (Acanthaceae) and *Lumnitzera racemosa* (Combretaceae). These fungi were cultivated in a liquid medium containing 1% w/v glucose, 0.1% w/v yeast extract and 0.2% w/v peptone during 3 weeks for isolation of secondary metabolites. Cytochalasin D exhibited potent activity against *Plasmodium falciparum* (3D7) with an IC_{50} value of 0.0258 μ M while cytochalasins H, J and O presented IC_{50} values of <0.02 μ M (Calcul et al., 2013). In this study, the positive standard used was chloroquine presenting an IC_{50} value of 0.0045 μ M and dihydroartemisinin with an IC_{50} value of 0.0003 μ M.

Epoxychochalasin H

This compound (**112**) was first isolated from *Phomopsis sojae* isolated from soybean seeds (Cole et al., 1982) and later it was also obtained from the endophytic fungus *Diaporthe miriciae* isolated from the plant *Vellozia gigantea* (Velloziaceae) showing potent antiplasmodial activity. Endophytic fungus *Diaporthe miriciae* was cultured in Potato dextrose agar at 25°C during 15 days for compound extraction. Epoxychochalasin H exhibited an IC₅₀ value of 0.1 and 0.07 μM against D6 and W2 strains of *Plasmodium falciparum*, respectively while no toxicity was detected against Vero cells (mammalian kidney) (Ferreira et al., 2017). Chloroquine and artemisinin were used as positive standards for both plasmodial strains.

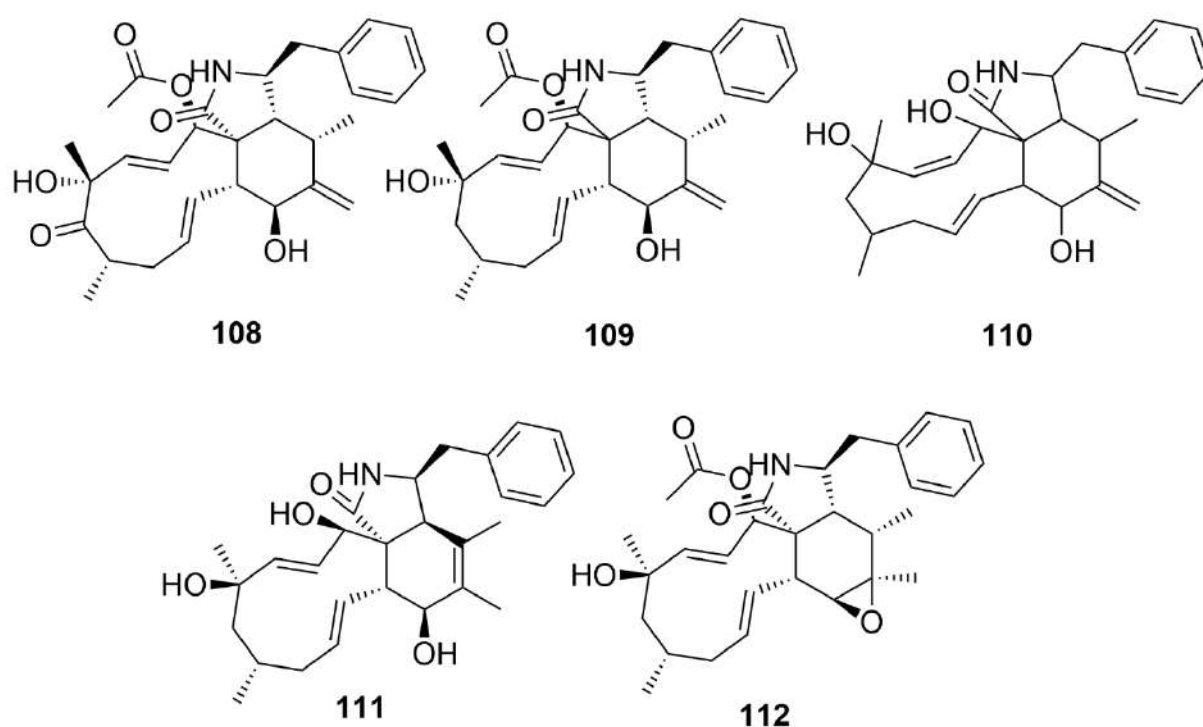


Figure 12. Chemical structures of cytochalasin D (108), cytochalasin H (109), cytochalasin J (110), cytochalasin O (111) and epoxychochalasin H (112).

- **Polyketides**

11-Hydroxymonocerin, monocerin

Monocerin (**132**) and a new analogue of monocerin (**113**) were isolated from endophytic fungus *Exserohilum rostratum* isolated from the leaves and roots of *Stemona* sp. (Stemonaceae) (Sappapan et al., 2008). Monocerin was first isolated from *Helminthosporium monoceras* along with other related benzopyrans (Alridge et al., 1970). Fungus *Exserohilum rostratum* was cultivated in yeast sucrose extract at 31°C during 21 days under static conditions for supernatant extraction. Compound 11-hydroxymonocerin was weakly active against *Plasmodium falciparum* K1 with an IC₅₀ value of 7.7 μM while monocerin exhibited a potent antiplasmodial activity with an

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IC₅₀ value of 0.68 μM. According to the authors, the presence of the additional –OH group in 11-hydroxymonocerin caused the lower activity compared to monocerin. Dihydroartemisinin was used as the positive standard with an IC₅₀ value of 0.004 μM. Additionally, the compounds showed no cytotoxicity against 5 human tumor cell lines BT474 (breast carcinoma), CHAGO (lung carcinoma), Hep-G2 (hepatocarcinoma), KATO-3 (gastric carcinoma), and SW-620 (colon carcinoma) (Sappapan et al., 2008).

2-Chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione, xylariaquinone A

Two novel benzoquinones with antiplasmodial activity were isolated from fungal endophyte *Xylaria* sp. isolated from the leaves of *Sandoricum koetjape* (Meliaceae) (Tansuwan et al., 2007). Fungus was cultivated in malt extract broth at 30°C during 5 weeks under static conditions for supernatant extraction. Xylariaquinone A (**141**) was weakly active against *Plasmodium falciparum* K1 with an IC₅₀ value of 6.68 μM while 2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione (**114**) exhibited a moderate antiplasmodial activity with an IC₅₀ value of 1.84 μM and cytotoxic activity against Vero cells with an IC₅₀ value of 1.35 μM. (Tansuwan et al., 2007). Positive standard dihydroartemisinin exhibited an IC₅₀ value of 0.0033 μM.

7 Butyl-6,8-dihydroxy-3(R)-pentylochroman-1-one

A novel dihydroisocoumarin (**115**) was isolated from fungal endophyte *Geotrichum* sp. isolated from the healthy stems of *Crassocephalum crepidioides* (Asteraceae). The fungus was cultivated in Czapek broth at 25°C during 21 days for supernatant extraction. The 7 Butyl-6,8-dihydroxy-3(R)-pentylochroman-1-one was weakly active against *Plasmodium falciparum* K1 with an IC₅₀ value of 8.48 μM (Kongsaree et al., 2003). The standard compound Chloroquine presented an IC₅₀ value of 0.31 μM.

Asterric acid, preussiafuran A

Asterric acid (**117**) and a novel dibenzofuran (**138**) were isolated from fungal endophyte *Preussia* sp. isolated from the healthy leaves and barks of *Enantia chlorantha* (Annonaceae) (Talontsi et al., 2014). Asterric acid was first isolated from *Aspergillus terreus* (Curtis et al., 1960). The endophytic strain of *Preussia* sp. was cultivated in a rice medium at 25°C during 30 days under static conditions. Asterric acid and preussiafuran A were weakly active against *Plasmodium falciparum* NF54 and they exhibited IC₅₀ values of 8.67 and 8.76 μM, respectively. Chloroquine was used as the positive standard. Moreover, asterric acid and preussiafuran A presented moderate cytotoxicity against L6 cell lines (rat skeletal myoblasts) with IC₅₀ values of 14.8 and 36.7 μM, respectively.

Chaetoxanthone B

A new compound (**120**) was isolated from the marine-derived fungus *Chaetomium* sp. isolated from an algal species (taxonomy not determined) (Pontius et al., 2008). This fungus was cultivated in malt extract yeast agar at room temperature for 15 days for compound extraction. Chaetoxanthone B exhibited moderate activity against *Plasmodium falciparum* K1 with an IC₅₀ value of 1.41 μM while it showed no cytotoxicity against L6 cells (rat skeletal myoblasts). Chloroquine was used as the positive standard.

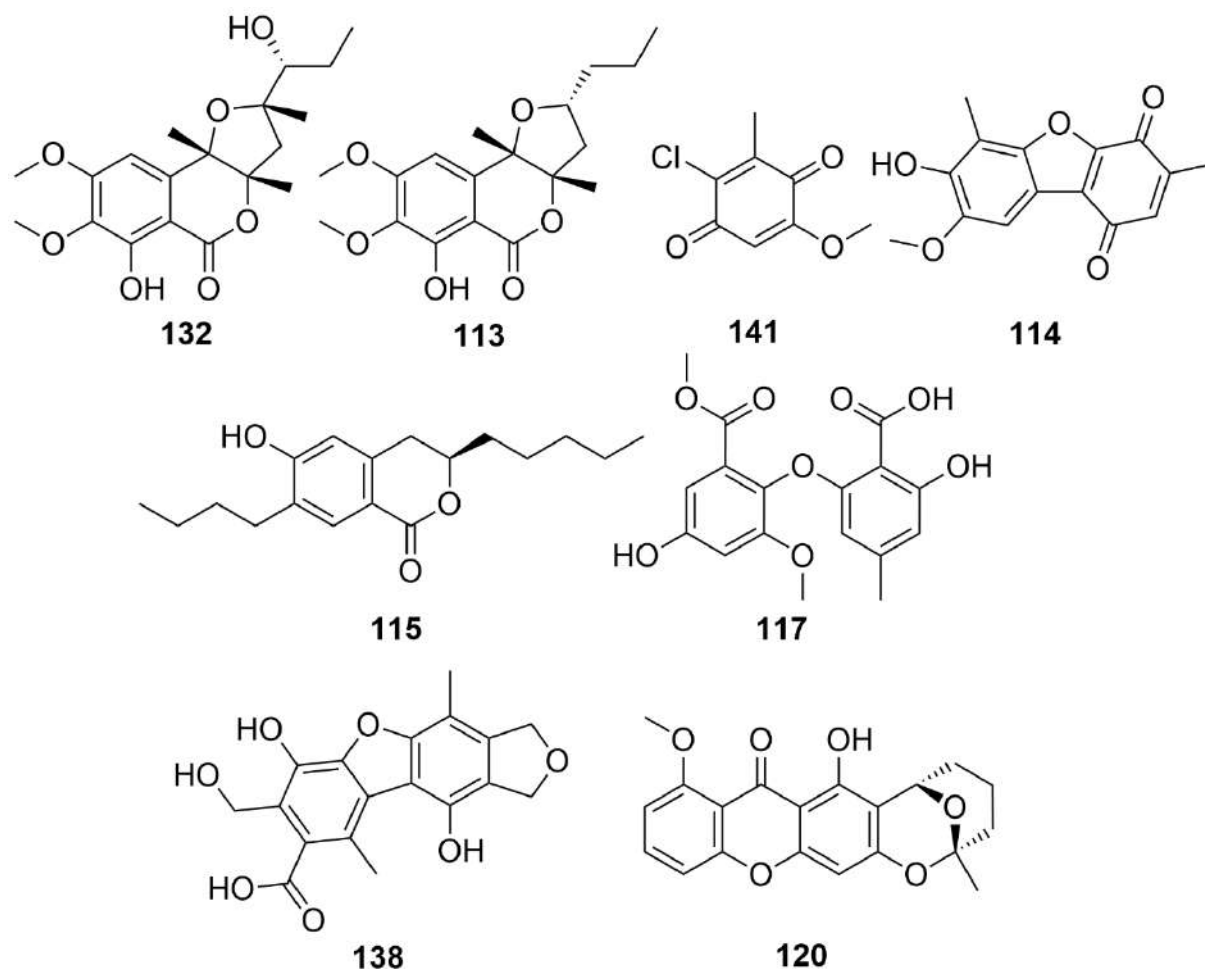


Figure 13. Chemical structures of 11-hydroxymonocerin (113), monocerin (132), 2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione (114), xylariaquinone A (141), dihydroisocoumarin (115), asterric acid (117), preussiafuran A (138) chaetoxanthone B (120).

Phomoxanthone A, phomoxanthone B

Two novel xanthone dimers were isolated from the fungal endophyte *Phomopsis sp.* isolated from the leaves of *Tectona crispa* (Lamiaceae). This fungus was cultivated in bacto-malt extract broth at 22°C during 20 days for mycelium extraction. Phomoxanthone A (**136**) and B (**137**) exhibited potent activity against *Plasmodium falciparum* K1 with IC_{50} values of 0.15 and 0.44 μ M, respectively. However, they presented elevated cytotoxicity against 3 cancer cell lines, KB cells, BC-1 cells and Vero cells (Isaka et al., 2001). Chloroquine diphosphate and artemisinin were used as positive standards.

Dicerandrol D

A new dimeric tetrahydroxanthone was isolated from the fungal endophyte *Diaporthe sp.* along with other known cytochalasins previously mentioned (Calcul et al., 2013). Dicerandrol D (**124**) exhibited a potent activity against

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Plasmodium falciparum 3D7 with an IC₅₀ value of 0.6 μM and a low cytotoxicity against A549 cells (adenocarcinomic human alveolar epithelial cells) with an IC₅₀ value of 7.8 μM.

KS-501a

KS-501a (**127**) was first isolated as a potent inhibitor of Ca²⁺ from *Sporothrix* sp. from a fallen leaf in Japan (Nakanishi et al., 1989). Later, it was also obtained from the culture of *Acremonium* sp. isolated from a palm leaf in Thailand. Here, *Acremonium* sp. was cultivated in Bacto-malt extract broth at 25°C during 32 days at 200 rpm for the isolation of compounds from the supernatant. This compound exhibited a weak activity against *Plasmodium falciparum* K1 with an IC₅₀ value of 9.9 μM while its cytotoxic activity possessed an IC₅₀ value of 8.8 μM against BC (human breast cancer) cell lines (Bunyapaiboonsri et al., 2008). Dihydroartemisinin was used as the positive standard with an IC₅₀ value of 0.004 μM.

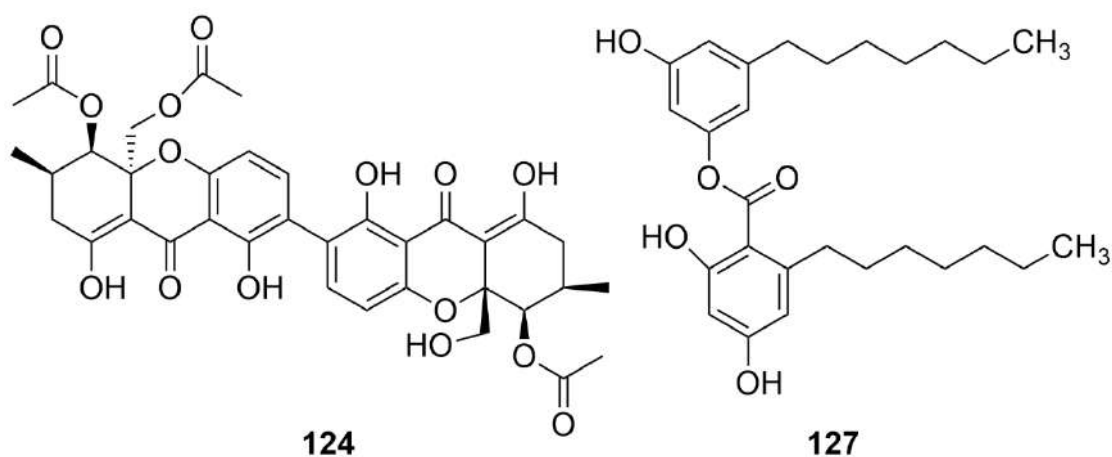


Figure 14. Chemical structures of dicerandrol D (**124**) and KS-501a (**127**).

Mollicellin E, mollicellin K, mollicellin L, mollicellin M

Three novel depsidones (mollicellin K, L and M, **129**, **130**, **131**) and one known (mollicellin E, **128**) were isolated from endophytic fungus *Chaetomium brasiliense*, isolated from a leaf collected in the Hala-Bala evergreen forest (Thailand) (Khumkomkhet et al., 2009). Mollicellin E was first isolated from *Chaetomium mollicellum* and its mutagenic and bactericidal activity against *Salmonella typhimurium* was reported (Stark et al., 1978). The strain of *C. brasiliense* was cultured in potato dextrose broth between 25-28 °C during 4 weeks under static conditions for mycelium extraction. Mollicellin E, L and M presented a weak activity against *Plasmodium falciparum* K1, exhibiting IC₅₀ values of 7.16, 8.6 and 6.95 μM, respectively. Mollicellin K presented a moderate antiplasmodial activity with an IC₅₀ value of 3.13 μM and cytotoxicity against KB cells (human epidermoid carcinoma of the mouth) and NCI-H187 (human small cell lung cancer) with IC₅₀ values of 1.9 and 0.35 μM, respectively (Khumkomkhet et al., 2009). The standard compound used was artemisinin. Additionally, the 4 compounds exhibited significant cytotoxicity against 5 cholangiocarcinoma cell lines (KKU-100, KKU-M139, KKU-M156, KKU-M213, and KKUM214).

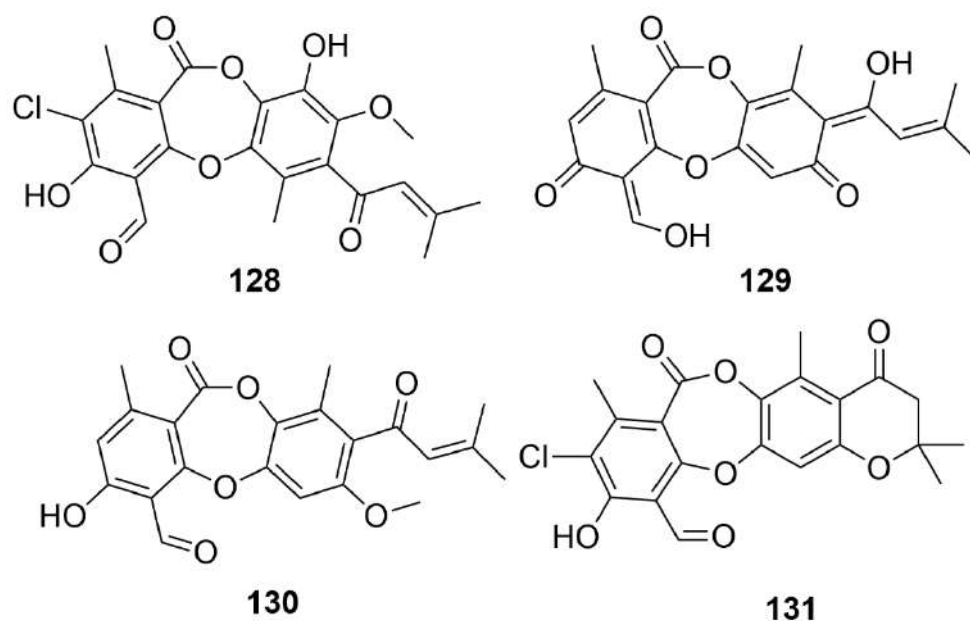


Figure 15. Chemical structures of mollicellin E (128), mollicellin K (129), mollicellin L (130) and mollicellin M (131).

- **Polyketide-Alkaloid**

Codinaeopsin

A novel tryptophan-polyketide hybrid (**143**) was isolated from fungal endophyte *Codinaeopsis gonytrichoidesi* isolated from the tree *Vochysia guatemalensis* (Vochysiaceae). This fungus was cultivated in a rich seed medium at 25°C during 21 days for compound extraction. Codinaeopsin exhibited moderate activity against *Plasmodium falciparum* 3D7 with an IC_{50} value of 4.7 μ M (Kontnik et al., 2008).

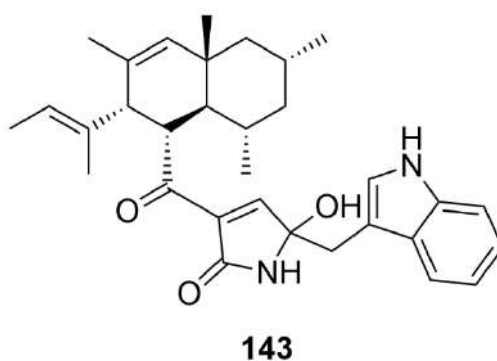


Figure 16. Chemical structure of codinaeopsin (143).

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- **Polypeptides**

12,12a-Dihydroantibiotic PI 016

A novel compound (**147**) with antimalarial activity was isolated from fungal endophyte *Menisporopsis theobromae* isolated from the seeds of a plant (not specified) in Thailand. This fungus was grown in potato dextrose broth at 25°C during 24 days at 200 rpm for whole culture extraction. The compound 12,12a-dihydroantibiotic PI 016 exhibited a moderate activity against *Plasmodium falciparum* K1 with an IC₅₀ value of 2.95 µM (Chinworrungsee et al., 2006). Moreover, this compound presented cytotoxicity against NCI-H187 cell lines (small cell lung cancer) with an IC₅₀ value of 20.3 µM. Dihydroartemisinin was used as the positive standard with an IC₅₀ value between 0.004 – 0.014 µM.

Apicidin B, apicidin C

Two novel cyclic tetrapeptides with antiplasmodial activity were isolated from fungal endophyte *Fusarium pallidorozeum* isolated from the branches of *Acacia* sp. (Fabaceae). Apicidin B (**148**) and C (**149**) exhibited significant activity against *Plasmodium falciparum* with MIC values of 0.189 and 0.069 µM, respectively (Singh et al., 2001).

Fusaripeptide A

A novel cyclodepsipeptide was isolated from endophytic fungus *Fusarium* sp. isolated from the roots of *Mentha longifolia* (Lamiaceae). Fungus was cultivated in rice solid medium at room temperature for 30 days. Fusaripeptide A (**151**) exhibited a potent activity against *Plasmodium falciparum* (D6) with an IC₅₀ value of 0.34 µM (Ibrahim et al., 2018). Additionally, this compound presented significant cytotoxicity against L5178Y (mouse lymphoma) and PC12 (rat brain cancer) cell lines with IC₅₀ values of 5.71 and 9.55 µM, respectively. Fusaripeptide A also presented high antifungal activity against 3 different species of *Candida* and one strain of *Aspergillus fumigatus*. Positive control artemisinin presented an IC₅₀ value of 0.57 µM.

Pullularin A, pullularin B

Two new cyclohexadepsipeptides with antiplasmodial activity were isolated from endophytic fungus *Pullularia* sp. (yeast-like) isolated from the leaves of *Calophyllum* sp. (Clusiaceae). This endophyte was cultivated in potato dextrose broth at 25°C during 19 days at static conditions for supernatant extraction. Pullularins A (**152**) and B (**153**) exhibited moderate activity against *Plasmodium falciparum* K1 with IC₅₀ values of 4.64 and 4.18 µM, respectively (Isaka et al., 2007). Pullularin A presented cytotoxic activity against Vero cells with an IC₅₀ value of 36 µM. Dihydroartemisinin was used as the positive standard.

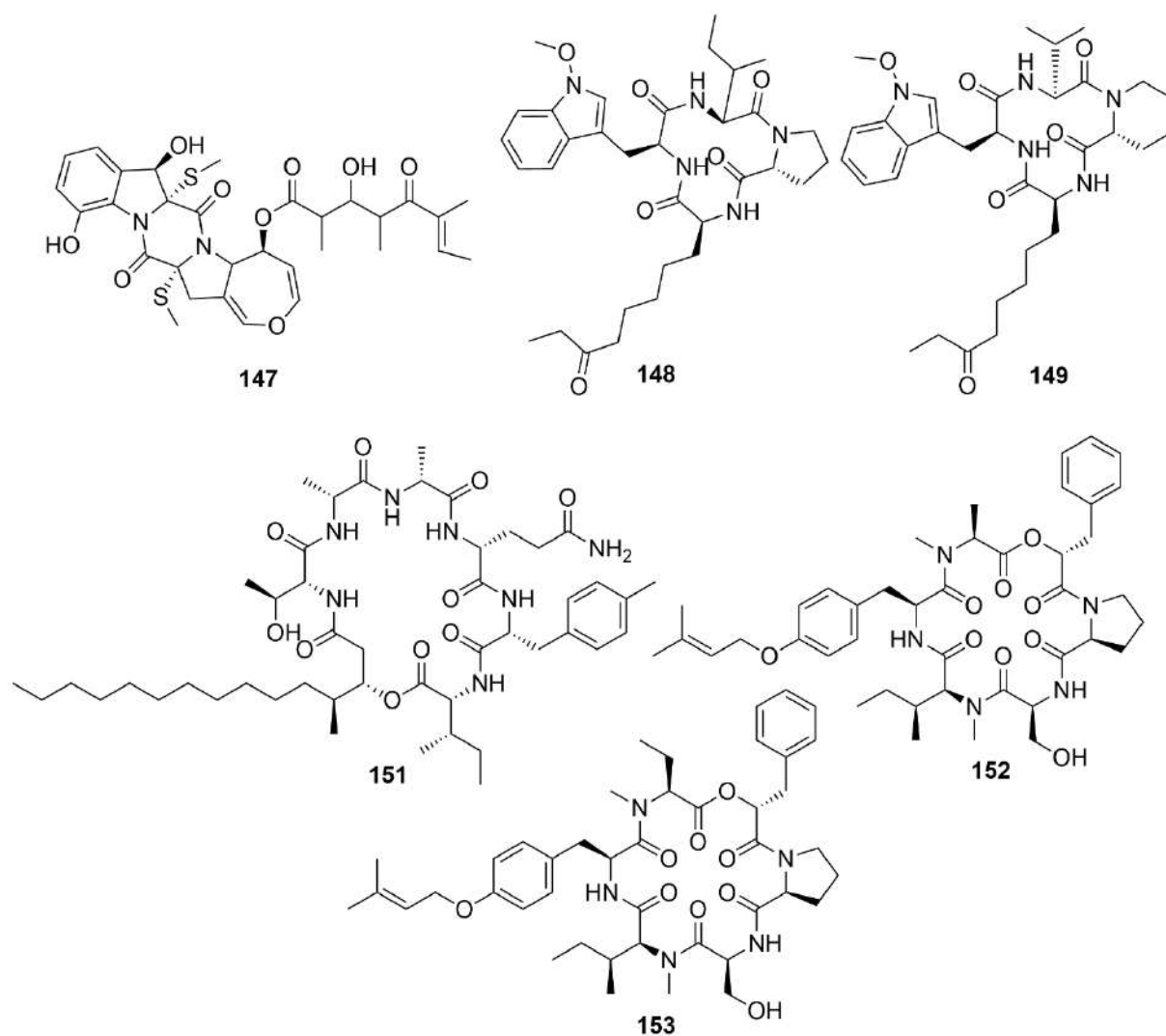


Figure 17. Chemical structures of 12,12a-dihydroantibiotic PI 016 (147), apicidin B (148), apicidin C (149), fusaripeptide A (151), pullularin A (152) and pullularin B (153).

- **Terpenoids**

7 α ,10 α -Dihydroxy-1 β -methoxyeremophil-11(13)-en-12,8 β olide

A novel eremophilanolide sesquiterpenoid was isolated from *Xylaria* sp. isolated from the palm *Licuala spinosa* (Arecaceae). This fungus was cultivated in potato dextrose broth at 25°C during 30 days at 200 rpm. The compound (**155**) exhibited weak activity against *Plasmodium falciparum* K1 with an IC₅₀ value of 8.1 μ M (Isaka et al., 2010). Moreover, it presented cytotoxic activity against KB (oral human epidermoid carcinoma), MCF-7 (human breast cancer), NCI-H187 (human small cell lung cancer) and Vero cells (African green monkey kidney fibroblasts) with IC₅₀ values of 21, 15, 7.2 and 8.5 μ M, respectively. Dihydroartemisinin was used as the positive standard with an IC₅₀ value of 0.004 μ M.

Phomoarcherin B

A new sesquiterpene was isolated from fungal endophyte *Phomopsis archeri* isolated from the cortex stem of *Vanilla albidia* (Orchidaceae). The fungus was cultivated in potato dextrose broth at 25-28°C during 4 weeks for mycelium extraction. Phomoarcherin B (**158**) exhibited moderate activity against *Plasmodium falciparum* K1 with an IC₅₀ value of 2.05 μM (Hemtasin et al., 2011). Additionally, it presented low cytotoxicity against the KB cell line with an IC₅₀ value of 9.4 μM. However, it possessed significant toxicity against KKU-M139 and KKU-M156 (cholangiocarcinoma cell lines) with IC₅₀ values of 0.1 and 2 μM, respectively. Dihydroartemisinin was used as the positive standard with an IC₅₀ value of 0.015 μM.

12,13-Deoxyroridin E, roridin E

Along with the Cytochalasins D, H, J and O and dicerandrol D previously mentioned, two already known terpenoids with potent antiplasmodial activity were isolated from a non-identified strain (CY-3923) isolated from the barks and leaves of 3 different plants; *Kandelia obovata* (Rhizophoraceae), *Avicennia marina* (Acanthaceae) and *Lumnitzera racemosa* (Combretaceae) (Calcul et al., 2013). The compounds 12,13-deoxyroridin E (**154**) and roridin E (**159**) exhibited activity against *Plasmodium falciparum* 3D7, both with an IC₅₀ value <0.02 μM. Moreover, they showed no cytotoxicity against A549 cell lines (adenocarcinomic human alveolar epithelial cells).

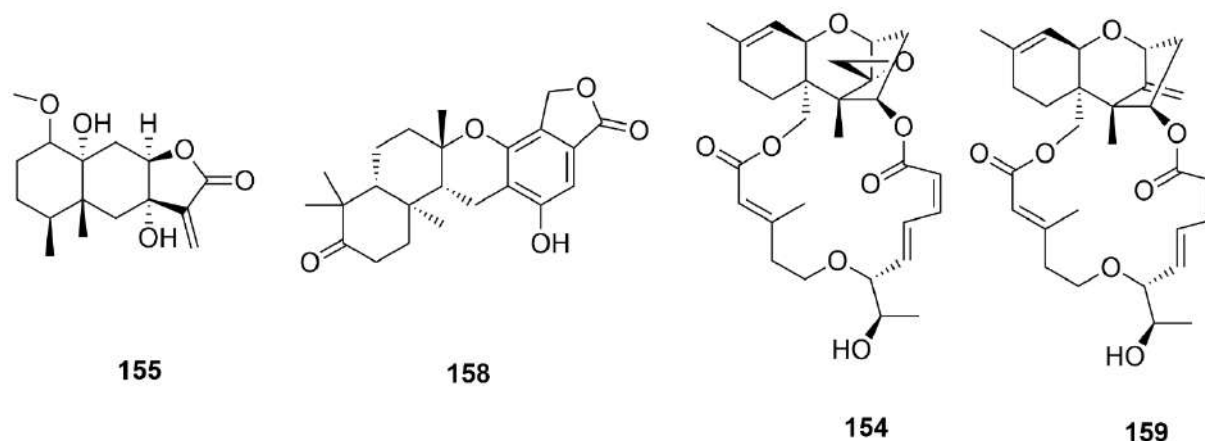


Figure 18. Chemical structures of 7 α ,10 α -dihydroxy-1 β -methoxyeremophil-11(13)-en-12,8 β olide (**155**), phomoarcherin B (**158**), 12,13-deoxyroridin E (**154**) and roridin E (**159**).

2.2.3. Antitrypanosomal / Antiplasmodial / Antileishmanial

The most active compounds against *Trypanosoma cruzi* are cercosporin, exhibiting an IC₅₀ value of 1.08 μM while its semisynthetic tetra-acetylated derivative presented an IC₅₀ value of 0.78 μM. Cercosporin and its tetra-acetylated derivative also exhibited activity against *Leishmania donovani* with IC₅₀ values of 0.46 and 0.64 μM, respectively and activity against *Plasmodium falciparum* with IC₅₀ values of 1.03 and 2.99 μM, respectively. Moreover, compound Beauvericin exhibited a moderate activity against *T. cruzi* with an IC₅₀ value of 2.43 μM followed by chaetoxanthone C with an IC₅₀ value of 3.83 μM. Altenusin was tested in an assay with the

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recombinant enzyme trypanothione reductase, considered as a drug target (Cota et al., 2008) and it exhibited IC₅₀ value of 4.3 µM.

- **Polyketides**

Altenusin

Altenusin (**116**) was first isolated from *Alternaria tenuis* (Thomas, 1961). It was also obtained from endophytic fungus *Alternaria* sp. isolated from the leaves of *Trixis vauthieri* DC (Asteraceae). *Alternaria* sp. was cultivated in malt extract agar at 28°C during 9 days at 150 rpm. Altenusin exhibited a moderate activity against the recombinant enzyme trypanothione reductase (TR) from *Trypanosoma cruzi* with an IC₅₀ value of 4.3 µM but this activity was not observed for the amastigotes of *Leishmania amazonensis* (Cota et al., 2008). It is suggested that altenusin might not be able to reach the intracellular compartments in *Leishmania* parasites in sufficient quantities to inhibit parasite survival. In this assay, clomipramine was used as a standard TR inhibitor at a concentration of 6 µg/mL.

Chaetoxanthone C

Chaetoxanthone C (**121**) was isolated from endophytic fungus *Chaetomium* sp. along with chaetoxanthone B, which exhibited a moderate antiplasmodial activity as it was previously mentioned (Pontius et al., 2008). Chaetoxanthone C also exhibited a moderate activity against *Trypanosoma cruzi* Tulahuen C4 with an IC₅₀ value of 3.83 µM and a low cytotoxicity against L6 cells (rat skeletal myoblasts) with an IC₅₀ value of 46.7 µM. Benznidazole was used as a standard compound showing an IC₅₀ value of 1.15 µM.

Cercosporin, cercosporin derivative tetra-acetylated

Cercosporin (**118**) was first isolated as a pigment from the cultured mycelia of *Cercospora kikuchii* (Kuyama & Tamura, 1957). Later, it was also obtained from the endophytic fungus *Mycosphaerella* sp. isolated from *Psychotria horizontalis* (Rubiaceae). The fungus was cultivated in potato dextrose at 28°C during 15 days at 200 rpm for mycelium extraction. In this study, cercosporin was acetylated to obtain a tetra-acetylated derivative (**119**), both with potent anti-parasitic activities. Cercosporin and its tetra-acetylated derivative exhibited activity against *Leishmania donovani* with IC₅₀ values of 0.46 and 0.64 µM, respectively, against *Plasmodium falciparum* with IC₅₀ values of 1.03 and 2.99 µM, respectively and activity against *Trypanosoma cruzi* with IC₅₀ values of 1.08 and 0.78 µM, respectively (Moreno et al., 2011). The drugs used as positive controls were amphotericin (IC₅₀ 0.08 – 0.13 µM) for *L. donovani*, chloroquine (IC₅₀ 0.08 – 0.1 µM) for *P. falciparum* and nifurtimox (IC₅₀ 10 - 16 µM) for *T. cruzi*. Additionally, cercosporin and its tetra-acetylated derivative presented cytotoxic activities against MCF-7 (human breast cancer) with IC₅₀ values of 4.68 and 3.56 µM, respectively and against Vero cells (African green monkey kidney fibroblasts) with IC₅₀ values of 1.54 and 1.24 µM, respectively.

- Polypeptides

Beauvericin

The depsipeptide beauvericin (**150**) was first isolated from the fungus *Beauveria bassiana*, known to be a potent insect pathogen (Hamill et al., 1969). This compound was also isolated from the endophytic fungus *Fusarium* sp. isolated from the stems and barks of the endangered species *Caesalpinia echinata* Lam (Fabaceae) (Campos et al., 2015). This fungus was cultivated in potato dextrose agar at 28°C during 14 days for compound extraction. Beauvericin exhibited a moderate activity against *Trypanosoma cruzi* with an IC₅₀ value of 2.43 μM. Benznidazole was used as a standard compound showing an IC₅₀ value of 3.8 μM. Beauvericin showed cytotoxic activity against L929 (mouse fibroblasts) with an IC₅₀ value of 6.38 μM.

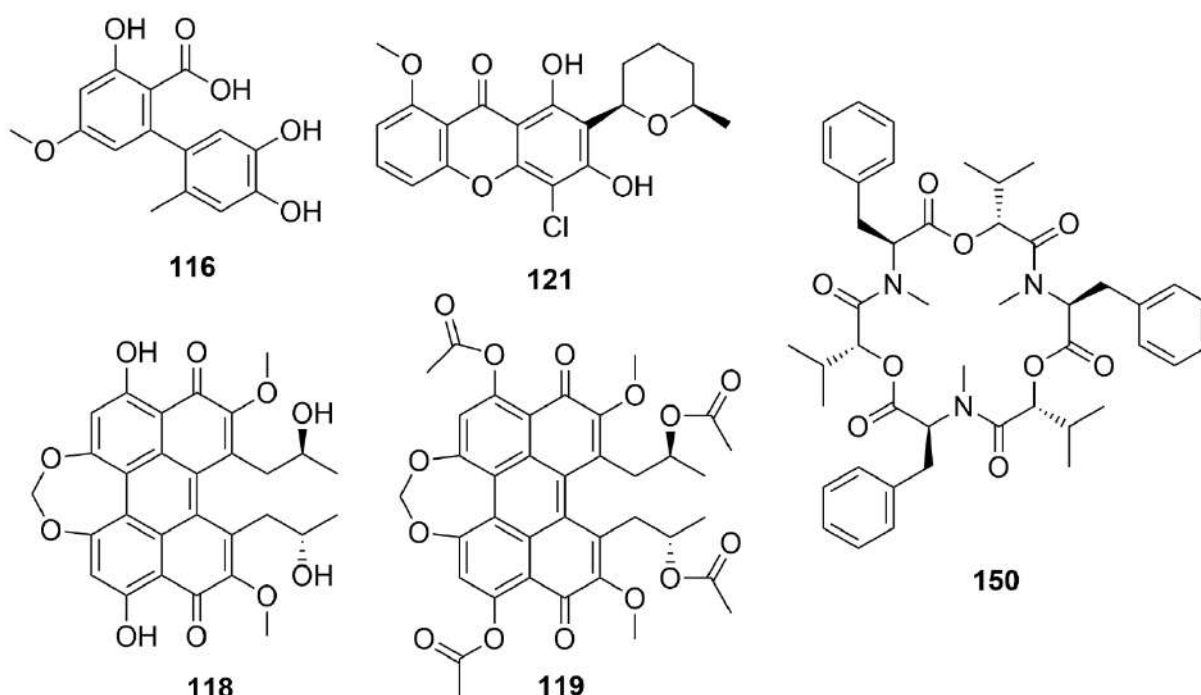


Figure 19. Chemical structures of altenusin (116), chaetoxanthione C (121), cercosporin (118), tetra-acetyl cercosporin derivative (119) and beauvericin (150).

3. Discussions and Conclusions

This work listed 101 antibacterial and 58 antiparasitic compounds isolated from endophytic fungi over the last two decades (2000-2019). These endophytic fungi were obtained from host plants of different botanical families and as shown in Figure 20, Fabaceae, Lamiaceae and Asteraceae are among the most represented families. These families are known to contain a large number of species producing bioactive compounds, as medicinal or toxic plants (Moraes Neto et al., 2019 ; Sharma et al., 2017 ; Bessada et al., 2015 ; Setzer and Setzer, 2006) and they may represent a great target for the researchers bioprospecting for endophytes. More generally, without focusing on antibacterial and antiparasitic properties, most of the bioactive endophytes studied between 2006 and 2016 were mainly isolated from plants corresponding to the families Fabaceae, Lamiaceae, Asteraceae and Araceae

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(Martinez-Klimova et al., 2016) while in a recent study, between 2016 and 2019, 254 plants families were studied for their endophytes, with Poaceae, Fabaceae, Pinaceae and Asteraceae as the most studied plant families (Harrison et al., 2020).

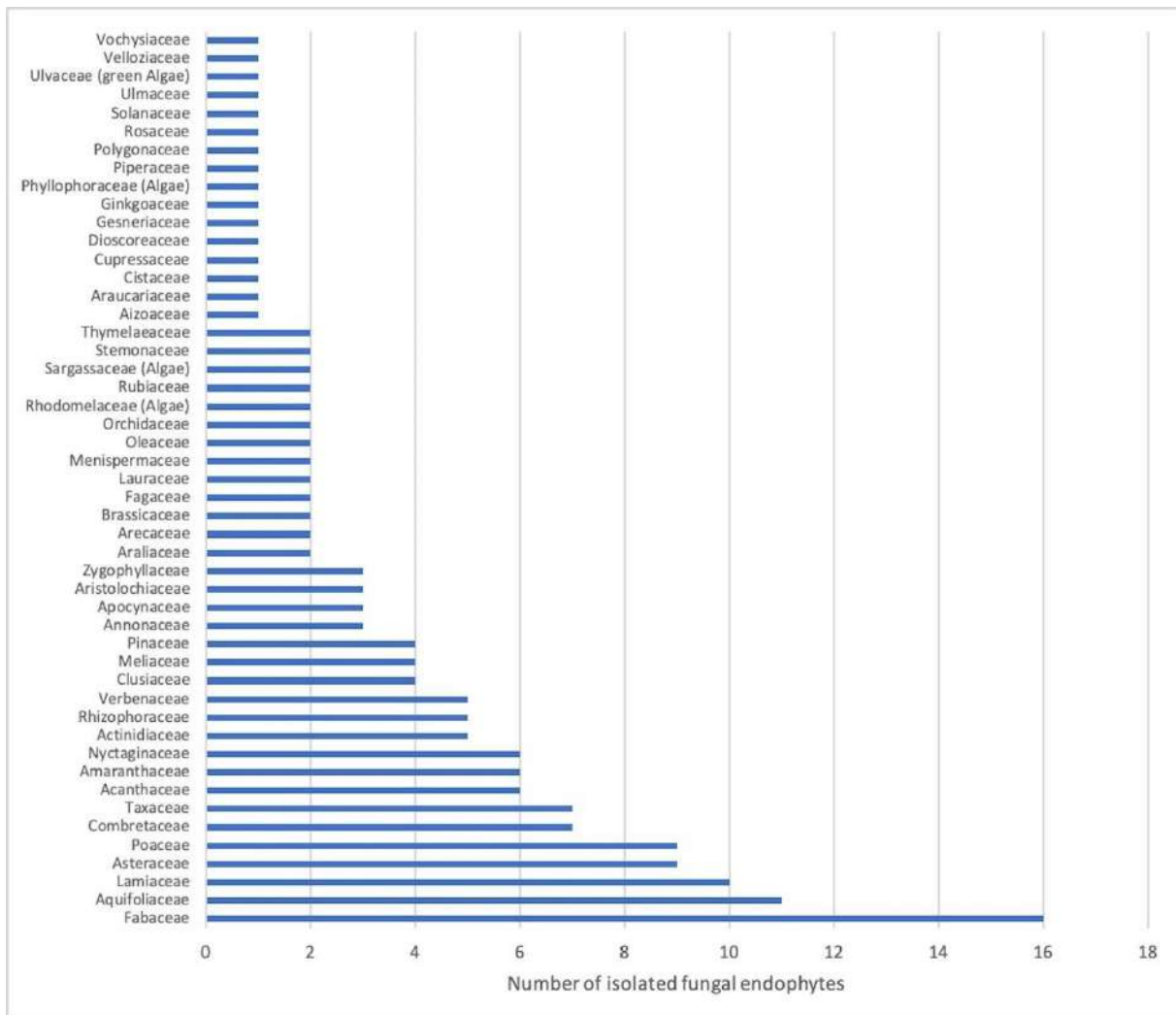


Figure 20. Botanical families of host-plants reported in this work

In this review, endophytes were mostly isolated from leaves tissues, which represent one of the largest microbial habitats on the planet (Oono et al., 2017). Most of the endophytic fungal strains belong to the Pezizomycotina subdivision of Ascomycetes: 53% of the compounds were isolated from Sordariomycetes (85 compounds from 48 fungal strains), 28% from Dothideomycetes (45 compounds from 27 fungal strains) and 12% from Eurotiomycetes (19 compounds from 12 fungal strains). According to Arnold et al. (Arnold, 2007), Dothideomycetes, Sordariomycetes and Eurotiomycetes are the three most common classes of foliar endophytes found from Canadian arctic to the lowland tropical forest of central Panama. Only one Basidiomycete, a strain belonging to the genus *Rhizoctonia*, has been listed here, as a producer of an antibacterial compound, monomethylsulochrin, a polyketide active against *Helicobacter pylori* (MIC = 10 ug/mL).

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Almost 60% of the compounds described herein are polyketides (94 over the 159), 16% are alkaloids (26/159), 9% are polypeptides (15/159) and 8% belong to the terpenoids (13/159). Polyketides is a large class of secondary metabolites with diverse bioactivities, including immunosuppressive, anticancer, and antibacterial properties (Yin and Dickschat, 2021).

More than half of the 58 antiparasitic compounds (30 over the 58) and almost 40% (40 over the 101) of the antibacterial compounds listed here were described for the first time, highlighting the potential of novelty of endophytic fungal metabolites. Almost all the listed compounds were isolated after traditional axenic cultures. Two hypotheses could be made: due to their specific environment these endophytic fungal strains present specific metabolism leading to novel compounds; indeed, interactions between endophytes and their host-plant could enhance the biosynthesis of bioactive specialized metabolites. The second hypothesis is that fungal endophytes listed here may belong to species chemically understudied. Only 4 antibacterial compounds, neospergillilic acid, lateropyrone, enniatin A1 and enniatin B1, were isolated from extracts of co-cultures with other fungi or bacteria. Surprisingly, all of them were already known, and no new antibacterial or antiparasitic compounds seemed to be *de novo* induced by co-cultures. Nevertheless, interestingly their production rate could be increased by these co-culture conditions, such as enniatin A1. Nowadays, the development of new methods is commonly used in order to reveal some biosynthetic pathway and to induce the production of original specialized metabolites.

Despite the great number of gene clusters found in fungi thanks to molecular techniques, the conditions for gene expression are not fully understood and the presence of silent or cryptic genes and lowly-expressed genes represent one of the main challenges for the isolation of novel secondary metabolites (Brakhage et al., 2011). For the activation of cryptic genes, many approaches are proposed, broadly classified in culture-independent and culture-dependent techniques. Regarding the culture-based techniques, the modification of cultivation conditions focuses on providing a better environment to trigger the expression of cryptic gene clusters.

The set of variations of culture conditions is known as the OSMAC approach (One strain – Many compounds) which is based on the potential of a single strain to expand its chemical diversity under small changes (Bode et al., 2002). These small changes involve different carbon or nitrogen sources, solid or liquid media, temperature variations, aeration or shaking conditions, salinity or pH levels, light conditions and time of development in culture (Romano et al., 2018). Under this approach, many endophytic fungi have already shown to expand their chemical diversity, being able to produce novel secondary metabolites. For example, the endophytic fungus *Trichocladium* sp. was cultured under the OSMAC approach, considering variations of culture media, rice medium and peas medium, allowing the isolation of a new amidepsine derivative and a new reduced spiro azaphilone derivative (Tran-Cong et al., 2019).

In addition to this, chemical elicitation during fungal culture such as treatment with epigenetic modifiers have also shown to elicit secondary metabolism. Biosynthetic gene clusters in fungi are transcriptionally regulated by epigenetic regulators, mainly by DNA methyltransferases (DNMT) and histone deacetylases (HDAC), which change the condensation state of the chromosomes (Brakhage et al., 2013). The use of inhibitors of these enzymes induces the transcription and expression of silenced genes. For instance, the treatment of the endophytic fungus

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Dimorphoporicola tragani with 5-azacytidine (DNMT inhibitor) and valproic acid (HDAC inhibitor) elicited the production of 3 different mycotoxins dendrodolides (González-Menéndez et al., 2019).

Similarly, the addition of biosynthetic precursors such as the use of amino acids has been used for the induction of cryptic biosynthetic pathways in fungi. The culture of the marine-derived fungus *Dichotomomyces cejpii* was supplemented with the amino acids L-tryptophan and L-phenylalanine allowing the production of 2 new polyketides, dichocetides B and C and 2 new alkaloids, dichotomocejs E and F and the induction of other known compounds (Wu et al., 2018).

Moreover, considering the natural conditions under which endophytic fungi are found, constant interactions with other microorganisms are occurring, situations that induce the production of different secondary metabolites useful for competition, communication or defense. For this reason, the co-culture of endophytic fungi with another bacterium or fungus might mimic microbial interactions, triggering different signaling pathways and responses. The co-culture of the endophytic fungus *Bionectria* sp. with *Bacillus subtilis* or *Streptomyces lividans* triggered the production of the new molecules, bionectriamines A and B and two other known compounds (Kamdem et al., 2018). To conclude, this review shows the great potential of fungal endophytes as renewable sources of antiparasitic and antibacterial compounds. With the advent of original cultures techniques, fungal endophytes are far from having revealed all their secrets.

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| ID | Compound names | Chemical Formula | Endophytic fungal strain | Host plant | Botanical family | Activity | References |
|--------------------|--|---|---|--|----------------------|---|------------------------------|
| Alkaloids | | | | | | | |
| 1 | (+)-Flavipucine | C ₁₂ H ₁₅ NO ₄ | <i>Phoma</i> sp. 7204* | <i>Salsola oppositifolia</i> Desf. | Amaranthaceae | 6, 17, and 11 mm zone inhibition against <i>B. subtilis</i> , <i>S. aureus</i> and <i>E. coli</i> | Loessen et al., 2011 |
| 2 | Brevianamide M | C ₁₈ H ₁₅ N ₃ O ₃ | <i>Aspergillus versicolor</i> <i>Chaetomium globosum</i> SNB-GTC2114 | <i>Sargassum thunbergii</i> (Mertens ex Kuhn) Kuntze | Sargassaceae (Algae) | 11-10 mm inhibition against <i>E. coli</i> and <i>S. aureus</i> at 30 µg/disk | Miao et al., 2012 |
| 3 | Cochlodinol | C ₂₁ H ₃₀ N ₅ O ₄ | | <i>Paspalum virgatum</i> L. | Poaceae | 4 µg/mL MIC against <i>S. aureus</i> | Caecilia et al., 2013 |
| 4 | Fumigaclavine C | C ₂₃ H ₃₀ N ₅ O ₂ | <i>Aspergillus</i> sp. EIC08 | <i>Bauhinia guianensis</i> Aubl. | Fabaceae | 7.81 µg/mL MIC against <i>B. subtilis</i> | Pinheiro et al., 2012 |
| 5 | Fusapyridin A | C ₂₉ H ₄₃ NO ₃ | <i>Fusarium</i> sp. YG-45 <i>Aspergillus</i> sp. FSY-01/ <i>Aspergillus</i> sp. FSW-02 (co-culture)* | <i>Maackia chinensis</i> Takeda | Fabaceae | 6.25 µg/mL MIC against <i>P. aeruginosa</i> | Tsuchihari et al., 2007 |
| 6 | Neospergilline acid | C ₁₂ H ₂₀ N ₃ O ₂ | | <i>Avicennia marina</i> (Forsk.) Vieh. | Acanthaceae | 0.98-7.80 µg/mL MIC against <i>S. aureus</i> , <i>S. epidermidis</i> , <i>B. subtilis</i> , <i>B. dysenteriae</i> , <i>B. proteus</i> | Zhu et al., 2011 |
| 7 | Phomapyrolidone B | C ₂₄ H ₄₁ NO ₄ | <i>Phoma</i> sp. NRRL 46751 * | <i>Saurauia scaberrima</i> Lauterb. | Actinidiaceae | 5.9 µM IC50 against <i>M. tuberculosis</i> H37Pv | Wijetane et al., 2013 |
| 8 | Phomapyrolidone C | C ₂₃ H ₄₁ NO ₃ | <i>Phoma</i> sp. NRRL 46751 * | <i>Saurauia scaberrima</i> Lauterb. | Actinidiaceae | 5.2 µM IC50 against <i>M. tuberculosis</i> H37Pv | Wijetane et al., 2013 |
| 9 | Phomoenamidine A | C ₁₄ H ₂₄ N ₃ O ₄ | <i>Phomopsis</i> PSU-1D15* | <i>Garcinia dulcis</i> (Roxb.) Kurz | Clusiaceae | 6.25 µg/ml MIC against <i>M. tuberculosis</i> H37Ra 2.62 and 1.74 MIC against <i>M. tuberculosis</i> and <i>M. smegmatis</i> | Rukachaisirikul et al., 2008 |
| 10 | Piperine | C ₁₇ H ₁₉ NO ₃ | <i>Piperonia</i> sp | <i>Piper longum</i> L. | Piperaceae | | Verma et al., 2011 |
| 11 | Pseudo A | C ₂₂ H ₂₅ NO ₈ | <i>Aspergillus</i> sp. EIC08 | <i>Bauhinia guianensis</i> Aubl. | Fabaceae | 15.62 µg/mL MIC against <i>B. subtilis</i> | Pinheiro et al., 2012 |
| 12 | Pyrocidine A | C ₁₁ H ₁₇ NO ₄ | <i>Acronium zae</i> NRRL 13540 | <i>Zea mays</i> L. | Poaceae | 0.25-2 µg/mL MIC against <i>S. aureus</i> , <i>S. haemolyticus</i> , <i>E. faecalis</i> , <i>E. faecium</i> | Wicklow et al., 2008 |
| 13 | Pyrocidine B | C ₁₁ H ₁₉ NO ₄ | <i>Acronium zae</i> NRRL 13540 | <i>Zea mays</i> L. | Poaceae | 4 µg/mL MIC against <i>S. aureus</i> , <i>S. haemolyticus</i> , <i>E. faecalis</i> , <i>E. faecium</i> | Wicklow et al., 2008 |
| 14 | Pyrocidine C | C ₂₄ H ₄₁ NO ₄ | <i>Lewisia tilfactoria</i> SNB-GTC2402* | <i>Besleria insohla</i> C. V. Morton | Gesneriaceae | 2 µg/mL MIC against <i>S. aureus</i> | Caecilia et al., 2013 |
| 15 | Semiochlorinol A | C ₂₇ H ₃₂ N ₂ O ₄ | <i>Chaetomium globosum</i> SNB-GTC2114 | <i>Paspalum virgatum</i> L. | Poaceae | 2 µg/mL MIC against <i>S. aureus</i> | Caecilia et al., 2013 |
| Fatty acids | | | | | | | |
| 16 | Cerebroside 1 | C ₄₁ H ₇₇ NO ₉ | <i>Fusarium</i> sp. IFB-121* | <i>Quercus variabilis</i> Blume | Fagaceae | 7.8-3.9 MIC against <i>B. subtilis</i> , <i>E. coli</i> , <i>P. fluorescens</i> | Shu et al., 2004 |
| 17 | Cerebroside 2 | C ₄₁ H ₇₅ NO ₉ | <i>Fusarium</i> sp. IFB-122 | <i>Quercus variabilis</i> Blume | Fagaceae | 1.9-3.9 MIC against <i>B. subtilis</i> , <i>E. coli</i> , <i>P. fluorescens</i> | Shu et al., 2004 |
| Poliketides | | | | | | | |
| 18 | (12S)-12-hydroxymonocerin | C ₁₆ H ₂₇ O ₇ | <i>Microdochium bolleyi</i> * | <i>Fagonia cretica</i> L. | Zygophyllaceae | 10 mm zone inhibition against <i>E. coli</i> at 50 µg/disk | Zhang et al., 2008 |
| 19 | (3R)-5-methylmellemn (4R,5R,6S)-6-acetoxy-4,5-dihydroxy-2-(hydroxymethyl)cyclohex-2-en-1-one | C ₁₁ H ₁₂ O ₃ | <i>Cytospora</i> sp. | <i>Ilex canariensis</i> Poir. | Aquifoliaceae | 20 mm zone inhibition against <i>B. megaterium</i> at 50 µg/disk | Lu et al., 2011 |
| 20 | (R)-5-(S)-acetate(phenyl)-methyl)dihydrofuran-2(3H)-one | C ₁₃ H ₁₄ O ₄ | <i>Phoma</i> sp. 8889 | <i>Salsola oppositifolia</i> Desf. | Amaranthaceae | 10 mm zone inhibition 0.05 mg against <i>B. megaterium</i> | Qin et al., 2010 |
| 21 | | | <i>Cytospora</i> sp. * | <i>Ilex canariensis</i> Poir. | Aquifoliaceae | 20 mm zone inhibition against <i>B. megaterium</i> at 50 µg/disk | Lu et al., 2011 |

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|----|--|---|--|--|---|---|--|
| 22 | (R)-5-(S)-hydroxy(phenyl)-methyl)dihydrofuran-2(3H)-one | C ₁₁ H ₁₂ O ₃ | <i>Cytospora</i> sp. * | <i>Ilex canariensis</i> Poit. | Aquifoliaceae | 20 mm zone inhibition against <i>B. megaterium</i> at 50 µg/disk | Lu et al., 2011 |
| 23 | (S)-5-(S)-hydroxy(phenyl)-methyl)dihydrofuran-2(3H)-one | C ₁₁ H ₁₂ O ₃ | <i>Cytospora</i> sp. | <i>Ilex canariensis</i> Poit. | Aquifoliaceae | 18 mm zone inhibition against <i>B. megaterium</i> at 50 µg/disk | Lu et al., 2011 |
| 24 | (S)-5-benzyl-dihydrofuran-2(3H)-one | C ₁₁ H ₁₂ O ₂ | <i>Cytospora</i> sp. * | <i>Ilex canariensis</i> Poit. | Aquifoliaceae | 20 mm zone inhibition against <i>B. megaterium</i> at 50 µg/disk | Lu et al., 2011 |
| 25 | (S)-5-hydroxy-4-oxo-1,2,3,4-tetrahydronaphthalen-1-yl acetate | C ₁₂ H ₁₂ O ₄ | <i>Cytospora</i> sp. * | <i>Ilex canariensis</i> Poit. | Aquifoliaceae | 15 mm zone inhibition against <i>B. megaterium</i> at 50 µg/disk | Lu et al., 2011 |
| 26 | 2-hydroxy-3-(hydroxymethyl)anthraquinone | C ₁₅ H ₁₀ O ₄ | <i>Coniothyrium</i> sp. Zw86* | <i>Salsola oppositifolia</i> Desf. | Amaranthaceae | 16 and 15 mm zone inhibition against <i>E. coli</i> and <i>B. megaterium</i> at 50 µg per disk | Sun et al., 2012 |
| 27 | 2,3-didehydro-1,9a-hydroxy-14-epi-cecloquinone B | C ₈ H ₈ O ₇ | <i>Nigrospora</i> sp. MA75* | <i>Pongamia pinnata</i> (L.) Pierre | Fabaceae | 8-0.5 µg/mL MIC against MRSA, <i>E. coli</i> , <i>P. aeruginosa</i> , <i>P. fluorescens</i> | Shang et al., 2012 |
| 28 | 3-(2,5-dihydro-4-hydroxy-5-oxo-3-phenyl-2-furyl)propanoic acid | C ₁₃ H ₁₂ O ₅ | <i>Cytospora</i> sp. | <i>Ilex canariensis</i> Poit. | Aquifoliaceae | 15 mm zone inhibition against <i>B. megaterium</i> at 50 µg/disk | Lu et al., 2011 |
| 29 | 3-O-methylalaternin | C ₁₆ H ₁₂ O ₆ | <i>Ampelomyces</i> sp. EU143250 | <i>Urospermum pteroides</i> (L.) Scop. Ex F. W. Schmidt | Asteraceae | 12.5 µg/mL MIC <i>S. epidermis</i> , <i>E. faecalis</i> , <i>S. aureus</i> | Aly et al., 2008 |
| 30 | 4-deoxyxyetrahydrobostroycin | C ₁₆ H ₂₀ O ₇ | <i>Nigrospora</i> sp. MA75 | <i>Pongamia pinnata</i> (L.) Pierre | Fabaceae | 4 µg/mL MIC against <i>E. coli</i> | Shang et al., 2012 |
| 31 | 5-phenyl-4-oxopentanoic acid | C ₁₁ H ₁₂ O ₃ | <i>Cytospora</i> sp. * | <i>Ilex canariensis</i> Poit. | Aquifoliaceae | 15 mm zone inhibition against <i>B. megaterium</i> at 50 µg/disk | Lu et al., 2011 |
| 32 | 6-hydroxy-2-methyl-4-chromanone | C ₁₀ H ₁₀ O ₃ | <i>Periconia sinensis</i> CMUG5015 | <i>Thysanolaena latifolia</i> (Roxb. Ex Hornem.) Honda | Paaceae | 12.5-6.25 µg/mL MIC against <i>B. subtilis</i> , <i>L. monocytogenes</i> , <i>P. aeruginosa</i> | Bhlabura et al., 2007 |
| 33 | 6-hydroxyterrefuranone | C ₁₄ H ₂₀ O ₄ | <i>Microdiplodia</i> sp. KS 75-1* | <i>Pinus</i> sp. | Pinaceae | 16 mm zone inhibition against <i>S. aureus</i> at 40 µg/disk | Shiono et al., 2012 |
| 34 | 6(7)-dehydro-8-hydroxyterrefuranone | C ₁₄ H ₁₈ O ₃ | <i>Microdiplodia</i> sp. KS 75-1* | <i>Pinus</i> sp. | Pinaceae | 15 mm zone inhibition against <i>S. aureus</i> at 40 µg/disk | Shiono et al., 2012 |
| 35 | 7-amino-4-methylcoumarin | C ₁₀ H ₈ NO ₂ | <i>Xylaria</i> sp. YX-28* | <i>Ginkgo biloba</i> L. | Ginkgoaceae | 15 mm zone inhibition against <i>S. aureus</i> at 40 µg/disk | Liu et al., 2008 |
| 36 | 7,8-dihydrovitlanone A | C ₁₅ H ₂₀ O ₄ | <i>Microdiplodia</i> sp. KS 75-1* | <i>Pinus</i> sp. | Pinaceae | 3.9, 3.9, 1.8, and 3.9 µg/mL MICs against <i>Bacillus subtilis</i> , <i>Escherichia coli</i> , <i>Pseudomonas fluorescens</i> and <i>Candida albicans</i> | Shiono et al., 2012 |
| 37 | Altechromone A | C ₁₁ H ₁₀ O ₃ | <i>Alternaria brassicicola</i> ML-P08 | <i>Malus halliana</i> Koehne | Rosaceae | 15 mm zone inhibition against <i>Xanthomonas oryzae pv. oryzae</i> | Shiono et al., 2012 |
| 38 | Alterlactone | C ₁₅ H ₁₂ O ₆ | <i>Alternaria alternata</i> ZHUG5 | <i>Cercis chinensis</i> Bunge | Fabaceae | 16 µg/mL MIC value against <i>Xanthomonas oryzae pv. oryzae</i> | Gy et al., 2009 |
| 39 | Alternariol | C ₁₄ H ₁₀ O ₅ | <i>Alternaria alternata</i> ZHUG5 | <i>Cercis chinensis</i> Bunge | Fabaceae | 4 µg/mL MIC value against <i>Xanthomonas oryzae pv. oryzae</i> | Zhao et al., 20et al., 20 |
| 40 | Altersolanol A | C ₁₆ H ₁₆ O ₈ | <i>Ampelomyces</i> sp. EU143251 | <i>Urospermum pteroides</i> (L.) Scop. Ex F. W. Schmidt | Asteraceae | 12.5 µg/mL MIC <i>S. epidermis</i> , <i>E. faecalis</i> | Zhao et al., 20et al., 20 |
| 41 | Ambucic acid | C ₁₀ H ₁₆ O ₆ | 4 strains of <i>Pestalotiopsis microspora</i> , <i>P. guenpinii</i> MSU 214, <i>Monochaetia</i> sp. MSU-NC 202 | <i>Taxus baccata</i> L., <i>Torreya taxifolia</i> Arn., <i>Taxodium distichum</i> (L.) Rich., <i>Dendrobium speciosum</i> Sm., <i>Wollemia nobilis</i> W. G. Jones, K. D Hill & J.M. Allen, <i>Taxus wallichiana</i> Zucc. | Taxaceae, Taxaceae, Cupressaceae, Orchidaceae, Araliaceae, Taxaceae | 10 µM IC50 inhibited the production of gelatinase by <i>E. faecalis</i> OG1RF (anti-OS) | Nakayama et al., 2009, Li et al., 2001 |
| 42 | Cercosporamide | C ₁₆ H ₁₃ NO ₇ | <i>Phoma</i> sp. NG-25 | <i>Saurauia scaberrima</i> Lauterb. | Actinidiaceae | 2.0 µg/mL MIC against <i>S. aureus</i> | Hoffman et al., 2008 |
| 43 | Citreoselin | C ₁₅ H ₁₀ O ₆ | <i>Microsplophaeopsis</i> sp. (strain 8875) | <i>Lycium intricatum</i> Boiss. | Solanaceae | 9 mm zone inhibition against <i>E. coli</i> , 10 mm against <i>Bacillus megaterium</i> at 50 µg/disk | Krohn et al., 2009 |

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| 44 | Cyosporone D | C ₁₆ H ₂₂ O ₅ | <i>Cyospora</i> sp. CR200 and <i>Diaporthe</i> sp. CR146* | <i>Conocarpus erectus</i> L. and <i>Forsteronia spicata</i> (Jacq.) G. Mey. | Combretaceae and Apocynaceae | 8 µg/mL MIC against <i>S. aureus</i> and <i>E. faecalis</i> | Brady et al., 2000 |
| 45 | Cyosporone E | C ₁₅ H ₂₀ O ₅ | <i>Cyospora</i> sp. CR200 and <i>Diaporthe</i> sp. CR146* | <i>Conocarpus erectus</i> L. and <i>Forsteronia spicata</i> (Jacq.) G. Mey. | Combretaceae and Apocynaceae | 8 µg/mL MIC against <i>S. aureus</i> and <i>E. faecalis</i> | Brady et al., 2000 |
| 46 | Diclerandrol A | C ₃₁ H ₅₄ O ₁₄ | <i>Phomopsis longicolla</i> MMW29* | <i>Diclerandra frutescens</i> Shimmers | Lamiaceae | 11-10 mm inhibition against <i>B. subtilis</i> and <i>S. aureus</i> | Wagenar et al., 2001 |
| 47 | Diclerandrol B | C ₃₁ H ₅₆ O ₁₅ | <i>Phomopsis longicolla</i> MMW29* | <i>Diclerandra frutescens</i> Shimmers | Lamiaceae | 9.5-8.5 mm inhibition against <i>B. subtilis</i> and <i>S. aureus</i> | Wagenar et al., 2001 |
| 48 | Diclerandrol C | C ₃₈ H ₅₈ O ₁₆ | <i>Phomopsis longicolla</i> MMW29* | <i>Diclerandra frutescens</i> Shimmers | Lamiaceae | 8-7 mm inhibition against <i>B. subtilis</i> and <i>S. aureus</i> | Wagenar et al., 2001 |
| 49 | Grisospherone C | C ₁₆ H ₁₆ O ₆ | <i>Nigropora</i> sp. MA75 strain CR115* | <i>Pongamia pinnata</i> (L.) Pierre | Fabaceae | 2-0.5 µg/mL MIC against MRSA, <i>E. coli</i> , <i>P. aeruginosa</i> , <i>P. fluorescens</i> , <i>S. epidermis</i> | Shang et al., 2012 |
| 50 | Guamaacstepene A | C ₃₂ H ₅₀ O ₅ | <i>Nigropora</i> sp. MA75 strain CR115* | <i>Daphnopsis americana</i> (Mill.) J.R. Johnston | Thymelaeaceae | 6-8 mm inhibition zone at 6.25 µg per spot against <i>S. aureus</i> , <i>E. faecium</i> and <i>E. coli</i> | Singh et al., 2000 |
| 51 | Integracins A | C ₂₇ H ₅₆ O ₈ | <i>Cyospora</i> sp. | <i>Ilex canariensis</i> Poir. | Aquifoliaceae | 20 mm zone inhibition against <i>B. megaterium</i> at 50 µg/disk | Lu et al., 2011 |
| 52 | Integracins B | C ₃₅ H ₅₄ O ₇ | <i>Cyospora</i> sp. | <i>Ilex canariensis</i> Poir. | Aquifoliaceae | 20 mm zone inhibition against <i>B. megaterium</i> at 50 µg/disk | Lu et al., 2011 |
| 53 | Isotrisdiolenol A | C ₁₆ H ₁₂ O ₆ | <i>Chadara</i> sp. (strain 6661)* | <i>Artemisia vulgaris</i> L. | Asteraceae | 23 mm zone inhibition 0.015 mg against <i>B. subtilis</i> | Løsgen et al., 2008 |
| 54 | Isotrisdiolenol B | C ₁₆ H ₁₂ O ₇ | <i>Chadara</i> sp. (strain 6661)* | <i>Artemisia vulgaris</i> L. | Asteraceae | 22 mm zone inhibition 0.015 mg against <i>B. subtilis</i> | Løsgen et al., 2008 |
| 55 | Isorhodoptiometrin | C ₂₇ H ₄₄ O ₆ | <i>Penicillium restrictum</i> G851T5 | <i>Silybum marianum</i> (L.) Gaertn. | Asteraceae | 8.9 µgR P3lux IC50 <i>S. aureus</i> MRSA (anti-QS) | Figuerola et al., 2014 |
| 56 | Javanicin | C ₁₅ H ₁₄ O ₆ | <i>Chloridium</i> sp. <i>Fusarium trichinatum</i> / <i>Bacillus subtilis</i> 168 tppC2 (co-culture) | <i>Azadirachta indica</i> A. Juss. | Meliaceae | 10-2 µg/mL MIC against <i>Pseudomonas fluorescens</i> , <i>P. aeruginosa</i> | Khanwar et al., 2009 |
| 57 | Lateopyrone | C ₁₅ H ₁₀ O ₈ | <i>Periconia stamenis</i> CMU/GE015 | <i>Aristolochia paucifloris</i> Pomel | Aristolochiaceae | 8-2 µg/mL MIC against <i>B. subtilis</i> , <i>S. aureus</i> , <i>S. pneumoniae</i> and <i>E. faecalis</i> , MRSA | Ola et al., 2013 |
| 58 | Modiolide A | C ₁₀ H ₁₄ O ₄ | <i>Microdochium bolleyi</i> | <i>Thyrsanodelva latifolia</i> (Roxb. Ex Hornem.) Honda | Paaceae | 12.5-3.12 µg/mL MIC against <i>B. subtilis</i> , <i>L. monocytogenes</i> , <i>P. aeruginosa</i> | Bhilaratra et al., 2007 |
| 59 | Monocecin | C ₁₆ H ₂₀ O ₆ | <i>Rhizoctonia</i> sp. Cy064 | <i>Fegonia cretica</i> L. | Zygophyllaceae | 10 mm zone inhibition against <i>E. coli</i> at 50 µg/disk | Zhang et al., 2008 |
| 60 | Monomethylsulochrin | C ₁₈ H ₁₈ O ₇ | <i>Microdidymia</i> sp. KS 75-1 | <i>Cynodon dactylon</i> (L.) Pers. | Paaceae | 10 µg/mL MIC against <i>H. pylori</i> ATCC 43504 | Ma et al., 2004 |
| 61 | Nivecuranone A | C ₁₅ H ₁₈ O ₄ | <i>Penicillium radiclecola</i> (IBT 10696) and <i>Penicillium coprobium</i> (IBT 6895) | <i>Pinus</i> sp. | Pinaceae | 15 mm zone inhibition against <i>S. aureus</i> at 40 µg/disk | Shiono et al., 2012 |
| 62 | Norlichexanhone | C ₁₄ H ₁₆ O ₅ | <i>Penicillium radiclecola</i> (IBT 10696) and <i>Penicillium coprobium</i> (IBT 6895) | <i>Pongamia pinnata</i> (L.) Pierre | Fabaceae | 0.5 µg/mL MIC against <i>S. epidermis</i> | Shang et al., 2012 |
| 63 | Patulin | C ₇ H ₆ O ₄ | <i>Penicillium radiclecola</i> (IBT 10696) and <i>Penicillium coprobium</i> (IBT 6895) | <i>Ammoracia rusticana</i> P. Gaertn., B. Mey. & Scherb./- | Brassicaceae / - | Abolish of QS-controlled lasB induction at 80 µM (anti-QS) | Rasmussen et al., 2005 |
| 64 | Penicillie acid | C ₃₀ H ₃₂ O ₈ | <i>Phoma</i> sp. NG-25* | <i>Ammoracia rusticana</i> P. Gaertn., B. Mey. & Scherb./- | Brassicaceae / - | Abolish of QS-controlled lasB induction at 40 µM (anti-QS) | Rasmussen et al., 2005 |
| 65 | Phomolone | C ₃₂ H ₃₆ O ₇ | <i>Phomopsis</i> sp. strain E02018 * | <i>Saurauia scaberrima</i> Lauterb. | Actinidiaceae | MIC 1.6 µg/mL <i>S. aureus</i> | Hoffman et al., 2008 |
| 66 | Phomol | C ₃₂ H ₃₆ O ₇ | <i>Phomopsis</i> sp. strain E02018 * | <i>Erythrina crista-galli</i> L. | Fabaceae | 10 µg/mL MIC against <i>Corynebacterium insidiosum</i> | Weber et al., 2004 |

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| 67 | Phomosine A | C ₈ H ₁₈ O ₇ | <i>Phomopsis</i> sp 5686 | <i>Ligustrum vulgare</i> L. | Oleaceae | 11 mm zone inhibition against <i>B. megaterium</i> at 50 µg/disk | Krton et al., 2011 | |
| 68 | Phomosine B | C ₉ H ₂₂ O ₇ | <i>Phomopsis</i> sp 5686 | <i>Ligustrum vulgare</i> L. | Oleaceae | 11 mm zone inhibition against <i>B. megaterium</i> at 50 µg/disk | Krton et al., 2011 | |
| 69 | Phomoxanthone A | C ₈ H ₁₈ O ₆ | <i>Phomopsis</i> sp. BCC 1323* | <i>Tectona grandis</i> L.f. | Lamiaceae | 0.5 µg/mL against <i>M. tuberculosis</i> H37Ra | Isaka et al., 2001 | |
| 70 | Phomoxanthone B | C ₈ H ₁₈ O ₆ | <i>Phomopsis</i> sp. BCC 1323* | <i>Tectona grandis</i> L.f. | Lamiaceae | 0.5 µg/mL against <i>M. tuberculosis</i> H37Ra | Isaka et al., 2001 | |
| 71 | Phyllostine | C ₇ H ₁₀ O ₄ | <i>Phomopsis</i> sp <i>Botryosphaeria mamane</i> | <i>Notobasis syriaca</i> (L.) Cass. | Asteraceae | 10 mm zone inhibition against <i>E. coli</i> at 50 µg/disk | Hussain et al., 2011 | |
| 72 | Primin | C ₁₂ H ₁₆ O ₃ | PSU-M76 | <i>Garcinia x mangostana</i> L. | Clusiaceae | 8 µg/mL MIC against <i>S. aureus</i> and MRSA | Pongcharoen et al., 2007 | |
| 73 | Pyrenocine L | C ₁₄ H ₂₀ O ₆ | <i>Phomopsis</i> sp* | <i>Cistus salvifolius</i> L. | Cistaceae | 10 mm zone inhibition against <i>E. coli</i> at 50 µg/disk | Hussain et al., 2012 | |
| 74 | Secalonic acid B | C ₃₂ H ₅₀ O ₄ | <i>Bienertia</i> sp. 7064 | <i>Carpobrotus edulis</i> (L.) N.E.Br. | Aizoaceae | 15 mm zone inhibition against <i>B. megaterium</i> at 50 µg/disk | Zhang et al., 2008 | |
| 75 | Spiropressione A | C ₉ H ₁₂ O ₅ | <i>Preussia</i> sp. CGMCC 2022 | <i>Agulitaria sinensis</i> (Lour.) Spreng | Thymelaeaceae | 16.4 mm zone inhibition against <i>S. aureus</i> at 5 µg/disk | Chen et al., 2009 | |
| 76 | Tetrahydrobotrycyn | C ₆ H ₁₀ O ₈ | <i>Nigrospora</i> sp. MA75 | <i>Pongamia pinnata</i> (L.) Pierre | Fabaceae | 2-0.5 µg/mL MIC against MRSA, <i>E. coli</i> 18, 14, and 12 mm, against <i>Mycobacterium phlei</i> , <i>S. aureus</i> and <i>E.coli</i> at 20 µg/disk | Shang et al., 2012 | |
| 77 | Trypethelone | C ₆ H ₁₆ O ₄ | <i>Coniohyrium cereale</i> | <i>Enteromorpha</i> sp | Ulvacae (green Algae) | | Eisebati et al., 2011 | |
| 78 | Utric acid | C ₈ H ₁₆ O ₇ | <i>Phoma</i> sp. NG-25 | <i>Saurauia scoberrima</i> Lauterb. | Actinidiaceae | MIC 2.0 µg/mL against <i>S. aureus</i> | Hoffman et al., 2008 | |
| 79 | xanthone 8-hydroxy-6-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ester | C ₆ H ₁₂ O ₅ | <i>Microspheeropsis</i> sp. (7177) | <i>Zygophyllum fontanesii</i> Webb & Berthel | Zygophyllaceae | 13 mm zone inhibition against <i>B. megaterium</i> at 50 µg/disk | Krton et al., 2009 | |
| 80 | Yecathin C | C ₆ H ₁₂ O ₆ | <i>Aspergillus wentii</i> pt-1* | <i>Gymnogonrus flabelliformis</i> Harvey | Phylophoraceae (Algae) | 12 mm inhibition against <i>E. coli</i> at 10 µg/disk | Sun et al., 2012 | |
| 81 | γ-oxo-benzenepentanoic acid methyl ester | C ₂ H ₄ O ₃ | <i>Cytospora</i> sp. * | <i>Ilex canariensis</i> Poir. | Aquifoliaceae | 25 mm zone inhibition against <i>B. megaterium</i> at 50 µg/disk | Lu et al., 2011 | |
| 82 | ω-hydroxyemodin | C ₅ H ₁₀ O ₆ | <i>Penicillium restrictum</i> G851TS | <i>Silybum maritimum</i> (L.) Gaertn. | Asteraceae | 8.1 ager P3lux IC50 <i>S. aureus</i> MRSA (anti-QS) | Figuerola et al., 2014 | |
| Polyketides-Fatty acids | | | | | | | | |
| 83 | Bisogniazaphilone A | C ₃₄ H ₃₄ O ₄ | <i>Bisogniazia formosana</i> BRCRC 33718* | <i>Cinnamomum</i> sp. | Lauraceae | < 5.12 µg/mL against <i>M. tuberculosis</i> H37RV | Cheng et al., 2012 | |
| 84 | Bisogniazaphilone B | C ₃₅ H ₃₂ O ₅ | <i>Bisogniazia formosana</i> BRCRC 33718* | <i>Cinnamomum</i> sp. | Lauraceae | < 2.52 µg/mL against <i>M. tuberculosis</i> H37RV | Cheng et al., 2012 | |
| Polypeptides | | | | | | | | |
| 85 | 3,1'-didihydro-3[2'(3''',3'''-dimethyl-1-pirop-2-enyl)-3''',-indolyl)methyl]enyl-6-methyl piperazine-2,5-dione | C ₄ H ₁₃ N ₂ O ₂ | <i>Penicillium chrysogenum</i> MITCC 5108* | <i>Porteresia coarctata</i> (Roxb.) Tateoka | Raceae | 14-16 mm zone inhibition against <i>Vibrio cholerae</i> at 10 µg/disk | Devi et al., 2012 | |
| 86 | Beauvericin | C ₅ H ₁₇ N ₃ O ₉ | <i>Fusarium redolens</i> DstZ | <i>Wrightia zingiberensis</i> C.H. | Dioscoreaceae | 26-6-18.4 µg/mL MIC against <i>A. tumefaciens</i> , <i>X. vesicatoria</i> , <i>B. subtilis</i> , <i>S. haemolyticus</i> | Xu et al., 2010 | |
| 87 | Enniatin A1 | C ₅ H ₆ N ₂ O ₉ | <i>Fusarium trichinum</i> / <i>Bacillus subtilis</i> 168 tPC2 (co-culture) | <i>Aristolochia paucifloris</i> Pomel | Aristolochiaceae | 8-2 µg/mL MIC against <i>B. subtilis</i> , <i>S. aureus</i> , <i>S. pneumoniae</i> and <i>E. faecalis</i> , MRSA | Ola et al., 2013 | |
| 88 | Enniatin B | C ₅ H ₁₇ N ₃ O ₉ | <i>Fusarium trichinum</i> Salicorni 19 | <i>Salicornia bigelovii</i> Torr. | Amaranthaceae | 13-6 µM MIC against <i>B. subtilis</i> , <i>E. aerogenes</i> , <i>M. tetragenus</i> | Zhang et al., 2014 | |

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| 89 | Emmatin B1 | C ₃₄ H ₅₀ N ₆ O ₉ | <i>Fusarium trichinctum/Bacillus subtilis</i> 168 rPC2 (co-culture) | <i>Aristolochia paucifloris</i> Pomel <i>Panax notoginseng</i> (Burkhill) F. H. Chen <i>Panax notoginseng</i> (Burkhill) F. H. Chen | Aristolochiaceae Araliaceae | 16-4 µg/mL MIC against <i>B. subtilis</i> , <i>S. aureus</i> , <i>S. pneumoniae</i> and <i>E. faecalis</i> , MRSA 5.4 and 14.00 µg/mL IC50 against <i>E. faecium</i> and <i>S. aureus</i> | Ola et al., 2013 Ding et al., 2012 | |
| 90 | Halobacillin | C ₃₃ H ₆₄ N ₄ O ₁₂ | <i>Trichoderma asperellum</i> | <i>Panax notoginseng</i> (Burkhill) F. H. Chen | Araliaceae | 7.30 and 19.02 µg/mL IC50 against <i>E. faecium</i> and <i>S. aureus</i> | Ding et al., 2012 | |
| 91 | PF1022F | C ₄₀ H ₆₈ N ₄ O ₁₂ | <i>Trichoderma asperellum</i> | <i>Panax notoginseng</i> (Burkhill) F. H. Chen | Araliaceae | | Ding et al., 2012 | |
| 92 | Tardioxopiperazine A | C ₂₄ H ₃₁ N ₅ O ₂ | <i>Eurotium cristatum</i> EN-220 | <i>Sargassum thunbergii</i> (Mertens ex Kuhn) Kunze | Sargassaceae (Algae) | 8 µg/mL MIC against <i>S. aureus</i> and <i>E. coli</i> | Du et al., 2012 | |
| Steroids | | | | | | | | |
| 93 | Helvolic acid | C ₃₃ H ₄₄ O ₈ | <i>Aspergillus</i> sp. CY725 | <i>Gynodon dactylon</i> (L.) Pers. | Peaceae | 5.0 µg/mL MIC against <i>H. pylori</i> ATCC 43504 | Song et al., 2005 | |
| Terpenoids | | | | | | | | |
| 94 | 1β-Hydroxy-α-cyperone | C ₁₅ H ₂₂ O ₂ | <i>Metrosphaeropsis arundinis</i> | <i>Ulmus macrocarpa</i> Hance | Ulmaceae | 11.4 µg/mL MIC against <i>S. aureus</i> | Luo et al., 2013 | |
| 95 | Conidigenol | C ₃₀ H ₃₄ O ₂ | <i>Penicillium chrysogenum</i> QEN-24S | <i>Laurencia</i> sp. | Rhodomeleaceae (Algae) | 16 µg/mL MIC against <i>P. fluorescens</i> , <i>S. epidermis</i> | Gao et al., 2011 | |
| 96 | Conidigenone B | C ₃₀ H ₃₀ O | <i>Penicillium chrysogenum</i> QEN-24S | <i>Laurencia</i> sp. | Rhodomeleaceae (Algae) | 8 µg/mL MIC against MRSA, <i>P. aeruginosa</i> , <i>P. fluorescens</i> , <i>S. epidermis</i> | Gao et al., 2011 | |
| 97 | Fusarelin B | C ₂₅ H ₄₀ O ₅ | <i>Fusarium trichinctum</i> Salicorn 19 | <i>Salicornia bigelovii</i> Torr. | Amaranthaceae | 19-10 µM MIC against <i>M. smegmatis</i> , <i>B. subtilis</i> , <i>M. phlei</i> , <i>E. coli</i> | Zhang et al., 2014 | |
| 98 | Fusariumin C | C ₃₁ H ₅₃ O ₃ | <i>Fusarium oxysporum</i> ZZP-R1* | <i>Rumex madia</i> Makino | Polygonaceae | 6.25 µg/mL MIC value against <i>S. aureus</i> ATCC 25923 | Chen et al., 2019 | |
| 99 | Fusartrin | C ₂₁ H ₄₀ O ₈ | <i>Fusarium trichinctum</i> Salicorn 19* | <i>Salicornia bigelovii</i> Torr. | Amaranthaceae | 19 µM MIC against <i>E. aerogenes</i> , <i>M. tetragenus</i> | Zhang et al., 2014 | |
| 100 | Periconin A | C ₃₀ H ₃₈ O ₃ | <i>Periconia</i> sp. OBW-15* | <i>Taxus cuspidata</i> Siebold & Zucc. | Taxaceae | 12.5-3.12 µg/mL MIC against <i>S. aureus</i> , <i>S. epidermis</i> , <i>B. subtilis</i> , <i>K. pneumoniae</i> , <i>S. typhimurium</i> | Kim et al., 2004 | |
| Other | | | | | | | | |
| 101 | 3-nitropropionic acid | C ₃ H ₅ NO ₄ | <i>Phanerochaete longicolla</i> | <i>Trichilia elegans</i> A. Juss. | Meliaceae | 2.3-1.1 mm zone inhibition against <i>Xanthomonas axonopodis</i> , <i>Micrococcus luteus</i> , <i>Salmonella typhi</i> | Flores et al., 2013 | |

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| ID | Compound names | Chemical Formula | Endophytic fungal strain | Host plant | Botanical Family | Parasite strain | Activity | References |
|--------------------|---|--|--|---|---|---|---|------------------------|
| Alkaloids | | | | | | | | |
| 102 | 18-Deoxy-19,20-epoxycytochalasin C | C ₃₀ H ₃₇ NO ₆ | <i>Nematia</i> sp. UM10M | <i>Torreya taxifolia</i> Arn. | Taxaceae | <i>P. falciparum</i> (D6 and W2) | IC50 0.56 μM (280 ng/mL) in D6; 0.19 μM (100 ng/mL) in W2 | Kumarhany et al., 2019 |
| 103 | 19,20-Epoxycytochalasin C | C ₃₀ H ₃₇ NO ₇ | <i>Nematia</i> sp. UM10M | <i>Torreya taxifolia</i> Arn. | Taxaceae | <i>P. falciparum</i> (D6 and W2) | IC50 0.07 μM (37 ng/mL) in D6; 0.05 μM (28 ng/mL) in W2 | Kumarhany et al., 2019 |
| 104 | 19,20-Epoxycytochalasin D | C ₃₀ H ₃₇ NO ₇ | <i>Nematia</i> sp. UM10M | <i>Torreya taxifolia</i> Arn. | Taxaceae | <i>P. falciparum</i> (D6 and W2) | IC50 0.04 μM (22 ng/mL) in D6; 0.04 μM (20 ng/mL) in W2 | Kumarhany et al., 2019 |
| 105 | 2,5-Dihydroxy-1-(hydroxymethyl)pyridin-4-one | | <i>Diaporthe</i> sp. | <i>Guapira standleyana</i> Woodson | Nyctaginaceae | <i>P. falciparum</i> 3D7 | IC50 136 nM (0.136 μM) | Calcut et al., 2013 |
| 106 | 7-Hydroxy-3,4,5-trimethyl-6-on 2,3,4,6-tetrahydroisoquinoline-8-carboxylic acid | C ₁₅ H ₁₅ O ₅ N | Strain BB4* | <i>Timospora erispa</i> (L.) Hook. f. & Thomson | Menispermaceae | <i>P. falciparum</i> 3D7 | IC50 0.02 μg/mL (0.127 μM) | Elifita et al., 2011 |
| 107 | Gisserin | C ₁₅ H ₁₅ O ₅ N | Strain BB4* | <i>Timospora erispa</i> (L.) Hook. f. & Thomson | Menispermaceae | <i>P. falciparum</i> 3D7 | IC50 0.03 μg/mL (0.129 μM) | Elifita et al., 2011 |
| 108 | Cytochalasin D | C ₂₃ H ₃₁ NO ₅ | <i>Prevuscia</i> sp. | <i>Enantia chlorantha</i> Oliv. | Amaraceae | <i>P. falciparum</i> NF54 | IC50 10.3 μM | Talontsi et al., 2014 |
| 109 | Cytochalasin H | C ₃₀ H ₃₇ NO ₅ | <i>Diaporthe</i> sp. | <i>Kandelia obovata</i> Sheue, H.Y. Liu & J. Yong, <i>Avicennia marina</i> (Forssk.) Vietn., <i>Lumnitzera racemosa</i> Willd., <i>Kandelia obovata</i> Sheue, H.Y. Liu & J. Yong, <i>Avicennia marina</i> (Forssk.) Vietn., <i>Lumnitzera racemosa</i> Willd., <i>Lumnitzera racemosa</i> Willd., <i>Velozia gigantea</i> N. L. Menezes & Melo-Silva | Rhizophoraceae, Acanthaceae, Combretaceae | <i>P. falciparum</i> 3D7 | IC50 25.8 nM (0.0258 μM) | Calcut et al., 2013 |
| 110 | Cytochalasin J | C ₃₀ H ₃₇ NO ₄ | <i>Diaporthe</i> sp. | <i>Kandelia obovata</i> Sheue, H.Y. Liu & J. Yong, <i>Avicennia marina</i> (Forssk.) Vietn., <i>Lumnitzera racemosa</i> Willd., <i>Lumnitzera racemosa</i> Willd., <i>Lumnitzera racemosa</i> Willd., <i>Lumnitzera racemosa</i> Willd., <i>Velozia gigantea</i> N. L. Menezes & Melo-Silva | Rhizophoraceae, Acanthaceae, Combretaceae | <i>P. falciparum</i> 3D7 | IC50 <20 nM (<0.02 μM) | Calcut et al., 2013 |
| 111 | Cytochalasin O | C ₂₈ H ₃₇ NO ₄ | <i>Verticillium</i> sp. | <i>Kandelia obovata</i> Sheue, H.Y. Liu & J. Yong, <i>Avicennia marina</i> (Forssk.) Vietn., <i>Lumnitzera racemosa</i> Willd., <i>Lumnitzera racemosa</i> Willd., <i>Velozia gigantea</i> N. L. Menezes & Melo-Silva | Rhizophoraceae, Acanthaceae, Combretaceae | <i>P. falciparum</i> 3D7 | IC50 <20 nM (<0.02 μM) | Calcut et al., 2013 |
| 112 | Epoxycytochalasin H | C ₃₀ H ₃₉ NO ₅ | <i>Diaporthe mitricata</i> URM/GCB 9720 | <i>Kandelia obovata</i> Sheue, H.Y. Liu & J. Yong, <i>Avicennia marina</i> (Forssk.) Vietn., <i>Lumnitzera racemosa</i> Willd., <i>Lumnitzera racemosa</i> Willd., <i>Velozia gigantea</i> N. L. Menezes & Melo-Silva | Rhizophoraceae, Acanthaceae, Combretaceae | <i>P. falciparum</i> (D6 and W2) | IC50 51.70 ng/mL in D6 (0.1 μM) and 39.40 ng/mL in W2 (0.07 μM) | Ferreira et al., 2017 |
| Polycetides | | | | | | | | |
| 113 | 11-hydroxymonocerin | C ₆₁ H ₈₉ O ₇ | <i>Esserohium rostratum</i> * | <i>Stemona</i> sp. | Stemonaceae | <i>P. falciparum</i> K1 | IC50 7.70 μM | Sapapan et al., 2008 |
| 114 | 2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione | C ₈ H ₈ ClO ₃ | <i>Xylaria</i> sp. PB-30* | <i>Sandoricum koeijape</i> (Burm. f.) Merr. | Meliaceae | <i>P. falciparum</i> K1 | IC50 1.84 μM | Tansuwan et al., 2007 |
| 115 | 7-butyl-6,8-dihydroxy-3(R)-perilylisochroman-1-one | C ₁₈ H ₂₆ O ₄ | <i>Geotrichum</i> sp.* | <i>Crassecephalum erepidioides</i> (Benth.) S. Moore | Asteraceae | <i>P. falciparum</i> K1 | IC50 2.6 μg/mL (8.48 μM) | Kongsaree et al., 2003 |
| 116 | Altenuin | C ₁₅ H ₁₄ O ₆ | <i>Alternaria</i> sp. UFM/CGB55 | <i>Trixis vaudleri</i> DC. | Asteraceae | Inhibits trypanthione reductase | IC50 4.3 - 0.3 μM | Cota et al., 2008 |
| 117 | Asteric acid | C ₁₇ H ₁₆ O ₈ | <i>Prevuscia</i> sp. | <i>Enantia chlorantha</i> Oliv. | Amnonaceae | <i>P. falciparum</i> NF54 | IC50 8.67 μM | Talontsi et al., 2014 |
| 118 | Cercosporin | C ₂₉ H ₂₆ O ₁₀ | <i>Mycosphaerella</i> sp. nov. strain F2140 | <i>Psychotria horizontalis</i> Spreng. ex DC. | Rubiaceae | <i>L. donovani</i> , <i>P. falciparum</i> , <i>T. cruzi</i> | IC50 0.46 μM, 1.03 μM, 1.08 μM | Moreno et al., 2011 |
| 119 | Cercosporin derivative tetraacylated * | C ₂₇ H ₂₄ O ₁₄ | <i>Mycosphaerella</i> sp. nov. strain F2140* | <i>Psychotria horizontalis</i> Spreng. ex DC. | Rubiaceae | <i>L. donovani</i> , <i>P. falciparum</i> , <i>T. cruzi</i> | IC50 0.64 μM, 2.99 μM, 0.78 μM | Moreno et al., 2011 |

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|------------------------------|-------------------|--|---|--|---|---|---------------------------|-----------------------------|--|
| 120 | Chaetoxanthone B | C ₃₀ H ₄₈ O ₆ | <i>Chaetonium</i> sp* | <i>Algal species</i> | - | <i>P. falcatiparum</i> K1 | IC50 0.5 µg/mL (1.41 µM) | Pontius et al., 2008 | |
| 121 | Chaetoxanthone C | C ₃₀ H ₄₈ ClO ₆ | <i>Chaetonium</i> sp* | <i>Algal species</i> | - | <i>T. cruzi</i> C4 | IC50 1.5 µg/mL (3.83 µM) | Pontius et al., 2008 | |
| 122 | CJ-12,371 | C ₃₀ H ₄₆ O ₄ | <i>Ecleria</i> sp | <i>Persea volubilis</i> L. | Verbenaceae | <i>L. donovani</i> amastigotes | IC50 8.40 µM | Martinez-Luis et al., 2008 | |
| 123 | Cochliquinone A | C ₃₀ H ₄₈ O ₈ | <i>Cochliobolus</i> sp | <i>Pipradenia adamantoides</i> (Spreng.) J.F. Macbr. <i>Kandelia obovata</i> Sheue, H.Y. Lu & J. Yong, <i>Artemisia maritima</i> (Torresk.) Vethf., <i>Lumnitzera racemosa</i> Willd. | Fabaceae Rhizophoraceae, Acanthaceae, Combrataceae | <i>L. amazonensis</i> promastigotes | EC50 1.7 µM | Campos et al., 2008 | |
| 124 | Dicranolol D | C ₃₄ H ₅₂ O ₁₅ | <i>Diaporthe</i> sp. * | | | <i>P. falcatiparum</i> 3D7 | IC50 600 nM (0.6 µM) | Calcul et al., 2013 | |
| 125 | Fumiquinone B | C ₈ H ₈ O ₅ | <i>Aspergillus</i> sp. F1544 | <i>Guapira standleyana</i> Woodson | Nyctaginaceae | <i>L. donovani</i> ; <i>P. falcatiparum</i> | IC50 0.5 µM, 5.4 µM | Martinez-Luis et al., 2012 | |
| 126 | Isoochliquinone A | C ₃₀ H ₄₈ O ₈ | <i>Cochliobolus</i> sp | <i>Pipradenia adamantoides</i> (Spreng.) J.F. Macbr. | Fabaceae | <i>L. amazonensis</i> promastigotes | EC50 4.1 µM | Campos et al., 2008 | |
| 127 | KS-501a | C ₃₃ H ₄₈ O ₁₀ | <i>Acremonium</i> sp. BCC 14080 | Palm tree | Araceae | <i>P. falcatiparum</i> K1 | IC50 9.9 µM | Bunyapaboonsri et al., 2008 | |
| 128 | Mollicellin E | C ₃₂ H ₄₈ ClO ₈ | <i>Chaetonium brasiliense</i> | Hala-Bala evergreen forest | - | <i>P. falcatiparum</i> K1 | IC50 3.2 µg/mL (7.16 µM) | Khunmkomkhet et al., 2009 | |
| 129 | Mollicellin K | C ₃₃ H ₄₈ O ₇ | <i>Chaetonium brasiliense</i> * | Hala-Bala evergreen forest | - | <i>P. falcatiparum</i> K1 | IC50 1.2 µg/mL (3.13 µM) | Khunmkomkhet et al., 2009 | |
| 130 | Mollicellin L | C ₃₂ H ₄₈ O ₇ | <i>Chaetonium brasiliense</i> * | Hala-Bala evergreen forest | - | <i>P. falcatiparum</i> K1 | IC50 3.4 µg/mL (8.6 µM) | Khunmkomkhet et al., 2009 | |
| 131 | Mollicellin M | C ₃₃ H ₄₇ ClO ₇ | <i>Chaetonium brasiliense</i> * | Hala-Bala evergreen forest | - | <i>P. falcatiparum</i> K1 | IC50 2.9 µg/mL (6.95 µM) | Khunmkomkhet et al., 2009 | |
| 132 | Monocerin | C ₁₆ H ₂₆ O ₆ | <i>Esseverhodium rostratum</i> | <i>Stemona</i> sp | Stemoniaceae | <i>P. falcatiparum</i> K1 | IC50 0.68 µM | Sappapan et al., 2008 | |
| 133 | Palmarumycin CP17 | C ₃₀ H ₄₄ O ₅ | <i>Ecleria</i> sp* | <i>Persea volubilis</i> L. | Verbenaceae | <i>L. donovani</i> amastigotes | IC50 1.34 µM | Martinez-Luis et al., 2008 | |
| 134 | Palmarumycin CP18 | C ₃₀ H ₄₄ O ₅ | <i>Ecleria</i> sp* | <i>Persea volubilis</i> L. | Verbenaceae | <i>L. donovani</i> amastigotes | IC50 0.62 µM | Martinez-Luis et al., 2008 | |
| 135 | Palmarumycin CP2 | C ₃₀ H ₄₄ O ₄ | <i>Ecleria</i> sp | <i>Persea volubilis</i> L. | Verbenaceae | <i>L. donovani</i> amastigotes | IC50 3.93 µM | Martinez-Luis et al., 2008 | |
| 136 | Phomoxanthone A | C ₃₈ H ₅₈ O ₁₆ | <i>Phanopsis</i> sp. BCC 1323 | <i>Tectona grandis</i> L.f. | Lamiaceae | <i>P. falcatiparum</i> K1 | IC50 0.11 µg/mL (0.15 µM) | Isaka et al., 2001 | |
| 137 | Phomoxanthone B | C ₃₈ H ₅₈ O ₁₆ | <i>Phanopsis</i> sp. BCC 1323 | <i>Tectona grandis</i> L.f. | Lamiaceae | <i>P. falcatiparum</i> K1 | IC50 0.33 µg/mL (0.44 µM) | Isaka et al., 2001 | |
| 138 | Preussisafuran A | C ₁₈ H ₁₆ O ₇ | <i>Preussia</i> sp. * | <i>Enantia chlorantha</i> Oliv. | Amnaceae | <i>P. falcatiparum</i> NF54 | IC50 8.76 µM | Talontsi et al., 2014 | |
| 139 | Preussomerin EGI | C ₃₀ H ₄₂ O ₆ | <i>Ecleria</i> sp | <i>Persea volubilis</i> L. | Verbenaceae | <i>L. donovani</i> amastigotes | IC50 0.12 µM | Martinez-Luis et al., 2008 | |
| 140 | Purpureone | C ₃₂ H ₅₀ O ₁₄ | <i>Purpureocellium thiacinum</i> * | <i>Ranvolfia macrophylla</i> Ruiz & Pav. <i>Sandoricum koetjape</i> (Burm. f.) Merr. | Apocynaceae | <i>L. donovani</i> amastigotes | MIC 0.63 µg/mL (0.87 µM) | Lenta et al., 2016 | |
| 141 | Xylariaquinone A | C ₁₅ H ₁₂ O ₅ | <i>Xylaria</i> sp. PB-30* | | Melastaceae | <i>P. falcatiparum</i> K1 | IC50 6.68 µM | Tansuwan et al., 2007 | |
| Polyketides-alkaloids | | | | | | | | | |
| 142 | 14-Norpsseutin A | C ₃₁ H ₅₂ NO ₈ | <i>Aspergillus</i> sp. F1544 | <i>Guapira standleyana</i> Woodson | Nyctaginaceae | <i>L. donovani</i> | IC50 4.4 µM | Martinez-Luis et al., 2012 | |
| 143 | Codinaeopsin | C ₃₃ H ₄₈ NO ₃ | <i>Codinaeopsis gonyrrhichoides</i> CR127A* | <i>Tochystia guatemalensis</i> Donn. Sm. | Vochysiaceae | <i>P. falcatiparum</i> 3D7 | IC50 2.3 µg/mL (4.7 µM) | Kontnik et al., 2008 | |
| 144 | FD-838 | C ₃₂ H ₄₂ NO ₇ | <i>Aspergillus</i> sp. F1544 | <i>Guapira standleyana</i> Woodson | Nyctaginaceae | <i>L. donovani</i> | IC50 0.2 µM | Martinez-Luis et al., 2012 | |

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| 145 | Pseurotin A | C ₂₂ H ₂₈ N ₈ | <i>Aspergillus</i> sp. F1 544 | <i>Guapiha standleyana</i> Woodson | Nyctaginaceae | <i>L. donovani</i> | IC50 5.8 µM | Martinez-Luis et al., 2012 | |
| 146 | Pseurotin D | C ₂₂ H ₂₈ N ₈ | <i>Aspergillus</i> sp. F1 544 | <i>Guapiha standleyana</i> Woodson | Nyctaginaceae | <i>L. donovani</i> | IC50 0.5 µM | Martinez-Luis et al., 2012 | |
| Polypeptides | | | | | | | | | |
| 147 | 12,12a-dihydroantibiotic PI 016 | C ₃₁ H ₄₈ N ₂ O ₅ S ₂ | <i>Metsporopsis theobromae</i> BCC 3975* | Seed plant | - | <i>P. falciparum</i> K1 | IC50 2.95 µM | Chinworungsee et al 2006 | |
| 148 | Apicidin B | C ₃₃ H ₄₇ N ₅ O ₆ | <i>Fusarium pallidoreseum</i> * | <i>Acacia</i> sp. | Fabaceae | <i>P. falciparum</i> | MIC 1.89 nM (0.189 µM) | Singh et al., 2001 | |
| 149 | Apicidin C | C ₃₃ H ₄₇ N ₅ O ₆ | <i>Fusarium pallidoreseum</i> * | <i>Acacia</i> sp. | Fabaceae | <i>P. falciparum</i> | MIC 69 nM (0.069 µM) | Singh et al., 2001 | |
| 150 | Beauvericin | C ₄₅ H ₅₇ N ₃ O ₉ | <i>Fusarium</i> sp. [KF611679] | <i>Caesalpinia echinata</i> Lam. | Fabaceae | <i>T. cruzi</i> | IC50 2.43 µM | Campos et al., 2015 | |
| 151 | Fusaripeptide A | C ₆₄ H ₇₅ N ₃ O ₁₁ | <i>Fusarium</i> sp. * | <i>Mentha longifolia</i> (L.) L. | Lamiaceae | <i>P. falciparum</i> (DC6) | IC50 0.34 µM | Ibrahim et al., 2017 | |
| 152 | Pullularin A | C ₂₃ H ₃₇ N ₅ O ₉ | <i>Pullularia</i> sp. BCC 8613* | <i>Catolophyllum</i> sp. | Clusiaceae | <i>P. falciparum</i> K1 | IC50 3.6 µg/mL (4.64 µM) | Isaka et al., 2007 | |
| 153 | Pullularin B | C ₂₃ H ₃₅ N ₅ O ₉ | <i>Pullularia</i> sp. BCC 8613* | <i>Catolophyllum</i> sp. | Clusiaceae | <i>P. falciparum</i> K1 | IC50 3.3 µg/mL (4.18µM) | Isaka et al., 2007 | |
| Terpenoids | | | | | | | | | |
| 154 | 12,13-Deoxyortidin E | C ₂₉ H ₃₈ O ₇ | CY-3923 (not identified) | <i>Kandelia obovata</i> Sheue, H.Y. Liu & I. Yong, <i>Avicennia marina</i> (Forssk.) Vietn., <i>Lumnitzera racemosa</i> Willd. | Rhizophoraceae, Acanthaceae, Combrataceae | <i>P. falciparum</i> 3D7 | IC50 <20 nM (<0.02 µM) | Caloul et al, 2013 | |
| 155 | 7α,10α-Dihydroxy-1β-methoxyeremophil-11(13)-en-12,8β-olide | C ₁₆ H ₂₄ O ₅ | <i>Xylaria</i> sp. BCC 21097* | <i>Licuala spinoxa</i> Wurnb | Arceaceae | <i>P. falciparum</i> K1 | IC50 8.1 µM | Isaka et al., 2010 | |
| 156 | Integracide H | C ₉ H ₁₄ O ₇ | <i>Fusarium</i> sp* | <i>Mentha longifolia</i> (L.) L. | Lamiaceae | <i>L. donovani</i> amastigotes | IC50 4.75 µM | Ibrahim et al., 2016 | |
| 157 | Integracide J | C ₉ H ₁₄ O ₇ | <i>Fusarium</i> sp* | <i>Mentha longifolia</i> (L.) L. | Lamiaceae | <i>L. donovani</i> amastigotes | IC50 3.29 µM | Ibrahim et al., 2016 | |
| 158 | Phomarcherin B | C ₃₃ H ₂₈ O ₅ | <i>Phomopsis archeri</i> | <i>Vanilla albidia</i> Blume | Ochnaceae | <i>P. falciparum</i> K1 | IC50 0.79 µg/mL (2.05 µM) | Hernstein et al., 2011 | |
| 159 | Rordim E | C ₂₉ H ₃₈ O ₈ | CY-3923 (not identified) | <i>Kandelia obovata</i> Sheue, H.Y. Liu & I. Yong, <i>Avicennia marina</i> (Forssk.) Vietn., <i>Lumnitzera racemosa</i> Willd. | Rhizophoraceae, Acanthaceae, Combrataceae | <i>P. falciparum</i> 3D7 | IC50 <20 nM (<0.02 µM) | Caloul et al, 2013 | |

* First time isolation of the compound from the fungal strain

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