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Louis Bornancin

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THÈSE

Pour obtenir le grade de
Docteur

Délivré par **Université de Montpellier**

Préparée au sein de l'école doctorale
Sciences Chimiques Balard

Et de l'unité de recherche
**Centre de Recherche Insulaire et Observatoire de
l'Environnement (USR CNRS-EPHE-UPVD 3278)**

Spécialité : **Ingénierie des Biomolécules**

Présentée par **Louis BORNANCIN**

**Lipopeptides from Cyanobacteria :
Structure and Role in a Trophic Cascade**

Soutenue le 11 octobre 2016 devant le jury composé de



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Avant propos

Ce mémoire de doctorat est rédigé en anglais sous forme de thèse sur publications, publications acceptée (chapitre 3), à soumettre (chapitres 2 et 4) ou en préparation (chapitre 5). De ce fait les parties "Matériel et Méthodes" et les références bibliographiques sont associées à chaque chapitre.

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List of Abbreviations

At	<i>Anabaena cf torulosa</i>
DAD	Diode Array Detector
DMSO	Dimethyl sulfoxide
ELSD	Evaporative Light Scattering Detector
HMBC	Heteronuclear Multiple-Bond Connectivity
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single-Quantum Connectivity
LC-MS	Liquid Chromatography – Mass Spectrometry
Lm	<i>Lyngbya majuscula</i>
MDF	Mantle Dermal Formation
NMR	Nuclear Magnetic Resonance
NRPS	Non-Ribosomal Peptide Synthases
PKS	PolyKetide Synthases
ROESY	Rotating-frame Overhauser Effect Spectroscopy
RP HPLC	Reverse Phase High Performance Liquid Chromatography
TMS	Tetramethylsilane
TOCSY	Total Correlation Spectroscopy

Amino acids

Three letter code	Name
Ade	β -aminodecanoic acid
Ala	Alanine
Aoc	β -aminooctanoic acid
Asn	Asparagine
Dhb	α,β -didehydro- α -aminobutyric acid
Glp	Pyroglutamate acid
Glu	Glutamine
Gly	Glycine
Has	3-hydroxyasparagine
Hle	3-hydroxyleucine
Hmoaa	3-hydroxy-2-methyloct-7-anoic acid
Hmoea	3-hydroxy-2-methyloct-7-enoic acid
Hmoya	3-hydroxy-2-methyloct-7-ynoic acid
Hse	Homoserine
Htn	3-Hydroxy-threonine
Hyp	4-hydroxy-proline
Ile	Isoleucine
Leu	Leucine
N-Melle	<i>N</i> -methylisoleucine
N-MeVal	<i>N</i> -methylvaline
Phe	Phenylalanine
Pla	3-phenyllactic acid
Pro	Proline
Ser	Serine
Thr	Threonine
Tyr	Tyrosine
Val	Valine

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Chapter 1. General Introduction

Marine chemical ecology is an interdisciplinary science that has recently emerged in the last few decades and which aims to shed light on the role of chemistry in maintaining marine biodiversity. The study of marine biodiversity has led to the discovery of an immense diversity of marine natural products which has picqued the curiosity of chemists. Organisms such as sponges, algae, tunicates, bryozoans or cyanobacteria are among the greatest marine producers of secondary metabolites. Chemists were first interested in investigating new organic backbones, innovative biosynthetic pathways, and the biological activities of these novel compounds, mainly for pharmacological purposes. Later on, the ecological function of these compounds began to captivate chemists. Ecologists have always studied the interactions between and within species, but whether they were chemically mediated eluded them¹. Recently, chemists and ecologists have begun working together, discovering that some molecules, previously considered to have no function or to only have a function in chemical defense, are key to more complex interactions. Similarly, animal behaviors commonly studied by ecologists, such as mating, settlement or prey selection, appear to be chemically mediated. Currently, chemicals are known to be involved in defense against pathogens or generalist consumers, allelopathy, antifouling, feeding specializations, settlement or metamorphosis, and mating, as well as more complex interactions involving more than two species which thus have cascading affects on communities and even ecosystems.

Cyanobacteria are classified as a monophyletic phylum within the domain of Bacteria and represent a wide group of photoautotrophic prokaryotes. Cyanobacteria are photosynthetic organisms, sometimes nitrogen-fixing, and show a great tolerance to extreme and fluctuating conditions enabling them to adapt to a broad range of habitats. Moreover, this flexibility is a formidable asset for outcompeting eukaryotic algae or corals.

In the lagoon of Moorea in French Polynesia, *Lyngbya majuscula* and *Anabaena cf torulosa* are two benthic filamentous cyanobacteria that can proliferate across a wide sandy area and even on corals. Both species constitute prolific producers of secondary metabolites, mainly cyclic lipopeptides², which may either be toxic or act as feeding deterrents to potential consumers. *L. majuscula* is known for its extensive blooms found worldwide throughout the tropics and subtropics and for producing compounds involved in dermatitis and intoxication in humans, as well as causing other animal health problems^{3,4}.



Figure 1.1. Cyanobacteria blooms in the lagoon of Moorea (Left: *Lyngbya majuscula*, Right: *Anabaena cf torulosa*)

Among cyanobacteria, *L. majuscula* is the species that produces the greatest diversity of secondary metabolites, though the genus *Lyngbya* might need a taxonomic revision⁵. Indeed, phylogenetically different species but that share a similar morphology, might have been misidentified. Nevertheless, the production of secondary metabolites by *L. majuscula* remains impressive. In Moorea, *L. majuscula* mainly express the cyclic depsipeptides tiahuramides A-C⁶, while the closely-related trungapeptins A-C⁷, as well as the serinols 4a and 4b⁸ have been detected. Tiahuramides and trungapeptines are cyclic heptadepsipeptides containing a methyl hydroxyoctynoic acid residue and are part of a twenty seven compound family including antanapeptins A-D⁹, radamamide B¹⁰, hantupeptins A-C^{11,12}, veraguamides A-J^{13,14}, naopeptin¹⁵ and kulomo'Opunalides 1-2¹⁶ isolated from the cyanobacteria *Lyngbya majuscula*, *Symploca cf hydroides*, *Oscillatoria margaritifera*, *Moorea* sp. and the mollusc *Philinopsis speciosa*¹⁷. On the other hand, *A. cf torulosa* produces the cyclic lipopeptides laxaphycins A, B and B3¹⁸. Laxaphycins belong to a super family that includes the laxaphycin-A type sub-family which are undecapeptides, while the laxaphycin-B type sub-family are dodecapeptides, both sub-families with usual and non-proteinogenic amino acids such as the rare β -amino acid with an aliphatic side chain ranging from six (Aoc) to eight (Ade) carbons. Members of the laxaphycin-A type sub-family include laxaphycin A^{18,19}, hormothamnin A²⁰, laxaphycin E, lobocyclamide A²¹, scytocyclamide A²², trichormamides A²³ and D²⁴ produced by *Anabaena cf torulosa*, *Anabaena laxa*, *Hormothamnion enteromorphoides*, *Lyngbya confervoides*, *Scytonema hofmanni*, *Trichormus* sp. and *Oscillatoria* sp.. As regards the laxaphycin-B type subfamily, laxaphycins B, B2, B3, and D^{18,19}, lobocyclamides B and C²¹, trichormamides B²³ and C²⁴ and lyngbyacyclamides A and B²⁵ are produced by *Anabaena laxa*, *A. torulosa*, *Lyngbya confervoides*, *Trichormus* sp., *Oscillatoria* sp. and *Lyngbya* sp.².

Despite the putative repellent properties of their secondary metabolites, both cyanobacteria are consumed by mollusc herbivores. The cephalaspidea *Bulla orientalis* and the sea hare *Stylocheilus striatus* were observed feeding upon *L. majuscula*. Although *S. striatus* is considered to be a *L. majuscula* specialist, we also found it feeding on *A. cf*

torulosa along with *S. longicauda*. Interestingly, the nudibranch *Gymnodoris ceylonica*, a voracious feeder of *S. striatus*, and the crab *Thalamita coeruleipes*, that preys on mollusc species, were only found on *L. majuscula* (Fig. 1).

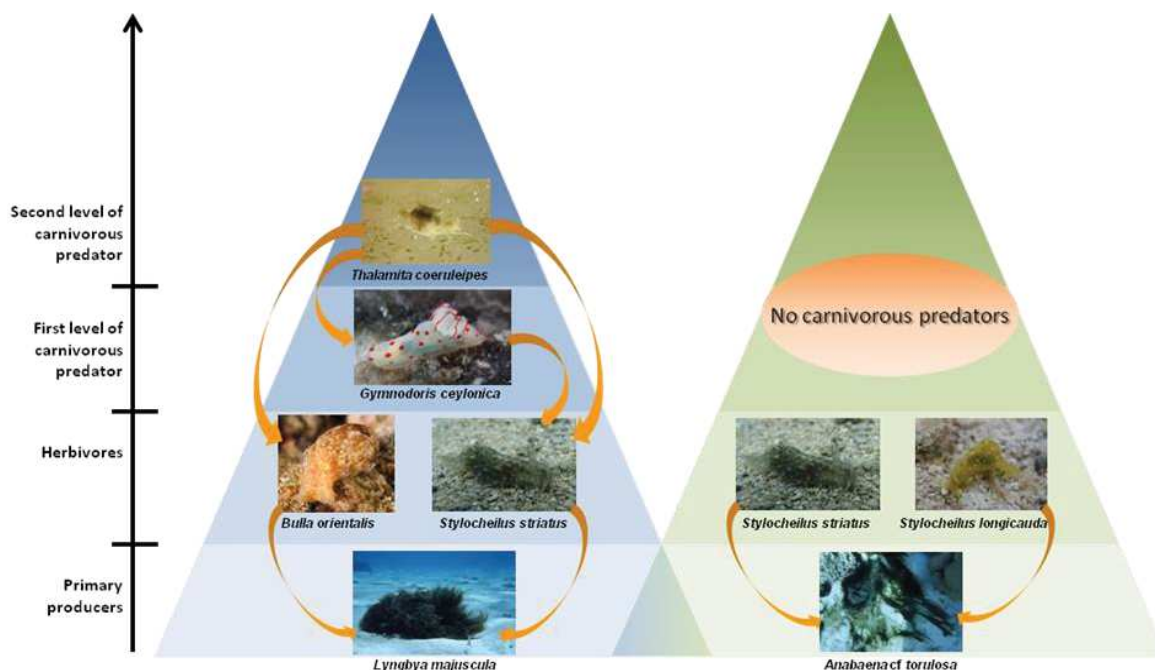


Figure 1. 2. Trophic interactions between primary producers, herbivorous molluscs and carnivorous predators

The aim of this thesis is to study the cascading effects of chemical mediators in multi-trophic relations, the sequestration and/or biotransformation of secondary metabolites acquired from dietary sources and the chemical recognition mechanisms in inter-specific relationships. To meet these objectives, it was first essential to have a thorough understanding of the secondary metabolites produced by the primary producers, the cyanobacteria *L. majuscula* and *A. cf. torulosa*.

This thesis is therefore structured as follows:

- Chapter 2 constitutes a bibliographic review of chemically mediated interactions between marine gastropods and their prey. Chapter 2 constitutes a review recently submitted to *Natural Product Reports*.

- In order to determine the complete metabolic profile of the cyanobacteria, we focused our attention on the chemical content of *A. cf. torulosa* and chapter 3 and 4 describe the isolation of new acyclic and cyclic laxaphycins from this species. Chapter 3 is part of an article published in 2015 (*Marine Drugs*, 2015, 13, 7285–7300). Chapter 4 will be soon submitted with the biological activities of the new laxaphycins.

- Chapter 5 focuses on the fate of cyanobacterial secondary metabolites along the trophic chain and their role in the ecosystem introduced above. Several questions were

raised by these two main topics. Regarding the fate of secondary metabolites acquired from cyanobacteria along the trophic chain we asked:

- Are the secondary metabolites produced by the cyanobacteria horizontally and vertically transmitted?

- If sequestration and/or biotransformation occur in molluscs what role does it play (detoxification, defense)?

- If sequestration occur, can the location of the sequestered metabolites inside the mollusc provide an indication of their role?

- Are the secondary metabolites produced by the cyanobacteria used as chemical cues, or signals, by the molluscs for food tracking or feeding choice?

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Chapter 2. Chemical Mediation as a Structuring Element in Marine Gastropod Predator-Prey Interactions

Abstract

Chemical mediation governs interactions between species and thus entire ecosystems. Marine gastropods are a well-diversified group of molluscs found worldwide, are slow-moving and often unprotected, and have therefore developed defense mechanisms to survive. Chemically defended prey such as algae, sponges, tunicates, bryozoans and cyanobacteria, constitute an important opportunity for molluscs either to enjoy the shelter they provide from predation pressure, or to steal and enhance their defensive weapons. In addition to defense, prey secondary metabolites are also used in complex chemical communication for prey detection, feeding preferences, settlement induction and their assimilation further provides the opportunity for interactions with conspecifics via diet-derived chemical cues or signals. This review intends to provide an overview of chemically mediated interactions between marine gastropods and their prey.

2.1. Introduction

Natural selection imposed by predators, pathogens and competitors has led to the evolution of chemical, physical/mechanical, and phenological defenses in organisms^{1,2}. In terms of chemical defenses, an enormous variety of adaptive chemical compounds exist, including those that ward off, inhibit or kill potential herbivores, are antimicrobial that kill viruses, bacteria, fungi, and still others that are allelopathic by suppressing competitors³⁻⁵. These compounds, known as secondary metabolites, are small molecules with no known function in the primary metabolism of the organisms that produce them⁶. In general, the use of secondary metabolites to deter predators has important implications for the success of individuals and populations. Moreover, in addition to facilitating escape from predators, secondary metabolites may mediate a wide range of other behaviors, such as finding prey, mating with suitable partners or interacting with congeners⁷. Chemicals are well known to influence intra- and inter-specific interactions as well as in shaping the structure of entire ecosystems⁸⁻¹⁰. Chemical communication therefore constitutes one of the most important languages used by Nature.

The multiple roles of chemicals are widespread in terrestrial systems. Some chemicals are repellent against predators but attractant to conspecifics. For instance, beetles emit secondary metabolites that defend them from potential predators and are used as intraspecific sex pheromones¹¹. However, the role of chemicals in structuring marine ecosystems is less well studied despite their invaluable function, such as their role in coral reef resilience. The multi-species interactions in which gobies defend Acroporid corals from

allelopathic algae, is one example of how chemical communication and defense underlie coral reef resilience. The responses of both the coral and fish are mediated by chemical signals and cues¹². It is not by accident that corals, sessile organisms, are armed with such highly evolved chemical defenses.

The majority of sessile organisms, unable to escape the pressures from other organisms, have evolved adaptive traits in order to protect them from predators, pathogens or competitors. In marine systems, primary producers such as cyanobacteria or algae, as well as other sessile animals such as corals, sponges, bryozoans or tunicates, are known to biosynthesize a broad range of different compounds that have cascading effects across trophic levels and shape communities^{13–15}. The defenses of these chemically defended organisms are on the whole adaptive, except to certain predators which have developed strategies of chemical-resistance, and even use chemical cues to locate their sessile prey. For example, while chlorodesmin produced by the seaweed *Chlorodesmis fastigiata* deters feeding by most fish species, it strongly stimulates feeding by the specialist crab *Caphyra rotundifrons*¹⁶. The use of chemical defenses that stimulate feeding by a specific predator, are known to influence specialist, rather than generalist, predator-prey interactions.

Another taxon that has developed strategies of chemical-resistance, but also the use of chemical cues to locate their sessile prey, are gastropods¹⁷. Marine gastropods are slow-moving, often unprotected (soft-bodied) benthic snails, and as such, strong selection pressures have led to the development of defense mechanisms enabling them to increase their chances of survival. Furthermore, in addition to their restricted vision, marine gastropods often live in environments where visual information is limited, but where chemical information abounds and they have evolved to use such information to their advantage. Herbivorous marine gastropods are able to consume chemically defended prey, such as the primary producers cyanobacteria and macroalgae. Similarly, carnivorous gastropods consume chemically defended herbivores or filter-feeding chemically-defended sessile invertebrates such as sponges, bryozoans or tunicates. Therefore, within their sphere of perception, marine gastropods must select useful chemical cues from the chemical noise in their surrounding smellscape.

The class Gastropoda is the most diversified class in the phylum Mollusca, with 60,000–80,000 snail and slug species and whose taxonomy is still under revision^{17,18}. Heterobranchia is a taxonomic clade of snails and slugs, which includes marine, aquatic and terrestrial gastropod molluscs. Jörger et al.¹⁷ have redefined major groups within the Heterobranchia. We will use the Jörger et al. classification for Heterobranchia and the classification of Bouchet & Rocroi¹⁸ for non-Heterobranchia gastropods.

Numerous publications have concentrated on either the sequestration and biotransformation of diet-derived compounds or on the role of prey secondary metabolites in foraging or settlement of marine gastropods, but rarely has data on both been synthesized together. Here we provide an integrative review of the role of secondary

metabolites in gastropod-prey interactions focusing on (i) the sequestration of secondary metabolites, (ii) the detoxification and biotransformation of secondary metabolites, and (iii) the role of secondary metabolites as chemical cues in foraging and settlement.

For all figures, we adopted a color code related to sequestration, biotransformation, feeding stimulation, settlement/metamorphosis induction and olfactory attraction (Figure 2. 1). Moreover, the molecules numbering is relating to the order of their appearance in the text.

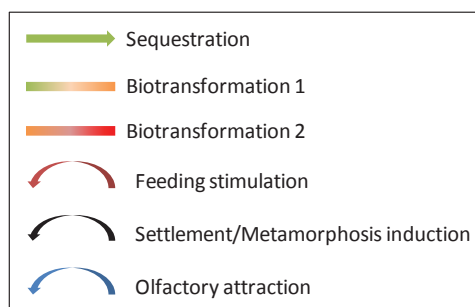


Figure 2. 1. Color code adopted for all figures

2.2. Gastropods capable of sequestering diet-derived chemicals

The role of secondary metabolites as a chemical defense strategy of algae, sponges, bryozoans, tunicates or cyanobacteria, has been widely studied^{19,20}. However, many consumers have developed counteradaptations that enable them to feed on chemically-defended prey without apparent negative effects. This evolutionary adaptation by terrestrial and marine species involves the development of mechanisms to process certain chemicals in order to tolerate prey secondary metabolites and even use them as an effective defense by sequestering and/or excreting them. Here we discuss the ways in which gastropods have become adapted to feeding on a particular chemically-defended diet by storing, concentrating and excreting diet-derived compounds. We also describe a few occasions of gastropods biosynthesising secondary metabolites *de novo* themselves.

2.2.1. Sequestration of diet-derived chemicals by sacoglossans

Sacoglossan mesograzers (Gastropoda, Heterobranchia, Euthyneura, Nudipleura, Euheterobranchia, Panpulmonata), a group of heterobranch molluscs, have a wide geographical distribution, being present in the majority of shallow tropical and temperate marine environments worldwide. They are generally cryptic and known to have a specific feeding habit: feeding suctorially and almost exclusively on the cell sap of macroalgae from the phylum Chlorophyta²¹. Interestingly, primitive species are shelled (Subclade Oxynoacea) and feed only upon the siphonalean green algal genus *Caulerpa*, while the more evolved species are shell-less (Subclade Plakobranchacea) and are found to feed on various algal genera²¹⁻²³. Both shelled, as well as the more primitive shell-less, sacoglossans are

kleptoplasts, having the ability to sequester functional chloroplasts with relatively high longevity from photosynthetic organelles in the absence of the original algal nucleus which enables the mollusc to be photosynthetic and fix carbon^{24,25}. *Elysia timida*, *E. chlorotica*, *E. clarki*, *Oxynoe viridis* and *Costasiella ocellifera* are known to store chloroplasts from their algal food via selective digestion so that digestive enzymes do not harm the chloroplasts. Furthermore, shelled species appear to acquire additional defense by sequestering secondary metabolites from their algal prey. Some shell-less species also concentrate algal secondary metabolites, and sometimes take this defense one step further by biotransforming them, while others are able to biosynthesize *de novo* toxic polypropionates²⁶⁻²⁸.

The Mediterranean shelled sacoglossan *Oxynoe panamensis*²⁹, specialist of the green algae, *Caulerpa* sp., is able to sequester four compounds that show toxic activity against mice and rats³⁰. Caulerpicin C-24 **1**, palmitic acid **2**, β -sitosterol **3** and caulerpin **4** are in fact more concentrated in the mollusc than in the original food, indicating a bioaccumulation effect (Fig. 2). Although when irritated or molested the sacoglossan mollusc secretes an astringent milky mucus that is toxic to predatory fish, none of the four accumulated algal compounds have been found in this secretion³¹. Other shelled sacoglossans such as *Oxynoe olivacea* found on *Caulerpa prolifera* and *Lobiger souverbiei* found on *C. racemosa* sequester the toxic molecules caulerpenyne **5** (Figure 2. 2) and caulerpin **4** (Figure 2. 2) respectively²⁴. Interestingly, it can be noticed that caulerpin **4** and caulerpenyne **5** are two different compounds since the former is an alkaloid, probably a tryptophan dimer, while the latter is a sesquiterpene. However, the presence of these compounds in subsequent defense is not confined to shelled species.

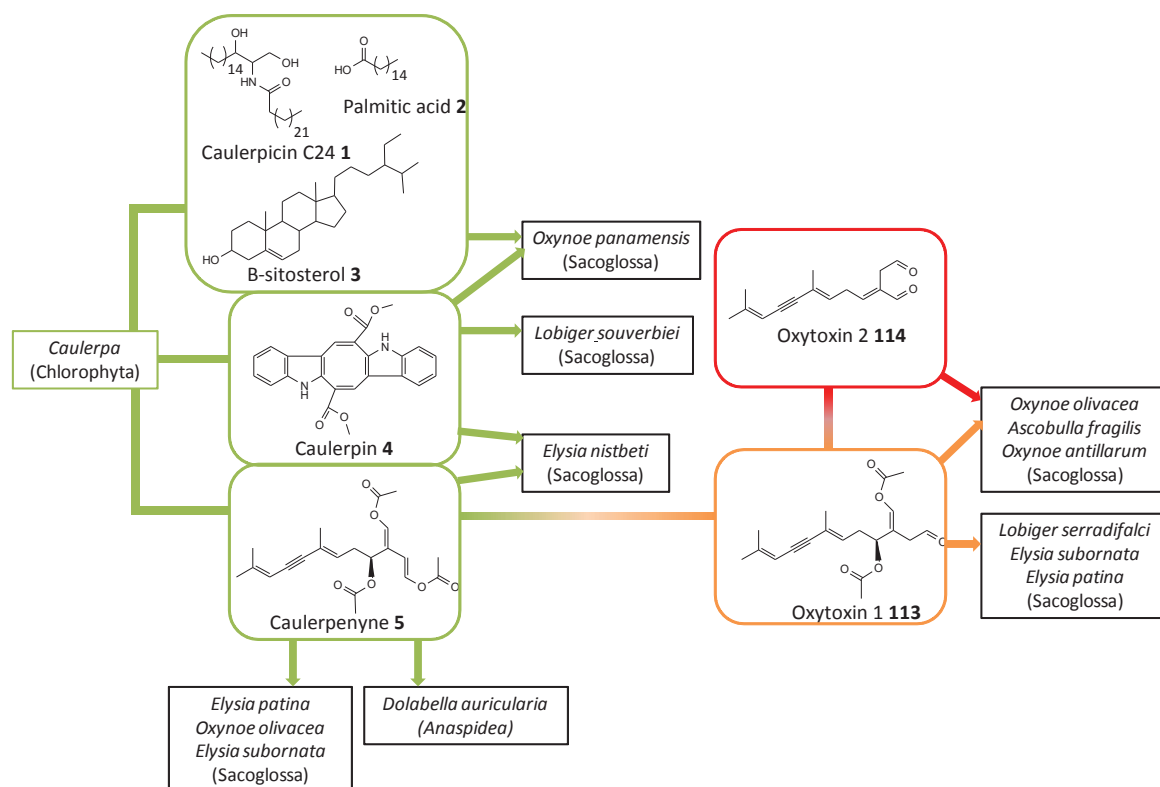


Figure 2. 2. Sequestration of algal secondary metabolites by *Oxynoe panamensis*, *Lobiger souverbiei*, *Elysia nistbeti*, *E. patina*, *O. olivacea*, *E. subornata* and *Dolabella auricularia* and biotransformation by *O. olivacea*, *Ascobulla fragilis*, *O. antillarum*, *L. serradifalci*, *E. subornata* and *E. patina*

Gastropods of the shell-less *Elysia* genera are often specialists of green algae. For example, the shell-less *Elysia translucens* that feeds upon *Udotea petiolata* and the shell-less *Bosellia mimetica* upon *Halimeda tuna*, store secondary metabolites from their algal food²⁸. *E. translucens* sequesters udoteal **6** (Figure 2. 3), while *B. mimetica* accumulates halimedatetraacetate **7** (Figure 2. 3), however the compounds do not show any ichthyotoxicity. Shell-less *Elysia* genera are also often found on *Halimeda* species, such as *Elysia tuca* that feeds on *Halimeda incrassata*. Besides the fact that *E. tuca* accumulates the diet-derived fish deterrent halimedatetraacetate **7**^{32,33} (Figure 2. 3), which confers it a chemical defense, the mollusc is also able to acquire chloroplasts from the algae^{34,35}. These combined strategies enable *Elysia* to photosynthesize and be cryptic and certainly increase its chances of survival.

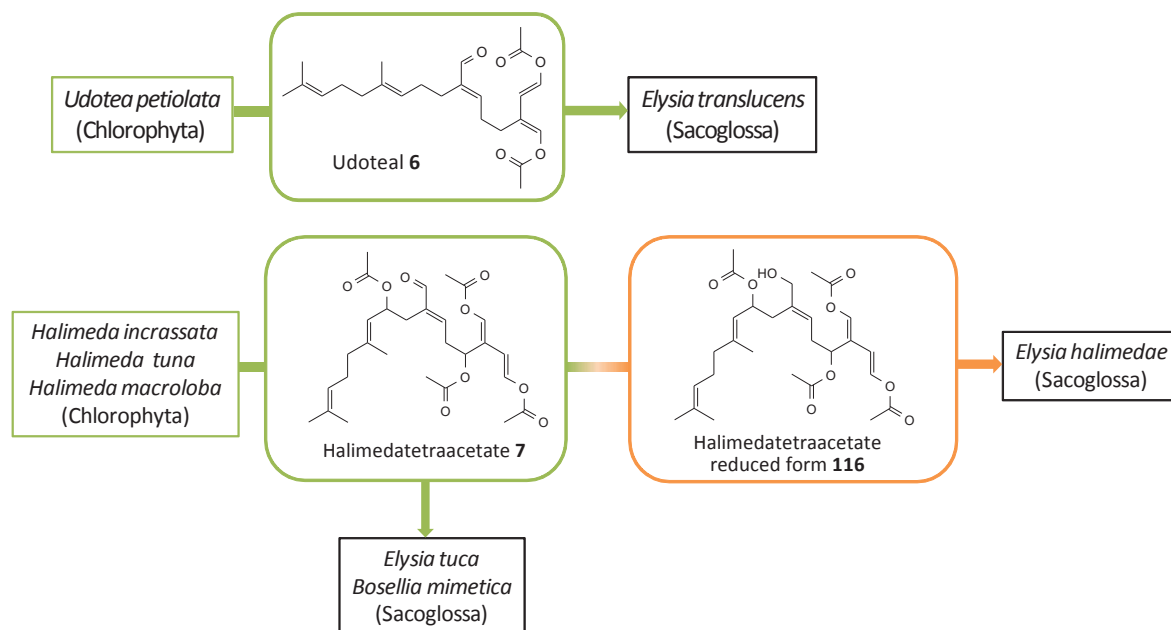


Figure 2. 3. Sequestration of algal secondary metabolites by *Elysia translucens*, *E. tuca* and *Bosellia mimetica* and biotransformation carried out by *E. halimeda*

Similarly, the shell-less *Elysia patina* and *Elysia subornata* reared on *C. racemosa* store caulerpenyne 5 (Figure 2. 2), while *E. nistbeti* found on the same species is able to sequester caulerpin 4 as well as caulerpenyne 5 (Figure 2. 2). However, in these examples, storage of these molecules is considered a chemical defensive strategy, in particularly for *E. subornata* in which caulerpenyne 5 constitutes the main component of the defensive mucus secretion. Furthermore, the shell-less *Elysia rufescens* feeds upon *Bryopsis* sp. and accumulates the algal secondary metabolite kahalalide F 8 (Figure 2. 4). The depsipeptide, present in mucus secretions, is cytotoxic against several cancer cell lines and a deterrent against reef fish which confers an effective defense to the mollusc^{36,37}. Kahalalides A 9, B 10, G 11 and K 12 are also produced by *Bryopsis* sp. and sequestered by *E. rufescens*, although the ecological functions have not been investigated³⁸⁻⁴⁰. Similarly, the presence of kahalalide O 13 has been detected both in *Elysia ornata* from Hawaii and *Bryopsis* sp., while kahalalide F is also present in *Elysia grandifolia*^{41,42}. However, the origin of kahalalides is unclear since kahalalide F has also been isolated from *Vibrio* sp. and the molluscs could acquire kahalalide-producing bacteria from the surface of *Bryopsis* sp. and retain them as symbionts^{43,44}.

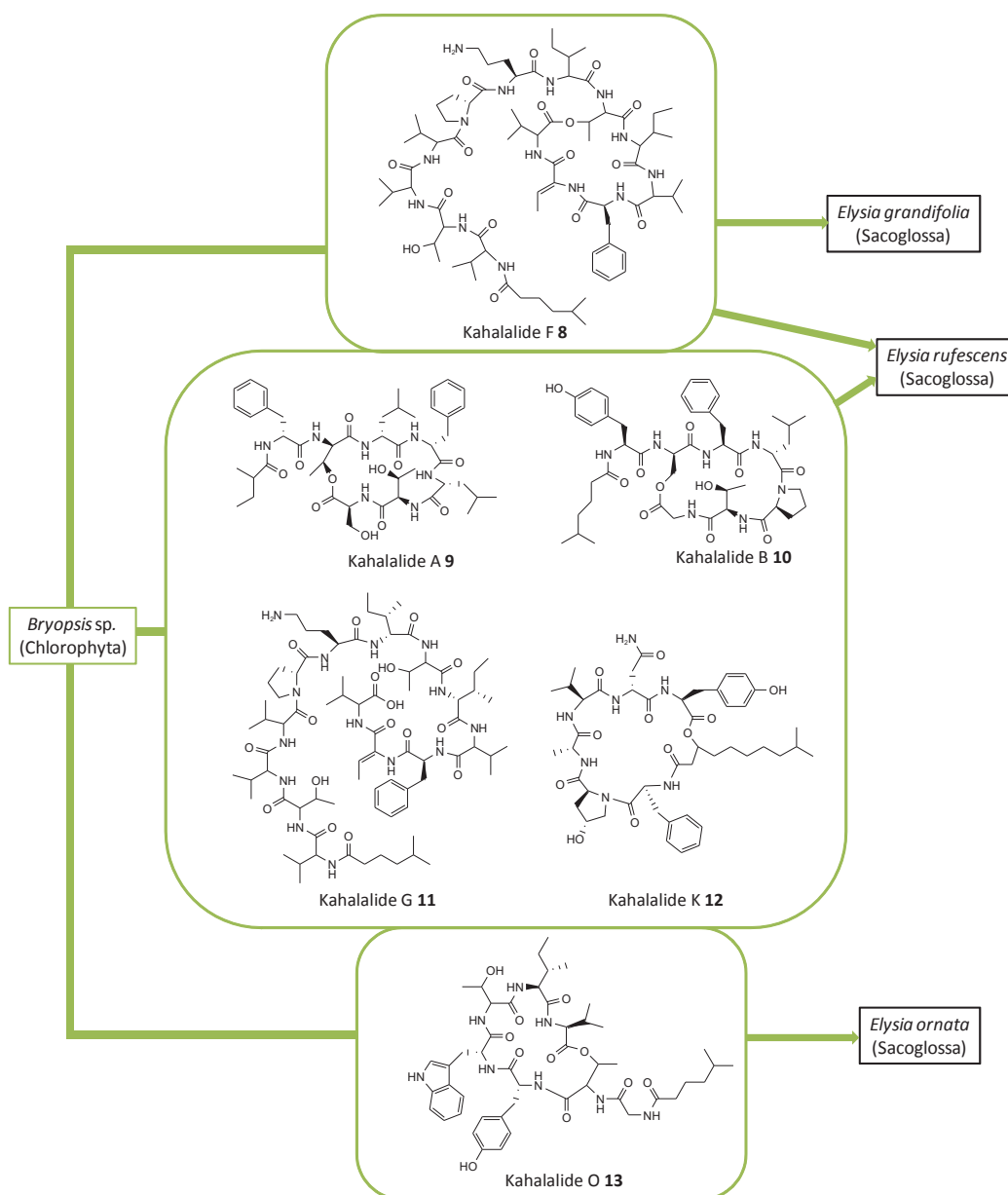


Figure 2. 4.. Sequestration of algal secondary metabolites by *Elysia grandifolia*, *E. rufescens* and *E. ornata*

Furthermore, the shell-less *Costasiella ocellifera* specifically consumes the chlorophyceae *Avrainvillea longicaulis*⁴⁵. Avrainvilleol **14**^{33,46}, a brominated diphenylmethane, is the main secondary metabolite produced by this green algae. The compound is toxic to reef fish and induces feeding avoidance behavior in the herbivorous damselfish, *Pomacentrus coeruleus*. Therefore, as *C. ocellifera* stores avrainvilleol **14** it may acquire an effective defense against predatory fishes (Figure 2. 5).

In addition, the shell-less gastropod *Mourgona germaineae* has developed an interesting defense mechanism in response to predator aggression⁴⁷. Some heterobranch molluscs possess cerata, dorsal and lateral excrescences on the upper body. *Mourgona germaineae* responds to a predatory attack by secreting a mucus and autotomizing cerata. The toxic secretion used in this defense is a non-fully identified water-soluble toxin produced

by the algae *Cymopolia barbata* and transferred to the specialist heterobranch during feeding⁴⁷.

However, some carnivorous predators are able to circumvent the defense strategies acquired by herbivores. The cytotoxic diterpenoid chlorodesmin **15**, which is the major secondary metabolite of the seaweed *Chlorodesmis fastigiata*, is a fish deterrent and confers an effective chemical defense to the algae³². However, it does not protect it from herbivory by two specialist herbivores, the shell-less *Elysia* sp. and *Cyerce nigricans*. Furthermore, although *Elysia* sp. and *C. nigricans* sequester chlorodesmin **15** (Figure 2. 5), *Gymnodoris* sp. is a specialized carnivorous predator on *Elysia* sp. indicating that chlorodesmin **15** does not affect the dorid nudibranch either. The diterpenoid is only found in small amounts in *C. nigricans*, which uses aposematism by displaying conspicuous color and biosynthesizing *de novo* toxic polypropionate compounds^{48,49} as alternative and efficient defense strategies.

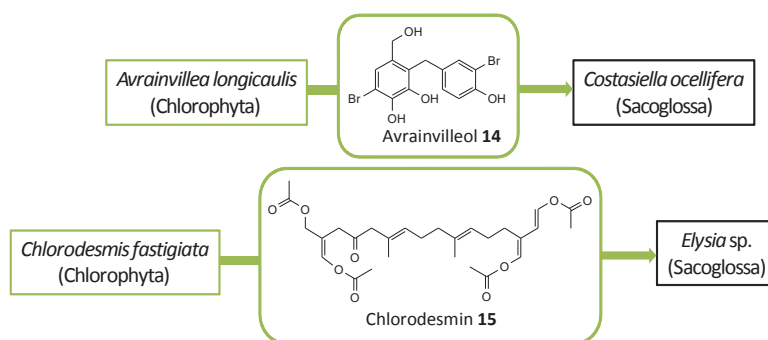


Figure 2. 5. Sequestration of algal secondary metabolites by *Costasiella ocellifera* and *Elysia* sp.

2.2.2. Sequestration of diet-derived chemicals by nudibranchs

Nudibranchia (Gastropoda, Heterobranchia, Euthyneura, Nudipleura) are a group of soft-bodied marine gastropod molluscs that shed their shells after their larval stage. They occur in seas worldwide, and counter to sacoglossans, which are herbivorous and generally cryptic, nudibranchs are carnivorous and are well known for their conspicuous colors and use of mimicry⁵⁰. Cryptic species, such as sacoglossans, emit information that is normally uninteresting for predators, rendering them difficult to locate and affording them safety from predation. This phenomenon may include a predator and its prey sharing the same color pattern or prey sharing the same color pattern as their habitat⁵¹ (Figure 2. 6). On the other hand, mimetic species, such as nudibranchs, emit cues of interest to a potential predator, either of an attractive or repellent nature. Species showing visual signals, such as conspicuous colors, coupled with an associated unpalatability are considered to form Mullerian mimicry complexes⁵² and could be associated with aposematism^{53,54} (Figure 2. 6). In contrast, species emitting a similar visible signal to another species, but lacking toxicity, show Batesian mimicry. This strategy consists of resembling a toxic species, using a similar color pattern and benefitting from reduced predation, without the associated costs of toxicity⁵⁵. Considering the reliance of mimicry on toxicity, and their conspicuous colors, we

would predict the sequestration of secondary metabolites to be common in nudibranch Mullerian, but not Batesian mimics.



Figure 2. 6. Left: the cryptic sacoglossan *Oxynoe olivacea* (credits: Enric Madrenas). Right: the aposematic nudibranch *Hexabranchnus sanguineus* (credits: Jason Jue)

Several examples of nudibranchs using conspicuous colors associated with toxicity have been described^{50,56}. Predatory fishes avoid prey that exhibit visual cues; for example, yellow, purple and green nudibranchs repel the bluehead wrasse, *Thalassoma bifasciatum*⁵⁷. In addition, the mummichog *Fundulus heteroclitus*, avoids unpalatable nudibranchs after tasting a single individual⁵⁸. In many cases, nudibranchs acquire toxicity by storing diet-derived metabolites. This sequestration generally occurs in specialized glands located on exposed parts of the body, such as mantle dermal formations (MDFs) and provides them with an effective chemical defense^{50,59,60}. The Chromodorid nudibranch, *Glossodoris pallida*, bioaccumulates two diet-derived diterpenoids, scalaradial **16** and desacetylscalaradial **17** from the sponges *Hyrtios erecta* and *Cacospongia* sp., and concentrates them in their MDFs^{61,62} (Figure 2. 7).

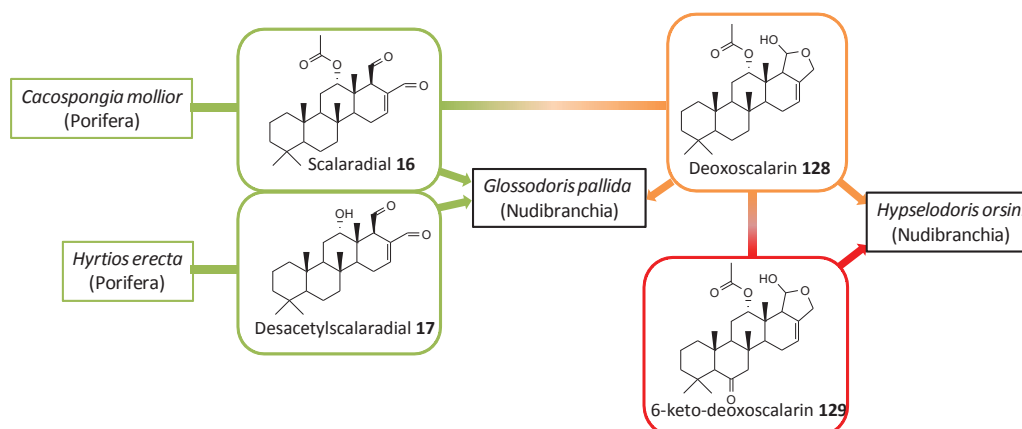


Figure 2. 7. Sequestration of sponge secondary metabolites by *Glossodoris pallida* and biotransformation carried out by *G. pallida* and *Hypselodoris orsini*

Similarly, *Hypselodoris webbi* sequesters seven sesquiterpenoids also in its MDFs, from three sponges, *Dysidea fragilis*, *Pleraplysilla spinifera* and *Microciona toxystila*⁵⁹ (Figure 2. 8). *H. webbi* stores Spiniferin **18** and **219** from *P. spinifera*, microcionins **1-4 20-23** from *M.*

toxystila and (-)-furodysin **24** from *D. fragilis* (Figure 2. 8). *H. webbi* rapidly transfers all of the sesquiterpenoids from the sponges to their MDFs⁵⁹.

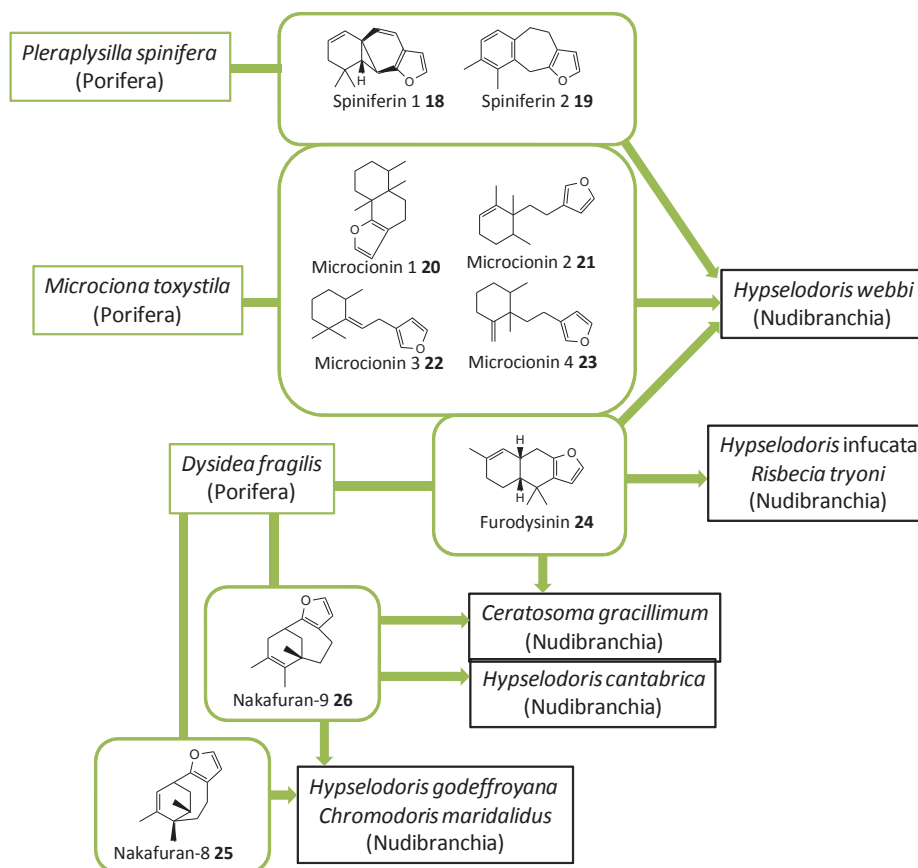


Figure 2. 8. Sequestration of sponge secondary metabolites by *Hypselodoris webbi*, *H. infucata*, *Risbecia tryoni*, *Ceratosoma gracillimum*, *H. cantabrica*, *H. godeffroyana* and *Chromodoris maridalidis*

Although nakafuran-8 **25** and nakafuran-9 **26** (Figure 2. 8) produced by the sponge *Dysidea fragilis* show anti-feeding activities, and confer protection against predators, such as the common reef fishes *Chaetodon* spp.⁶³, the nudibranch *Hypselodoris cantabrica* is able to circumvent this defense and bioaccumulate nakafuran-9 **26** in its MDFs. Furthermore, these sesquiterpenes are found at a higher concentration than in the sponge, indicating that the heterobranch is more protected than its prey⁶⁴. Two other nudibranchs, *Hypselodoris godeffroyana* and *Chromodoris maridalidis* are also able to store nakafuran-8 **25** and nakafuran-9 **26** from *D. fragilis*.

In order to determine whether the site of metabolite sequestration is important for nudibranch defense, six species of the chromodorid family were dissected into four parts including inner organs, mantle tissue devoid of MDFs, MDFs and dissection residuals⁴⁶. The deterrent activities of eight diet-derived terpenoids and their crude extracts were then determined for each body part using the general shrimp, *Palaemon elegans*⁶⁰. *P. elegans* is a potential predator of chromodorid nudibranchs, and in trials using artificial, and chemically unprotected nudibranchs sculpted from squid muscle, they preferentially attacked the edges of the model's mantle⁶⁰. These sites correspond to the location of MDFs in live nudibranchs

and which, on attack, would release high concentrations of repellent chemicals⁶⁰. We would therefore expect nudibranchs to sequester secondary metabolites in their MDFs, the most accessible and preferred part of the body to predators. Indeed, all six nudibranchs accumulate all but one of the eight terpenoids in their MDFs. *Chromodoris sinensis* and *Hypselodoris* sp. accumulate aplyroseol-2 **27** and the highly deterrent (+)-tetrahydrofurospingin-1 **28** respectively at high concentrations in their MDFs (Figure 2. 9). *Hypselodoris infucata* and *Risbecia tryoni* sequester (-)-furodysin **24** in their MDFs (Figure 2. 8) and these compounds show significant deterrent feeding activity even at lower concentrations than those found in the MDFs. Nakafuran-9 **26** and (-)-furodysin **24** are also found at high concentrations in the MDFs of *Ceratosoma gracillimum* (Figure 2. 8). However, interestingly, *Glossodoris atomarginata* accumulates the two deterrent terpenoids spongiatrioltriacetate **29** and spongiatriol-diacetate **30** in MDFs, while spongiatriol **31**, that does not show any deterrent activity, is only found in the mantle and viscera. This finding suggests that *G. atomarginata* selectively accumulates closely related secondary metabolites from sponges in different locations of its body as a function of their capacity as a feeding deterrent⁶⁰.

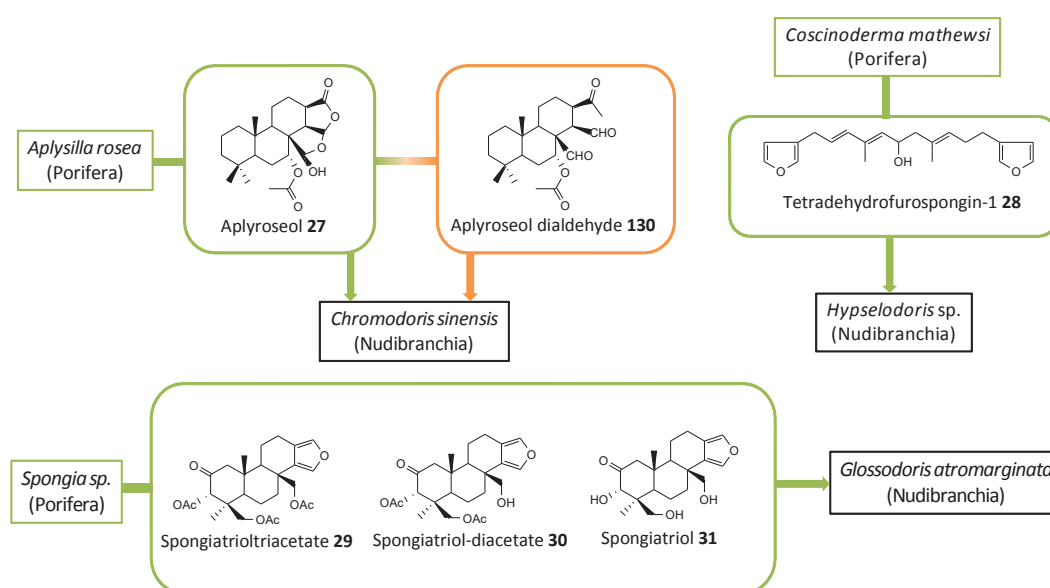


Figure 2. 9. Sequestration of sponge secondary metabolites by *Chromodoris sinensis*, *Hypselodoris* sp. and *Glossodoris atomarginata* and biotransformation carried out by *C. sinensis*

This strategy would be an effective defense mechanism, facilitating the release of toxic chemicals by preferentially storing them near the surface⁶². On the other hand, it is not unlikely that these molecules are stored in external tissues to avoid autotoxicity^{60,62} and MDFs could originally have had a purification function, similar to the role of the kidney, that later evolved into a defensive mechanism⁶⁵.

However, *Hypselodoris fontandraui* lacks MDFs yet has a similar color pattern to that of other *Hypselodoris* species that possess MDFs⁴⁰. To determine if this species lacks a chemical defense and thus uses Batesian mimicry as a defense mechanism, individuals were

studied histologically and chemically. The furanosesquiterpenoid tavacpalllescensin **32**, the main compound of a *Dysidea* sponge genus, was isolated from *H. fontandraui* and was found four times more concentrated along the mantle border than in other external parts and twenty times more concentrated than in inner parts (Figure 2. 10). This metabolite, also present in mucus secretions, repels the shrimp *P. elegans*. In addition, histological studies revealed structures with a granular component in the body wall, just below the border of the mantle whose function is comparable to those of MDFs⁵⁵. Therefore, *H. fontandraui* is indeed chemically defended and uses structures other than MDFs to store secondary metabolites, proving that it uses aposematic Mullerian mimicry similar to other *Hypselodoris* species.

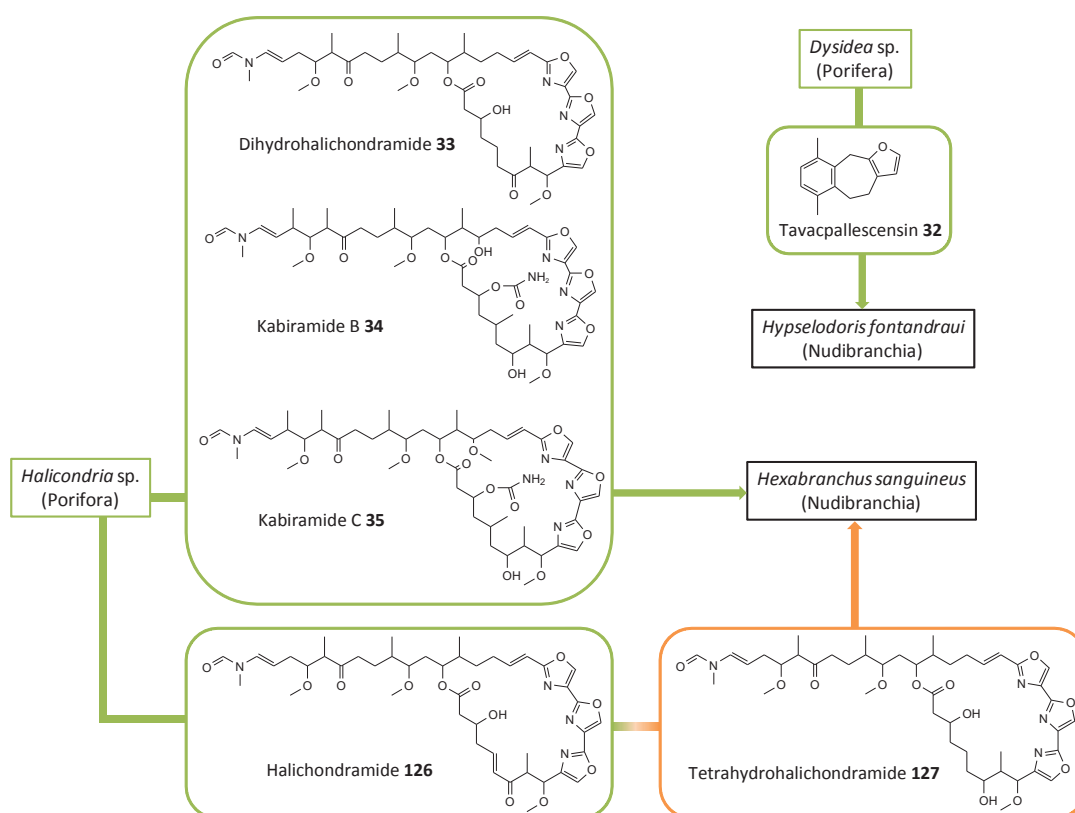


Figure 2. 10. Sequestration of sponge secondary metabolites by *Hypselodoris fontandraui* and *Hexabranchnus sanguineus* and biotransformation carried out by *H. sanguineus*

Nudibranch don't sequester only compounds from the terpene family as *Hexabranchnus sanguineus* sequesters three macrolides dihydrohalichondramide **33**, kabiramides B **34** and C **35** from two *Halichondria* sponges (Figure 2. 10), which are particularly concentrated in the dorsal mantle tissue, the most vulnerable part of the body and deters predation from the reef fish *Thalassoma lunare*. However, the metabolites are also sequestered in the digestive and gonad gland, which are in turn transferred to the eggs of the sea slug, an example of vertical transmission⁶⁶. As such, the deterrent activity of the metabolites provides both the adult nudibranch and its eggs, with chemical defenses.

Several diet-derived terpenoids have also been isolated from the dorsum of the generalist nudibranch *Cadlina luteomarginata*^{67,68}. Furodysin **36**, furodysin **24**, microcionin **2 21**, albicanol **37**, albicanyl acetate **38** and luteone **39** are found in the external parts of *C. luteomarginata* (Figure 2. 11). The former three are certainly produced by sponges, while the origin of the latter three remains unclear. However, no histological studies have been carried out to determine whether *C. luteomarginata* possesses MDFs or granular structures.

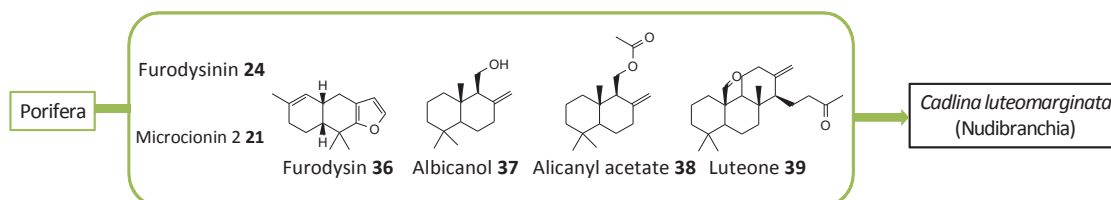


Figure 2. 11. Sequestration of sponge secondary metabolites by *Cadlina luteomarginata*

Metabolite sequestration has been found in four other nudibranch species based on chemical studies of the entire body, but the specific location is not known. *Anisodoris nobilis* sequesters N-methylnucleoside doridosine **40** originating from the sponge *Tedania digitata*⁶⁹ (Figure 2. 12) and *Phyllidia varicosa* stores the two isomer terpenoids 2-isocyanopupukeanane **41** and 9-isocyanopupukeanane **42**⁷⁰ (Figure 2. 12). Similarly, *Peltodoris atromaculata* accumulates unnamed polyacetylenes produced by the sponge *Petrosia ficiformis*⁷¹. Finally, the deterrent secondary metabolite heteronemin **43**, produced by the sponge *Hyrtios erecta*, is accumulated by *Glossodoris hikuensis* and *G. cincta*^{61,72} (Figure 2. 12).

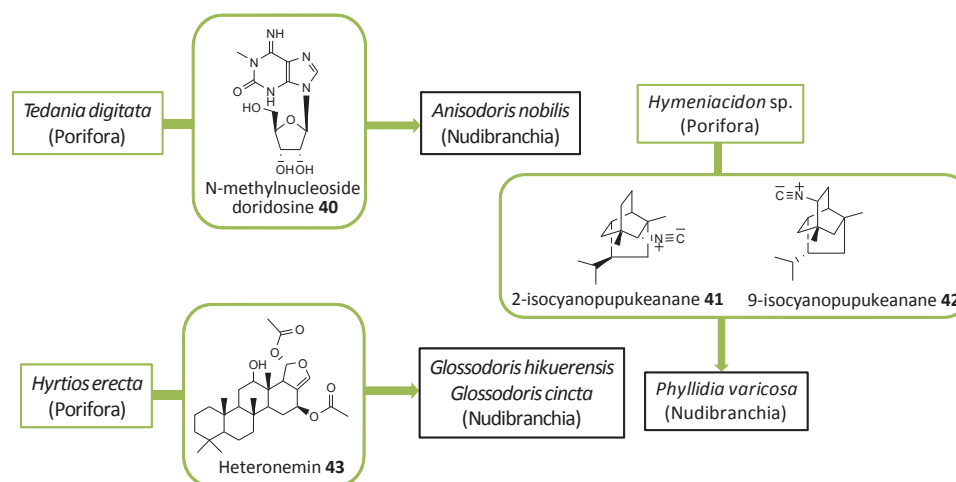


Figure 2. 12. Sequestration of sponge secondary metabolites by *Phyllidia varicosa*, *Anisodoris nobilis*, *Glossodoris hikuensis* and *G. cincta*

Nudibranchs not only sequester metabolites from sponges, but also from bryozoans and ascidians. *Tambja abdere* and *T. eliora* feed upon the bryozoan *Sessibugula translucens*⁷³ (Figure 2. 13). In turn, *Roboastra tigris*, another nudibranch, preys upon *T. abdere* and *T. eliora* and the alkaloids tambjamines A-D **44-47**, present in the bryozoan are sequestered by all three nudibranchs. As tambjamines deter feeding by the spotted kelpfish *Gibbonsia*

elegans, there is evidence for the transmission of both metabolites and the defense mechanism across two trophic levels. During an attack by *R. tigris*, *T. abdere* secretes a distasteful mucus containing deterrent chemicals which may cause the predator to abandon its attack, while *T. eliora* attempts to escape by swimming away. The cytotoxic tambjamine K **48** has also been isolated from the nudibranch *Tambja ceutae*, but appears to originate from the bryozoan *Bugula dentata* which contains this tambjamine in small amounts⁷⁴ (Figure 2. 13). Interestingly, tambjamins are also present in ascidians. The nudibranch *Nembrotha* spp., acquires tambjamins C **46**, E **49** and F **50** from its diet, the ascidian *Atapozoa* sp.⁷⁵ (Figure 2. 13). Whether tambjamins are biosynthesized by a common microorganism rather than a bryozoan or an ascidian is still being investigated.

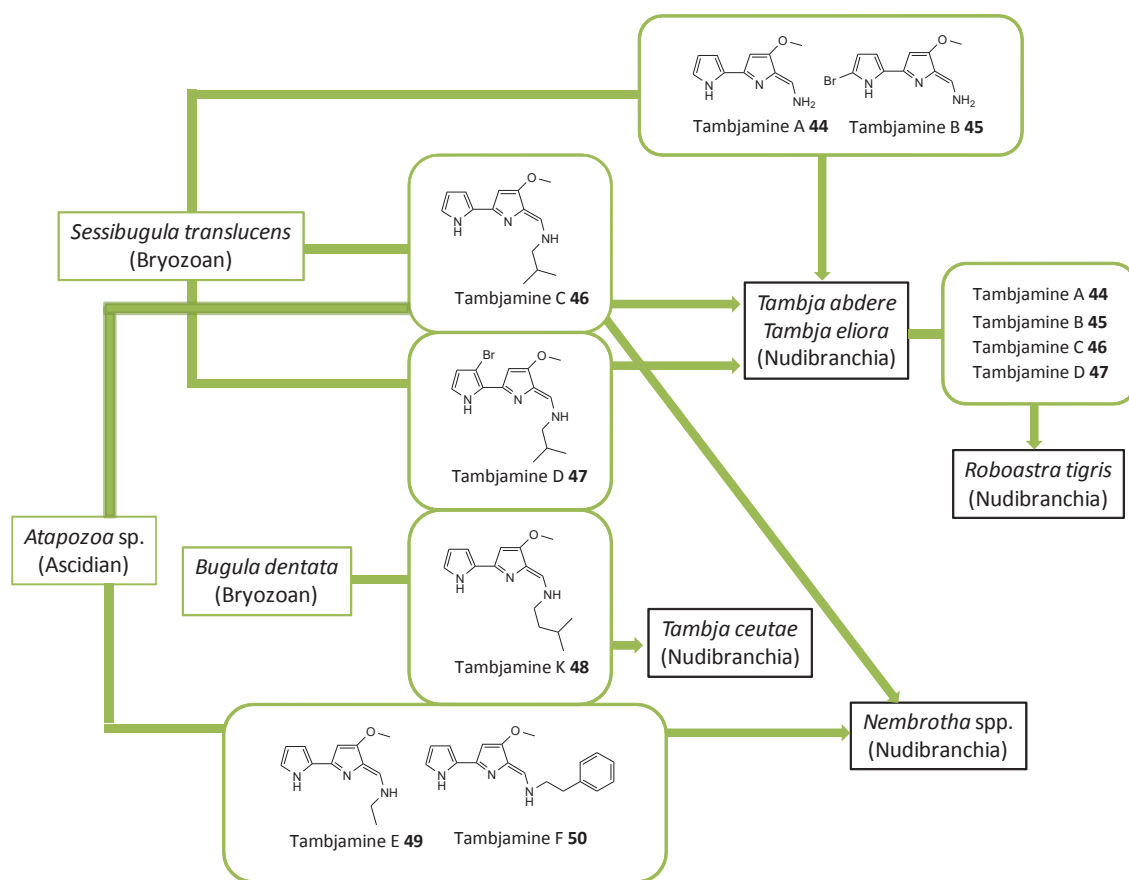


Figure 2. 13. Sequestration of bryozoan and ascidian secondary metabolites by *Tambja abdere*, *T. eliora*, *Roboastra tigris*, *T. ceutae* and *Nembrotha* spp.

Finally nudibranchs, in addition to sequestering secondary metabolites are also able to biosynthesize them *de novo*. The omnivorous nudibranch *Bathydoris hodgsoni* could be able to biosynthesize *de novo* hodgsonal **51** (Figure 2. 14), a sesquiterpene found to repel the sea urchin *Odontaster validus*^{76,77}. The compound, found at high concentration in the mantle tissue, presumably constitutes an effective form of defense and its absence in the viscera suggests that this molecule is the result of *de novo* biosynthesis. However, biosynthesis has not been studied in this species coupled with the closely related chemical structures of

hodgsonal **51** and the sponge secondary metabolite albicanol **37** have raised doubts about hodgsonal **51** origins.

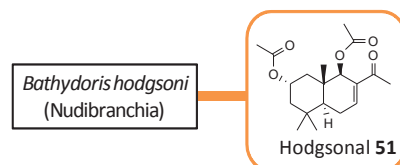


Figure 2. 14. *De novo* biosynthesis of hodgsonal **51** by *Bathydoris hodgsoni*

2.2.3. Sequestration of diet-derived chemicals by anaspideans (sea hares)

Anaspidea (Gastropoda, Heterobranchia, Euthyneura, Nudipleura) have soft bodies with an internal shell. They are herbivorous cryptic heterobranchs, similar to most sacoglossans, feeding on rich chemically-defended seaweeds or cyanobacteria and have developed specialized chemical defense strategies. Sea hares secrete an ink mixture which operates as an antipredator mechanism by acting on the olfactory and non-olfactory chemical senses of predators⁷⁸. The ink mixtures elicit aversive behavior in the sea anemone *Anthopleura sola*⁷⁹ and inhibits foraging and feeding in the crab *Callinectes sapidus*⁸⁰. This ink mixture is composed of both ink released by the ink gland, and opaline secreted by the opaline gland^{81,82}. The components of opaline and ink are diet-dependent or can be biosynthesized *de novo*⁸³ and some of the molecules appear to deter predators. The coloration of this ink mixture is often purple due to aplysiolavin **52** and phycoerythrobilin **53** derived from a light-harvesting protein present found in red algae (Rhodophyta) and cyanobacteria and which acts as a deterrent against the blue crab *Callinectes sapidus*^{80,84–86} (Figure 2. 15). Furthermore, five mycosporine-like amino acids (MAAs) have been isolated from the opaline secretion of the sea hare *Aplysia californica* including asterina-330 **54**, N-isopropanol palythine **55** and N-ethyl palythine **56** which act as an intraspecific alarm signal for juveniles warning them of a predatory attack on a conspecific⁸⁷. The other two MAAs, palythine **57** and N-methyl palythine **58** are also components of opaline, but do not act as alarm chemical signals. All five of these MAAs are derived from the red algae *Gracilaria ferox* and *Agardhiella subulata* (Figure 2. 15).

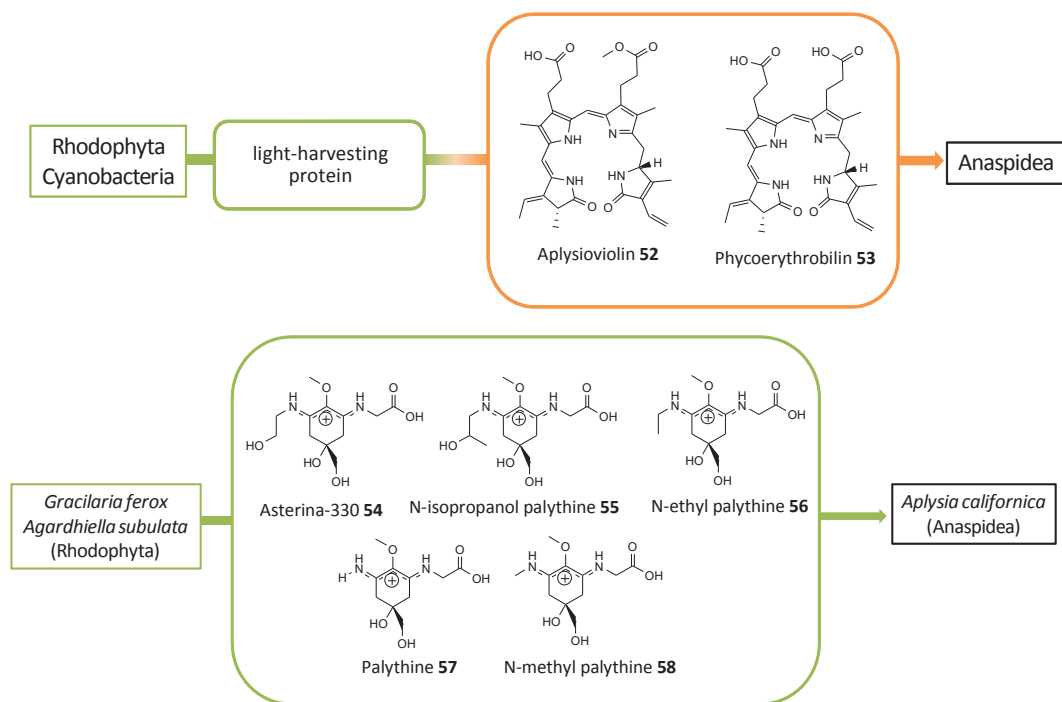


Figure 2. 15. Biotransformation of algal secondary metabolites by different Anaspidea and sequestration by *Aplysia californica*

Sea hares of the *Aplysia* genus are for the most part generalist herbivores of red algae, with geographical location dictating their preferred species. In Guam, *Aplysia parvula* prefers the red alga *Portieria hornemannii*, which produces the generally unpalatable compounds apakaochtodenes A **59** and B **60**, but also feeds on the red alga *Acanthophora spicifera* which does not contain any unpalatable compounds⁸⁸ (Figure 2. 16). However, *A. parvula* fed on *A. spicifera* are eaten by the fishes *Abudefduf saxatilis*, *Thalassoma lutescens* and *Arothron manilensis*, unlike *A. parvula* fed on *P. hornemannii*, providing evidence that the ingested unpalatable compounds defend the sea hare from predators. The two main algal secondary metabolites ingested, the tetrahalogenated monoterpenes apakaochtodenes A **59** and B **60**, are sequestered in the digestive gland, while small amounts are also detected in the mantle. In contrast, in Australia, *A. parvula* feeds upon two red algae: *Delisea pulchra* and *Laurencia obtusa*⁸⁹. *D. pulchra* produces the halogenated furanone Z-acetoxymimbrolide **61** at high concentrations, which deters reef fish, as well as mimbrolide(dibromo) **62**, Z-mimbrolide(bromo) **63** and Z-hydroxymimbrolide **64** at moderate concentrations (Figure 2. 14). *L. obtusa* produces the deterrent terpene palisadin A **65** at high concentrations as well as palisadin B **66**, aplysiastatin **67** and brasilenol **68** (Figure 2. 16). Thus, *A. parvula* fed on *D. pulchra* accumulates Z-acetoxymimbrolide **61**, mimbrolide(dibromo) **62**, Z-mimbrolide(bromo) **63** and Z-hydroxymimbrolide **64** in their digestive gland and moderate concentrations of Z-acetoxymimbrolide **61** and Z-hydroxymimbrolide **64** in the skin, the anterior mantle as well as in the opaline and ink glands⁹⁰ (Figure 2. 16). On the other hand, *A. parvula* fed on *L. obtusa* sequesters palisadin A **65** in the digestive gland, mucus and in opaline secretions, indicating its use as a defense strategy. Another sea hare, *Aplysia dactylomela*, sequesters the

sesquiterpene palisadin A **65** from the same red algae in its digestive gland, but not in mucus or opaline secretions.

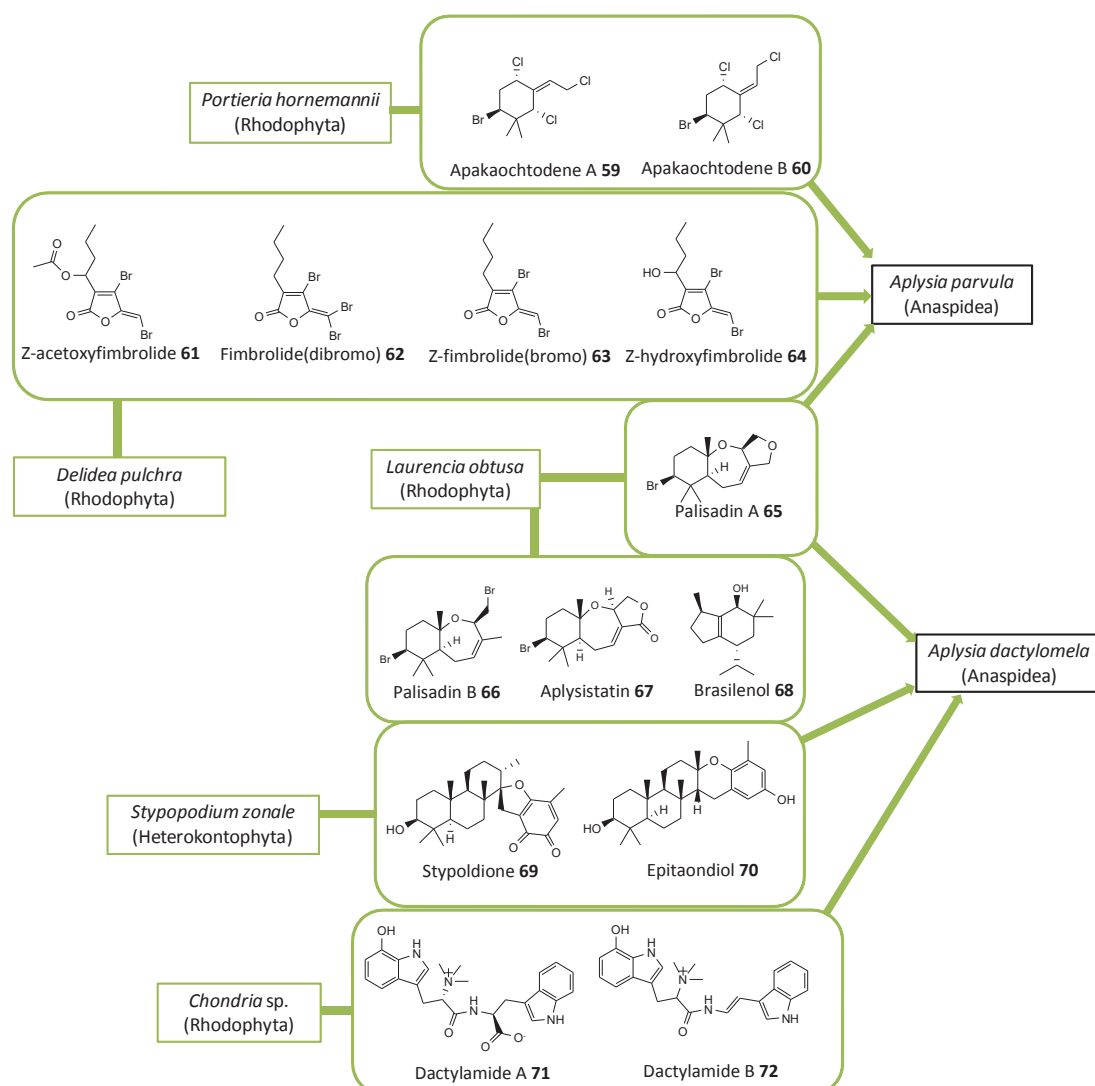


Figure 2. 16. Sequestration of algal secondary metabolites by *Aplysia parvula* and *A. dactylomela*

Similarly, *Aplysia dactylomela* accumulates stypoldione **69** and epitaondiol **70** from the brown alga (Heterokontophyta) *Styopodium zonale*⁹¹ (Figure 2. 16). In addition, dactylamide A **71**, dactylamide B **72** (Figure 2. 16), as well as isolaurenisol **73** and allolaurinterol **74** (Figure 2. 17) have been detected in the digestive gland of *A. dactylomela*⁸⁶. Isolaurenisol and allolaurinterol are produced by *Laurencia distichophylla* and *Laurencia filiformis* respectively (Figure 2. 17) while the dactylamides A **71** and B **72** could be biosynthetic precursors of chondriamides produced by the red algae genus *Chondria*.

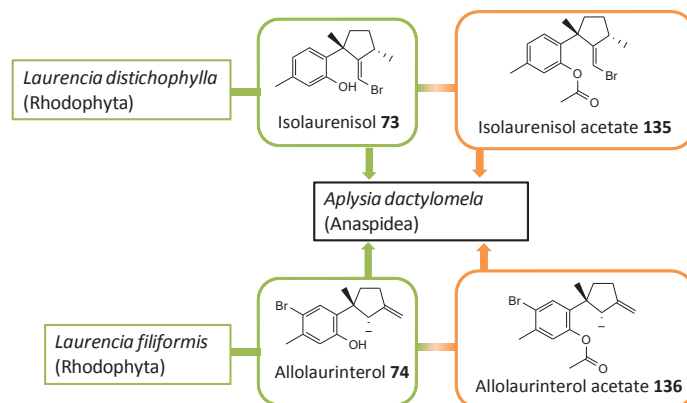


Figure 2. 17. Sequestration and biotransformation of algal secondary metabolites by *Aplysia dactylomela*

In another example of sequestered diet-derived compounds, seven cyclic halogenated monoterpenes are stored in the digestive gland and are found in trace levels in the opaline gland of *Aplysia punctata*⁹² (Figure 2. 18). These monoterpenes, mertensene **75**, 8-dechloro-8-bromo-coccinene **76**, 4-dechloro-4-bromo-violacene-2 **77**, violacene-2 **78**, coccinene **79**, 2-dechloro-2-bromo-coccinene **80** and 1-chloro-2-bromo-4-[2-chlorovinyl]-4,5-dehydro-1,5-dimethylcyclohexane **81** are produced by the red alga *Plocamium coccineum* and transferred to the sea hare during feeding (Figure 2. 18).

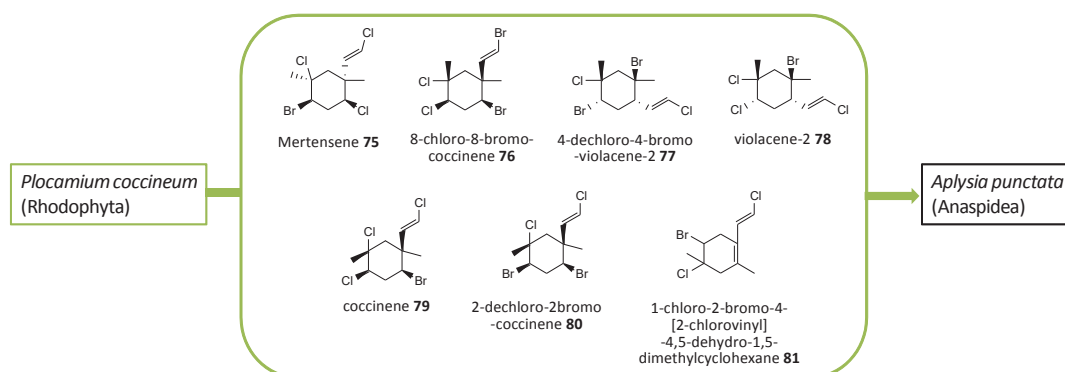


Figure 2. 18. Sequestration of algal secondary metabolites by *Aplysia punctata*

Two other sea hares, *Aplysia juliana* and *Aplysia kurodai*, were studied to determine their ability to sequester different secondary metabolites from various origins in their digestive glands⁹³. *A. juliana* sequesters the cyanobacterial compound malyngamide B **82** and the brown alga metabolite pachydietylol A **83** (Figure 2. 19), while *A. kurodai* sequesters pachydietylol A **83** and the sponge secondary metabolite luffariellolide **84** (Figure 2. 19). Similarly, the secondary metabolites aplysin **85**, debromoaplysin **86**, laurinterol **87**, pacifenol **88**, johnstonol **89** and pacifiadiene **90** produced by the alga *Laurencia pacifica* are found in the digestive gland of the generalist *A. californica*⁹⁴ (Figure 2. 19). Thus, multiple studies carried out on species in the *Aplysia* genus conclude that algal secondary metabolites are compartmentalized in the digestive gland. However, lower concentrations are also found in secretions, indicating their potential use as defense mechanisms.

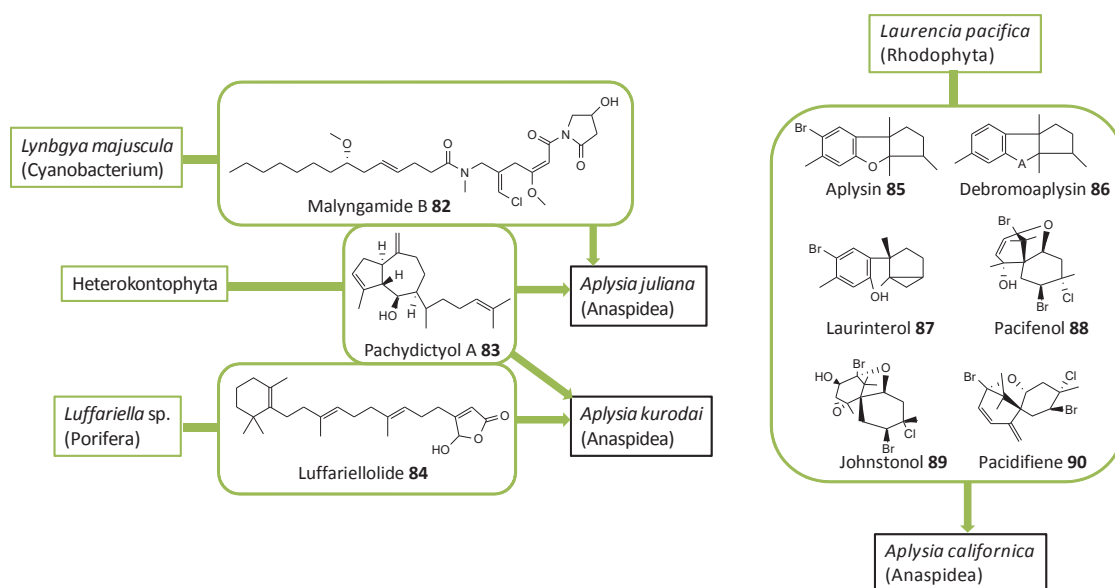


Figure 2. 19. Sequestration of cyanobacterial, algal and sponge secondary metabolites by *Aplysia juliana*, *A. kurodai* and *A. californica*

The fate of cyanobacterial secondary metabolites in *Stylocheilus striatus*, (previously identified as *Stylocheilus longicauda*), has sparked considerable interest in researchers. *S. striatus* is considered a specialist on the cyanobacterium *Lyngbya majuscula*^{95–98}, which appears to be a prolific source of chemicals, although due to misidentification of this cyanobacterium species it may not be so prolific as previously thought because all the chemicals are likely produced from a lot of different species thought of as *L. majuscula*. The fate of lyngbyatoxin A (LTA) 91 and debromoaplysiatoxin (DAT) 92 produced by *L. majuscula* collected in Moreton bay, Australia, was investigated in the digestive gland, the foot and the head and also in excretions of the sea hare, *S. striatus* (Figure 2. 20). The metabolites are present in high concentrations in the digestive gland and at low concentrations in the other sites, while LTA 91 is also present at very low concentrations in faecal matter, eggs and in ink⁹⁹. In addition, the malyngamides A 93 and B 82 isolated from *L. majuscula* collected from Guam are concentrated in the digestive gland of *S. striatus*^{95,100} and show deterrent activities against the puffer fish *Canthigaster solandri* and the crab *Leptodius* spp. (Figure 2. 20).

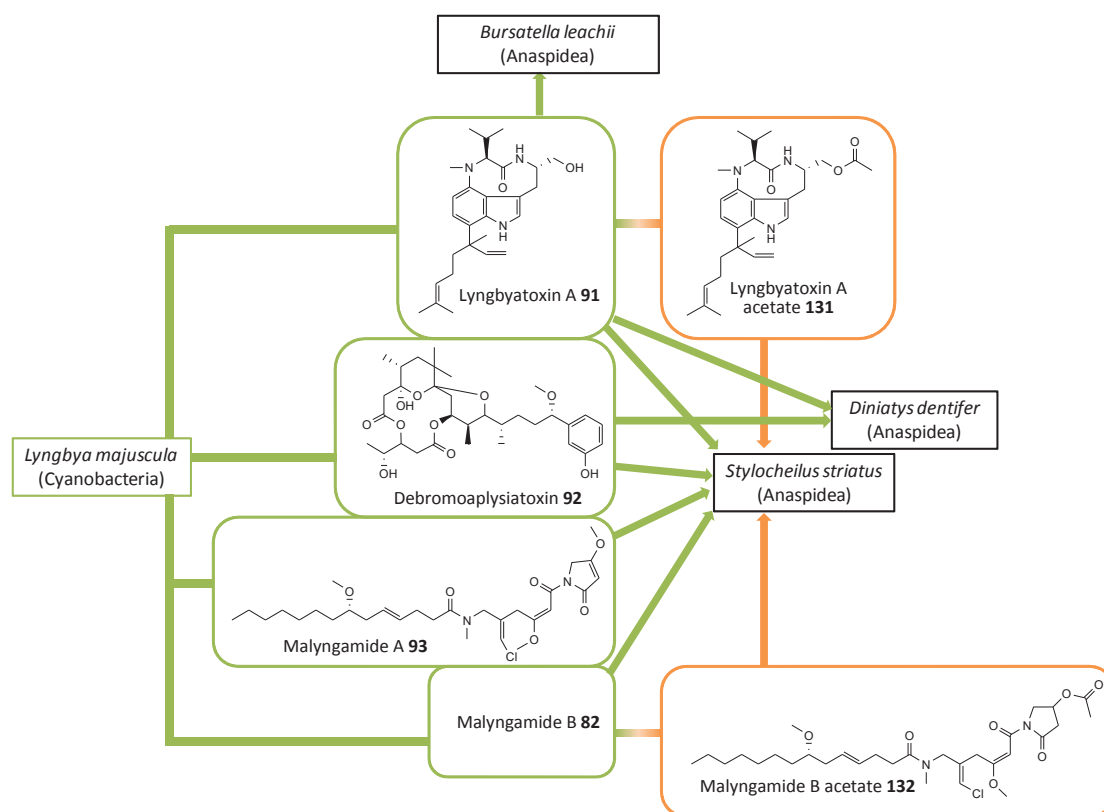


Figure 2. 20. Sequestration of cyanobacterial secondary metabolites by *Stylocheilus striatus*, *Diniatys dentifer* and *Bursatella leachii* and biotransformation carried out by *S. striatus*

Furthermore, *Dolabella auricularia*, a generalist sea hare, acts in the same way as *S. striatus* and stores caulerpenyne **5** from *Caulerpa*, pachydictyol A **83** from brown algae, malyngamide B **82**¹⁰⁰ from *L. majuscula*, johnstonol **89** from *Laurencia pacifica* and prepacifenol epoxide **94** from *L. nidifida* in its digestive gland⁸⁵ (Figure 2. 21). The ecological role of sequestered diet-derived metabolites remains unclear in *S. striatus* and *D. auricularia* as it is difficult to link the storage of deterrent compounds in an inner organ with a potential mechanism of defense, as the molecules are neither released in ink, or in mucus secretions^{99,101}. In contrast, although *Bursatella leachii*, another sea hare feeding on *L. majuscula*, sequesters LTA **91** also in the digestive gland, higher concentrations are found in ink, highlighting the existence of a potential defense strategy⁹⁹ (Figure 2. 20).

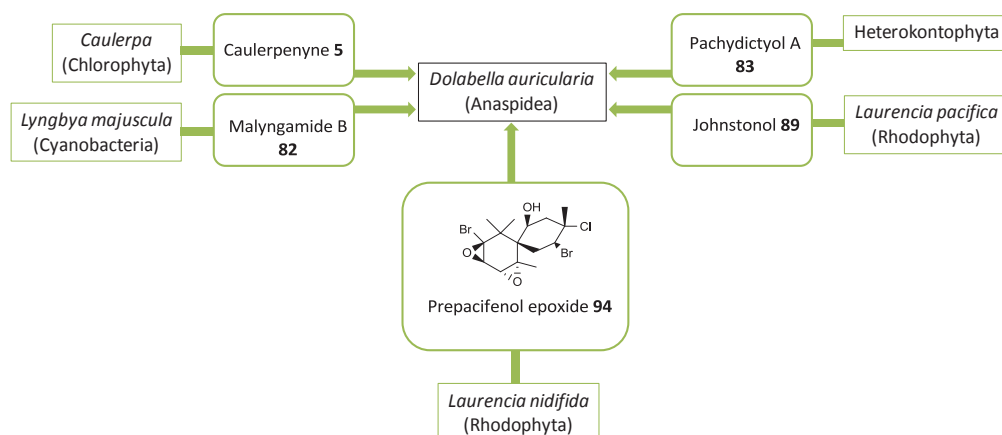


Figure 2. 21. Sequestration of algal and cyanobacterial secondary metabolites by *Dolabella auricularia*

2.2.4. Sequestration of diet-derived chemicals by other gastropods

Other instances of gastropod molluscs bioaccumulating compounds include *Diniatys dentifer* (order Cephalaspidea) which sequesters LTA **91** and DAT **92** from *L. majuscula*⁹⁹ (Figure 2. 20). However, whilst these compounds are found in high concentrations in the digestive glands of *S. striatus*, DAT **92** is only found in high concentrations in the head and the foot, and LTA **91** is present in low concentrations in the digestive gland in *D. dentifer*. In addition, the cephalaspidean mollusc *Philinopsis speciosa* (order Cephalaspidea) contains six cyclic depsipeptides; kulolide-1 **95**, kulolide-2 **96**, kulolide-3 **97**, kulokainalide-1 **98**, kulomo'opunalide-1 **99** and kulomo'opunalide-2 **100**, as well as the linear peptide pupukeamide **101** and the macrolide tolytoxin 23-acetate **102** (Figure 2. 22). *P. speciosa* is a generalist carnivore and consumes sea hares including *S. striatus* and *D. auricularia*. Thus, the above-mentioned diet-derived compounds are bioaccumulated across two trophic levels, as for example, kulolide-1 **95** is found in *S. striatus* originally sequestered from the cyanobacterium *L. majuscula*¹⁰² (Figure 2. 22).

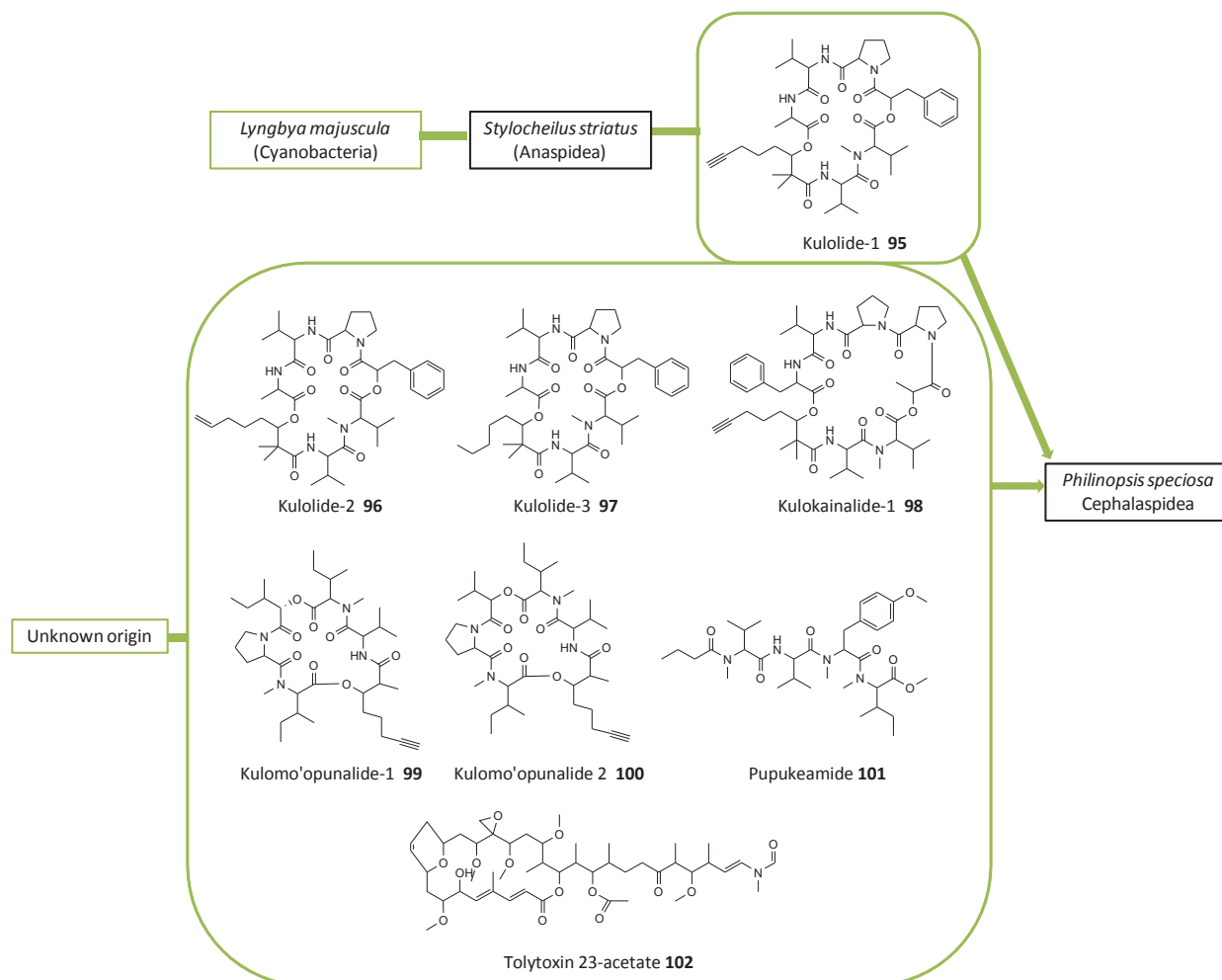


Figure 2. 22. Sequestration of secondary metabolites originating either from cyanobacteria or from an unknown origin by *Stylocheilus striatus* and *Philinopsis speciosa*

To compensate for the weak protection provided by its fragile shell, the cephalaspidean, *Bulla gouldiana*, biosynthesizes three polypropionates *de novo* as a defense strategy¹⁰³. 5,6-dehydroaglaïne-3 **103** and isopulo'upone **104**, which show considerable ichthyotoxicity to mosquito fish *Gambusia affinis*, are produced by the mollusc and stored in its mantle, and niuhinone-B **105** is also produced but for which no activity has been found (Figure 2. 23). *Navanax inermis*, another cephalaspidean heterobranch, feeds on *B. gouldiana* and also accumulates these three compounds. Therefore, although the presence of the diet-derived polypropionates in external parts of the body has not been determined, their storage could be linked to a chemical defense strategy¹⁰⁴.

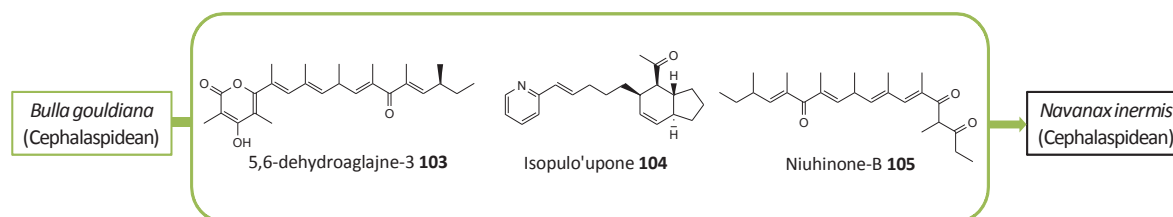


Figure 2. 23. Sequestration of mollusc secondary metabolites by *Navanax inermis*

The omnivorous mollusc *Tyrodina perversa* (order Umbraculoidea) provides a clear example of a predator using diet-derived compounds as a defense mechanism. *Aplysina aerophoba* and *A. cavernicola* are two sibling sponge species common in the Mediterranean sea, but *T. perversa* is always associated with *A. aerophoba* and never found on *A. cavernicola*. Sponges in the order Verongida are known to produce brominated isoxazoline alkaloids, and *A. aerophoba* contains considerable amounts of aplysinamisin-1 **106**, aerophobin-2 **107** and isofistularin-3 **108**^{105,106} (Figure 2. 24). *A. aerophoba* also lives in shallow waters, exposed to solar irradiation, enabling cyanobacteria to develop on its surface, whereas *A. cavernicola* thrives in shady environments such as deep underwater caves¹⁰⁷. The mollusc mostly consumes the cyanobacteria, only eating a fraction of the sponge¹⁰⁸. Nevertheless, the sponge-derived brominated alkaloids act as strong deterrents to the blenny, *Blennius sphinx*, and are found in high concentrations in the mantle of the sea slug *T. perversa*, whereas concentrations in the hepatopancreas, mucus secretions, feces and eggs are lower¹⁰⁹. Interestingly, aerophobin-2 **107** is the main compound found in the mantle, indicating selection on the site at which diet-derived compounds are sequestered. The bioaccumulation of the deterrent alkaloids in external tissues, as well as in the eggs, likely demonstrates a defense strategy against predators. In addition, *T. perversa* sequesters the sponge pigment uranidine **109** in order to have the same yellow coloration as its prey, becoming cryptic (Figure 2. 24). Finally, aerothionin **110**, a major metabolite produced by *A. cavernicola* but also present as a minor compound in *A. aerophoba* (personal observations), is found in the sea slug even in the absence of the sponge (Figure 2. 24).

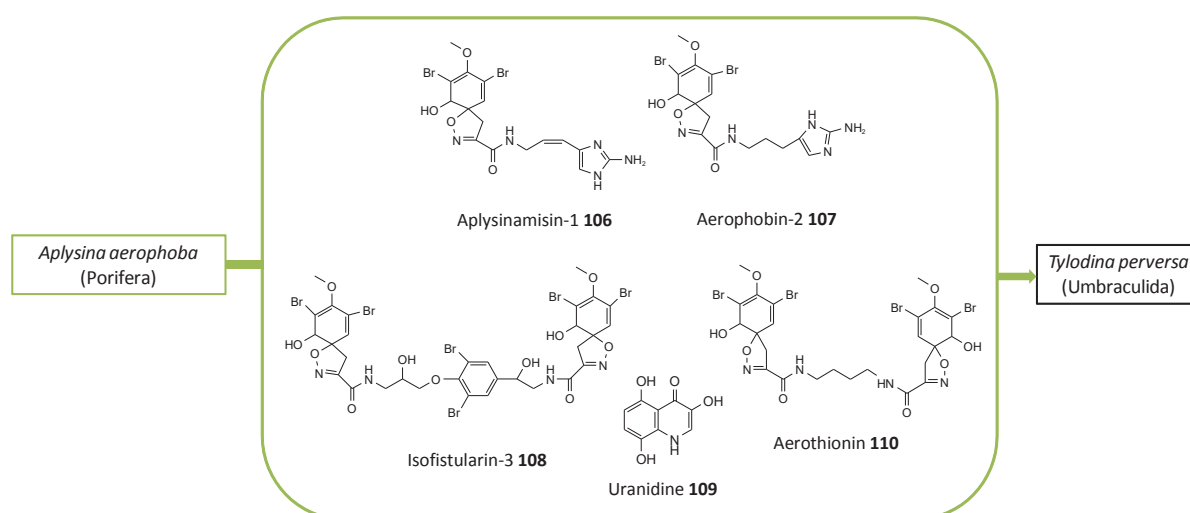


Figure 2. 24. Sequestration of sponge secondary metabolites by *Tyrodina perversa*

2.3. General mechanism of diet-origin secondary metabolites processing

Whether prey secondary metabolites deter potential predators or not can be explained by variation in post-ingestion responses¹¹⁰. Both terrestrial and marine species alike show general mechanisms for processing ingested secondary metabolites, which include the four parameters: Absorption, Distribution, Metabolism (detoxification or biotransformation) and Excretion (ADME). Sequestration and concentration or bioaccumulation, discussed in part 2, may be assimilated into absorption and distribution. The general mechanism of metabolism and excretion is separated into 3 phases according to their enzymatic architecture: phase I and phase II constitute the detoxification (or biotransformation) steps of xenobiotics and excretion occurs during phase III^{111,112}. Mechanisms of metabolism and excretion will be resumed in part 3.

2.3.1. Mechanism of metabolism and excretion: phases I, II and III

Ingested secondary metabolites are functionalized in phase I, during which multiple reactions introduce a functional group that reduces the lipophilicity of the compounds^{112,113}. In terrestrial and marine species, these reactions involve multiple families of enzymes that carry out various biotransformations such as hydroxylations, hydrolysis, reductions, oxidations, dehalogenations, dehydrogenations, heteroatom dealkylations, deaminations or epoxidations^{111,113,114}. Among them, cytochrome P450 monooxygenases (CYPs) located in cell microsomes, are an important phase I family of enzymes that add polar groups, such as a hydroxyl group, onto compounds. CYPs mediate the metabolism of endogenous compounds and catalyse the biosynthesis of signal molecules such as steroids, but are also able to functionalize various xenobiotics^{114,115}. The total level of P450s shows wide variation among different phyla; crustaceans have high levels compared to molluscs, echinoderms and polychaetes¹¹³. However, the amount of P450 is not correlated with its activity, since coenzymes and cofactors such as P450 reductase, cytochrome b5 and NADPH are also required^{114,116}. Cytochrome P450 is able to interact with a wide variety of lipophilic molecules and enables the organism to detoxify a considerable range of chemicals¹¹⁷. However, in some cases, reactions carried out by CYPs can lead to more toxic, mutagenic or carcinogenic compounds.

Phase II biotransformation reactions occur either with the functional group introduced during phase I, or directly with a functional group already present on xenobiotics. Phase II reactions generally result in a greater increase in hydrophilicity than achieved during phase I, in order to enable excretion in phase III^{112,113}. These reactions are controlled/mediated by multiple families of enzymes, allowing glucuronidation, sulfonation, acetylation, methylation, conjugation with amino acids and conjugation with glutathione¹¹³. Glutathione S-transferases (GSTs) form a major phase II family of enzymes which are located in the cell cytosol and in microsomes and are responsible for conjugative reactions by binding the tripeptide glutathione onto endogenous or exogenous electrophilic substrates^{111,118}.

Furthermore, GSTs appear to play an important role in marine herbivores, as they are involved in allelochemical biotransformations¹¹². The wide range of electrophilic xenobiotics, which can undergo reactions catalyzed by GSTs may be explained by the fact that each GST contains two kinds of ligand binding sites: one site shows strong specificity for glutathione, whereas the other is able to bind with a broad array of compounds. GSTs may also be responsible for sequestering exogenous compounds as a protective mechanism¹¹⁹⁻¹²². Indeed, some human and insect GSTs exert a strong binding affinity for exogenous compounds enabling them to be sequestered in the cytosol^{123,124}. In this way, the activity of the toxicant is inhibited by storing it away from target nuclear proteins thus preventing any toxic effects on gene regulation. Many studies have isolated GST activity in the presence of various exogenous compounds and proven that some induce GST activity. In contrast, some of them act as GST inhibitors or transcriptional repressors^{125,126}, indicating a potential prey defense strategy against consumers¹¹¹. GSTs also play a role in protection against oxidative stress by catalyzing glutathione peroxidase activity¹²⁷. This stress may be caused by exogenous compounds, such as secondary metabolites, increasing the amount of reactive oxygen species (ROS) in cells^{127,128}. GSTs are therefore involved in different mechanisms allowing the organism to increase its tolerance to a wide array of exogenous compounds.

Phase III excretion, the final step of ADME, is undertaken by membrane proteins known as ABC transporters (or multixenobiotic transporters) that play a role similar to that of a bouncer, by controlling the absorption, distribution and excretion at the cell gate, the membrane¹²⁹. These proteins, encoded by the ABC superfamily of genes, have been well studied in human pharmacology as they are responsible for multidrug resistance¹²⁹⁻¹³¹. ABC transporters are composed of seven subfamilies termed ABC-A to G. Among them, ABC-B, ABC-D and ABC-G appear to have a wide array of substrates including phase II conjugates and are responsible for trafficking molecules through cell membranes¹¹².

2.3.2. Examples of detoxification and biotransformation

Most of the studies regarding ADME have been carried out on humans, mammals¹¹³ or insects¹³² and research on the enzymatic architecture of marine gastropods is still in its infancy^{133,134}. The anatomical organization of digestion is different across gastropod molluscs. Caenogastropods (formerly prosobranchs) and heterobranchs have two main digestion organs: the stomach and the digestive gland¹³³. In heterobranchs, the digestive gland constitutes the primary site of enzymatic digestion, and is composed of various cell types, including rhodoplast digestive cells, specialized cells involved in the biotransformation of diet-derived secondary metabolites^{133,135}. In caenogastropods, the digestive enzymes are produced in the stomach, while the digestive gland is involved in the absorption and excretion of products^{136,137}.

2.3.2.1. ADME identified in marine gastropods: detoxification

A three phase enzymatic architecture has been identified in a marine gastropod mollusc, the generalist consumer *Cyphoma gibbosum* (Gastropoda, Caenogastropoda, Littorinomorpha), which feeds on chemically rich gorgonian corals¹³⁴. Prostaglandins constitute the main weapons of the gorgonian coral defense against predators, but are not fully efficient against *C. gibbosum*. Firstly, twelve genes encoding for phase I CYP enzymes have been identified in *C. gibbosum* and appear to be located in digestive gland cells. The gorgonian *Plexaura homomalla* produces deterrent prostaglandin A₂ (PGA₂) **111** analogs as its main chemical defense^{134,138} (Figure 2. 25) which likely induce the expression of *C. gibbosum* genes that produce CYP4BK and CYP4BL transcripts. CYP4BK and CYP4BL are closely related to vertebrate CYP4A and CYP4F that metabolize prostaglandins. Secondly, phase II GST enzymes have been characterized and located in the digestive gland cells of *C. gibbosum*¹³⁹. However, GST activity decreases when the mollusc is exposed to eight species of gorgonian corals¹¹⁹. A bioassay-guided fractionation identified PGA₂ **111** as the most GST inhibiting compound. *Cyphoma* GSTs can be saturated by PGA₂ **111** produced by *P. homomalla* and may explain why *C. gibbosum* favors a mixed diet. Finally, partial cDNA sequences encoding four ABC proteins, belonging to ABC-B and ABC-D families, have been identified in the digestive gland cells of *C. gibbosum*¹⁴⁰. These proteins show similarities with vertebrate ABC transporters that are in charge of glutathione conjugate transport through the cell membrane and could provide an activity complementary to that of GST enzymes.

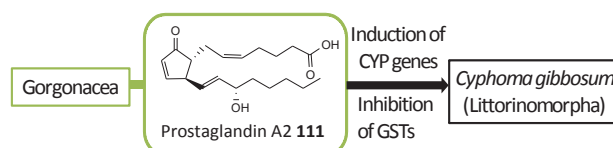


Figure 2. 25. Induction of CYP genes and inhibition of GSTs in *Cyphoma gibbosum* when exposed to prostaglandin A₂ **111**

The gastropoda *Bittium reticulatum* (Gastropoda, Caenogastropoda, Sorbeoconcha) feeds on the toxic alga *Caulerpa taxifolia* as well as the non toxic alga *Posidonia oceanica*¹²⁷. However, the activities of the antioxidant enzymes glutathione peroxidase, GST and glutathione reductase are higher in the case of the herbivore consuming *C. taxifolia*. Thus, antioxidant mechanisms, the response to oxidative stress, is induced in the presence of caulerpenyne **5**, the main defensive alga compound, and represents an adaptive response by the mollusc (Figure 2. 26).

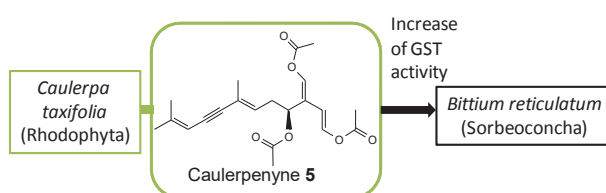


Figure 2. 26. Induction of an antioxidant mechanism in the presence of caulerpenyne **5**

The mean basal activities of GST in non-gastropod molluscs *Katharina tunicata* (Polyplacophora, Neoloricata) and *Cryptochiton stelleri* (Polyplacophora, Neoloricata), which frequently consume red algae containing the feeding deterrent lanosol **112**, increase significantly during feeding¹⁴¹. Phase I CYP3As and phase II GST are also found at very high concentrations in the digestive gland of the generalist consumer *Haliotis rufescens* (Gastropoda, Prosobranchia, Vetigastropoda)¹⁴² (Figure 2. 27). However, the mean basal activity of GST in *H. rufescens* was very high even before feeding on red alga i.e. before uptake of lanosol **112**. These results may demonstrate that the induction of detoxification enzymes is related to different feeding behaviors of marine herbivores, specialists versus generalists. The tolerance of specialist molluscs to secondary metabolites may be linked to their adaptive increase in GST activity. On the other hand, continuously high levels of GST activity may enable *H. rufescens* to detoxify a wider range of secondary metabolites, more adaptive for generalist species.

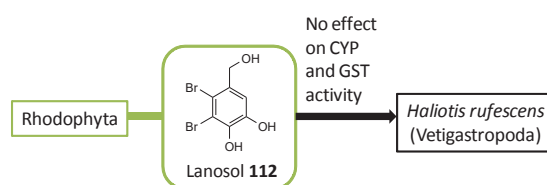


Figure 2. 27. Effect of lanosol 112 on CYP and GST activity in *Haliotis rufescens*

Sacoglossans and nudibranchs may use an enzymatic architecture to convert diet-derived compounds and this strategy could enable these molluscs to enhance their chemical defense and/or decrease the toxicity of absorbed molecules. Indeed, some compounds may show a specific toxicity against certain species but be harmless to others. The converted products could also be inoffensive for the sea slug, while maintaining or increasing their deterrent activities against predators.

2.3.2.2. Sacoglossans: biotransformation into more toxic forms for defense

Although no study has revealed the presence of a three-phase enzymatic architecture in sacoglossans, some instances of diet-derived compound biotransformations may indicate the presence of a similar process and this is an interesting field for future research. Firstly, the green alga *Caulerpa prolifera* is consumed by three shelled heterobranchs: *Oxynoe olivacea*, *Lobiger serradifalci*, and *Ascobulla fragilis*^{33,143}. Caulerpenyne **5** constitutes the major component of *C. prolifera* and is biotransformed into oxytoxin 1 **113** by three herbivores (Figure 2. 2). Consequently, the monoaldehyde oxytoxin 1 **113** is modified into the dialdehyde oxytoxin 2 **114** by *O. olivacea* and *A. fragilis* (Figure 2. 2). Interestingly, oxytoxins 1 **113** and 2 **114** are compartmentalized in the tail and in mucous secretions of *O. olivacea* and in external body parts of *A. fragilis*, while oxytoxin 1 **113** is located in the parapodia of *L. serradifalci*¹⁴⁴. Bioassays indicate that oxytoxins 1 **113** and 2 **114** are more toxic to the mosquito fish *Gambusia affinis* than caulerpenyne **5**. In addition, contrary to caulerpenyne **5**, oxytoxins 1 **113** and 2 **114** show antifeedant activities in the wrasse

Thalassoma pavo, the damselfish *Chromis chromis* and the sea bass *Serranus hepatus*. Thus, biotransformation of caulerpenyne **5** into two deterrent compounds that are then sequestered in external tissues and found in mucous secretions, provide clear examples of a defense mechanism in three shelled heterobranchs. Caulerpenyne **5** is also converted into oxytoxin 1 **113** by *Oxynoe antillarum*, *Elysia subornata* and *Elysia patina* and then to oxytoxin 2 **114** by *O. antillarum*³³ (Figure 2. 2).

Furthermore, *Elysia halimeda*, a specialist herbivore feeding on *Halimeda macroloba*, converts the algal diterpenoid halimedatetraacetate **7** into a reduced form **115**¹⁴⁵ (Figure 2. 3). This modified compound is then sequestered in high concentrations and transmitted to its eggs. Halimedatetraacetate **7** and its modified form **115** deter several herbivorous fishes and the *Elysia* diterpenoid is also deterrent towards a variety of carnivorous fishes. When attacked, the mollusc releases a mucous secretion containing the deterrent compounds providing evidence of a defense strategy using biotransformation.

Sacoglossans in the genus *Thuridilla* modify epoxy lactone **116**, an algal secondary metabolite produced by the green algae *Pseudochlorodesmis furcellata* and *Derbesia tenuissima*¹⁴⁶ (Figure 2. 28). The Mediterranean sea slug *T. hopei* converts epoxy lactone **116** into thuridillins A-C **117-119**^{28,147}, *nor*-thuridillonal **120**, dihydro-*nor*-thuridillonal **121** and deacetyl-dihydro-*nor*-thuridillonal **122**¹⁴⁸. Furthermore, the Australian mollusc, *T. splendens*, converts the algal metabolite into thuridillins A **117**, D **123**, E **124** and F **125**¹⁴⁹ (Figure 2. 28). Thuridillins A **117** and B **118** might result from an oxidation of epoxy lactone, while a reduction in the algal compound might lead to thuridillin C **119**¹⁵⁰ and phase I enzymes may be involved in these biotransformations. Thus, two species of *Thuridilla* genus are able to modify the algal secondary metabolite epoxy lactone into nine different compounds, revealing a wide diversity of biotransformation pathways. However, the activity of biotransformed compounds has not been investigated and do not enable us to link these biotransformations to a chemical defense increasing.

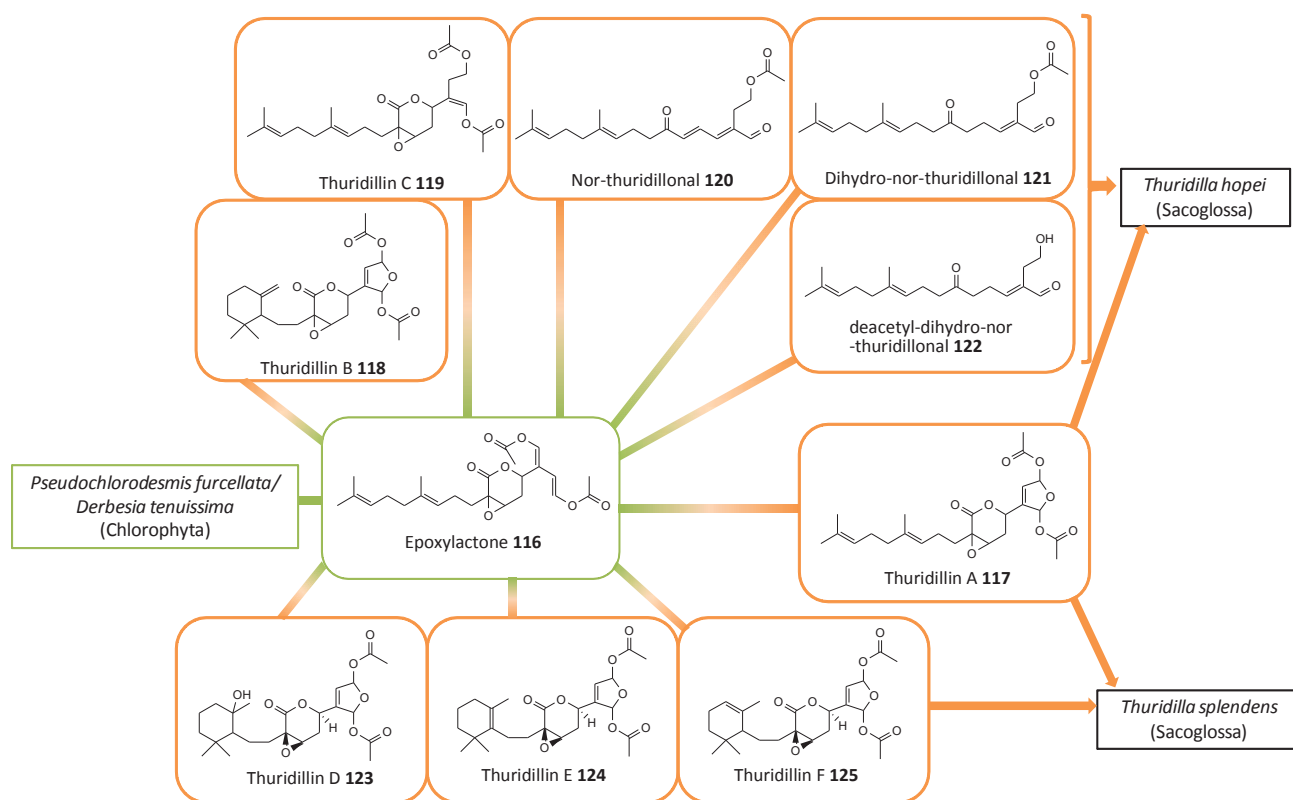


Figure 2. 28. Biotransformation of the algal secondary metabolites epoxy lactone 116 by *Thuridilla hopei* and *Thuridilla splendens*

2.3.2.3. Nudibranchs: biotransformation into more toxic forms for defense

As mentioned above, the nudibranch *Hexabranchnus sanguineus* sequesters secondary metabolites from its sponge prey (Figure 2. 10), but halichondramide **126**, the most abundant compound in *Halicondria* sp A, is not stored by the nudibranch⁶⁶. Rather, the mollusc converts halichondramide **126** into tetrahydrohalichondramide **127** (Figure 2. 10). This biotransformation likely occurs in the digestive system involving two reactions: double bond hydrogenation and carbonyl reduction, reactions that may be carried out by phase I CYP enzymes. The deterrent modified-compound is then sequestered in the mantle, digestive gland and eggs.

Many terpenoids produced by sponges are sequestered by nudibranchs. Furthermore, some nudibranchs are able to modify these terpenoids, such as *Hypselodoris orsini* that feeds on *Cacospongia mollior*¹⁵¹ (Figure 2. 7). The major sponge metabolite scalarial **16** is converted by selective aldehyde reduction into deoxoscalarin **128** which is found in the viscera and then oxidized into 6-keto-deoxoscalarin **129** which is compartmentalized in MDFs. The previously mentioned *Glossodoris pallida* also converts scalarial **16** into deoxoscalarin **128** and stores it in MDFs⁶² (Figure 2. 7). Once again, CYP may be involved in these reactions.

Chromodoris sinensis sequesters the sponge metabolite aplyroseol-2 **27** and biotransforms it into its dialdehyde derivative **130**⁶⁰ (Figure 2. 9). This modification may involve reductions carried out by phase I enzymes and methylation carried out by phase II enzymes. Aplyroseol-2 **27** and aplyroseol dialdehyde **130** are both concentrated in MDFs and the modified compound appears to be three times more concentrated than the original one. Interestingly, aplyroseol-2 **27** does not show any deterrent activity, while the dialdehyde derivative **130** induces considerable feeding-avoidance behavior. Thus, biotransformation may occur to increase the chemical defense of the sea slug. The specialist nudibranch, *Tritonia hamnerorum*, feeding on the chemically defended gorgonian *Gorgonia ventilina*, remains to date, the only nudibranch for which ABC transporters have been identified¹⁴⁰. Indeed, as is the case with *Cyphoma gibbosum*, ABC-B and ABC-C appear to transport glutathione conjugates through the cell membrane, suggesting the presence of GST in this nudibranch.

2.3.2.4. Anaspideans: biotransformation with loss of toxicity

Stylocheilus striatus sequesters diet-derived compounds from *Lyngbya majuscula* in its digestive gland and appears to biotransform some of them. LTA **91** from *S. striatus* collected in Moreton bay, Australia, undergoes acetylation leading to lyngbyatoxin A acetate **131** which is also sequestered in the digestive gland¹⁵² (Figure 2. 20). In addition, malyngamide B **82** is converted into malyngamide B acetate **132** by *S. striatus* in Guam (Figure 2. 20). Contrary to the parent molecule, malyngamide B acetate **132** does not repel the pufferfish *Canthigaster solandri* or the crab *Leptodius spp* and its toxicity on both brine shrimp *Artemia franciscana* and the sea urchin *Echinometra mathaei* is diminished. Sea hares in the *Aplysia* genus are also able to carry out acetylations on diet-derived compounds. For example, *Aplysia dactylomela* converts 14-keto epitaondiol **133**, produced by the brown alga *Styopodium zonale*, into 3-keto epitaondiol **134**⁹¹ (Figure 2. 29). Similarly, the algal metabolites isolaurenisol **73** and allolaurinterol **74** are converted into isolaurenisol acetate **135** and allolaurinterol acetate **136** respectively⁸⁶ (Figure 2. 17). Acetylation may be carried out by phase II enzymes and biotransformation by an adaptive detoxification mechanism. However, it is unlikely that the modified secondary metabolites are used in chemical defense as they are sequestered in an inner organ without being excreted and they also lose toxicity. Thus, sequestering metabolites and modifying the most toxic ones may be less energetically expensive than fully detoxifying and excreting them^{95,133}.

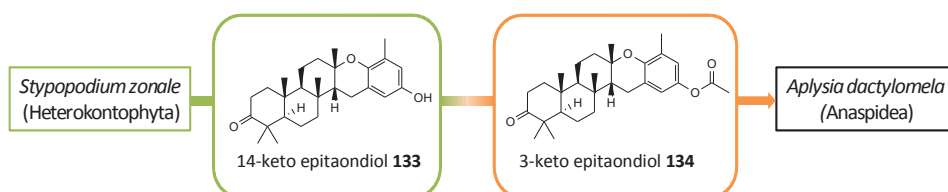


Figure 2. 29. Biotransformation of the algal secondary metabolites 14-keto epitaondiol **133** by *Aplysia dactylomela*

2.3.3. Detoxification limitation hypothesis and feeding choice

As vital GSTs can be saturated by diet-derived secondary metabolites during detoxification, there may be a limit to the number of metabolites that can be ingested before toxicity is incurred. Freeland and Janzen¹⁵³ introduced the detoxification limitation hypothesis (DLH) to understand generalist herbivore behavior and how secondary metabolites could limit feeding rate¹⁵⁴. This hypothesis predicts that generalist herbivores would select a mixed diet rather than a single one, to ingest different secondary metabolites with non-overlapping detoxification pathways, due to the constraints on GST saturation. Feeding rates, with a mixed diet compared to a single one, would be higher and overall consumer performance enhanced (growth, survival and/or fecundity)^{155,156}. However, few studies have confirmed DLH in marine ecosystems, and no study has investigated DLH in marine gastropod molluscs. Two marine herbivores, the urchin *Arbacia punctulata* and the amphipod *Amphithoe longimana* decrease feeding rates when their total secondary metabolite concentration increases¹⁵⁶. Feeding rate may also be influenced by the nutritional value of the prey consumed; a consumer feeding on a nutritionally low diet would increase foraging but would also consume more secondary metabolites than it can detoxify^{156,157}. Therefore, a mixed diet also offers a nutritional complement to the consumer as found in *Dolabella auricularia*, which grows faster on a mixed rather than on a single diet¹⁵⁸. Nitrogen concentration remains another factor that may play a role in foraging choice; for instance, the gastropod *Littorina sitkana* prefers to consume algae with a high nitrogen concentration regardless of the presence or absence of chemical defenses¹⁵⁹.

2.3.4. Induction of chemical defenses

Sedentary species or prey have adapted defense mechanisms to counter attacks by predators, but such defenses are costly^{160,161}. Therefore, the production of defenses is often linked to the rate of predation: if high then constitutive defenses are constantly produced even in the unusual absence of an attack. On the other hand, when predation rates are spatially or temporally variable then facultative defenses are only induced upon attack¹⁶². Induced resistance may be an adaptation to minimize costs by keeping defenses low until they are needed^{163,164}. Whilst predator-induced morphological defenses have been found in several marine taxa, including barnacles^{165,166}, bryozoa^{167,168}, and seaweeds^{169,170}, there are few examples of predator-induced chemical defenses in a marine organisms. The brown alga *Fucus distichus* responds to periwinkle *Littorina sitkana* grazing by increasing the concentration of polyphenolic compounds¹⁷⁰, to which the herbivores respond by grazing on unwounded algae. The brown alga *Ascophyllum nodosum* provides another clear example of chemical defenses induced by grazing by marine gastropods¹⁷¹. The concentration of phlorotannins is strongly increased in response to an enzyme present in the saliva of *Littorina obtusata*¹⁷². These chemicals reduce algal consumption by *L. obtusata*, increase feeding but decrease the amount ingested and increase the distance required to travel to find unwounded prey.

An activated defense (short-term inducible defense¹⁷³ or dynamic defense¹⁷⁴) is a chemical defense which involves the rapid conversion of one secondary metabolite into another more potent defensive compound upon attack¹⁷⁵. This conversion allows an organism to quickly produce potent feeding deterrents that are biologically active but unstable, thus minimising the risk of autotoxicity¹⁷⁵. For example, upon predator attack, the marine algae, *Halimeda* spp. are able to rapidly convert halimedatetraacetate to halimedatrial.

These are all examples of direct defenses that by themselves affect the susceptibility to attack¹⁷⁶. However, another form of defense, indirect defenses, serve as attractants to natural enemies of the attacking predator thus reducing damage to the prey. The only marine example involves gobies defending Acroporid corals from allelopathic algae in response to chemical cues from the coral¹². However, to date there are no examples with marine gastropods.

2.4. Chemically mediated interactions

As described by Mark Hay⁷, chemically mediated interactions have major impacts on population structure, community organization, and ecosystem function. Inter- and intra-species communication involves distinctive chemical signals and cues. Chemical signals are emitted intentionally by a sender towards a receiver, and are generally beneficial to the sender¹⁷⁷. Chemical signaling also occurs in intra-specific communication, for example, by pheromones. In contrast, chemical cues are released unintentionally by a sender and are intercepted by a receiver. Thus, the reaction to the cues is either neutral or disadvantageous to the sender. A compound released by prey and intercepted by a predator, allowing it to locate the prey, is considered as a chemical cue. The difference between these two types of chemical communication may be flexible over time in which communication could evolve via adaptation and exaptations¹⁷⁸. For example, senders could originally emit chemicals such as waste products, defensive molecules or by-products with non-communicative functions that could be precursors for the evolution of more complex communication. In addition, chemical cues can evolve into chemical signals; such exaptation is known as “chemical ritualization”¹⁷⁸. Conversely, the evolution of chemical signals into chemical cues can also occur as another type of exaptation^{179,180}. Marine gastropods have mastered the art and manner of using chemical cues and signals across a large range of spatial scales for feeding preference, foraging, mate attraction, and larval metamorphosis and settlement. Here we describe the role of secondary metabolites in palatability, olfaction and in mucus trail following in marine gastropods.

2.4.1. Prey chemicals as determinants of feeding preferences

Although a wide range of secondary metabolites produced by sponges, algae, cyanobacteria or tunicates act as feeding deterrents to potential predators, some consumers

are able to circumvent these chemical shields, and find putative unpalatable prey palatable. In these cases, secondary metabolites evolve into attractants to predators. However, only a few studies have demonstrated that feeding choice could be related to the palatability of secondary metabolites. Among them, the interaction between the sea hare *Stylocheilus striatus* and *Lyngbya majuscula* is a good predator-prey example, as the cyanobacterium is known to produce a broad range of compounds¹⁸¹. A comparison of the palatability of non polar and polar extracts of closely related cyanobacteria belonging to *Lyngbya*, *Moorea* and *Okeana* genera revealed that *S. striatus* is stimulated to feed on non polar and polar extracts of *Lyngbya* spp. as well as on the non polar extracts of *Moorea producens* (formerly *Lyngbya majuscula*)¹⁸². The majusculamides A **137** and B **138** (combined), and malyngamides A **93** and B **82** are deterrents against the pufferfish *Canthigaster solandri* and the crab *Leptodius* spp., yet appear to be palatable to the sea hare at natural concentrations^{97,98} (Figure 2. 30). However, at higher concentrations, these molecules also repel sea hares. Similarly, pitipeptolide A **139** induces *S. striatus* to feed on *L. majuscula*, yet it is repellent to the sea urchin *Echinometra mathaei*, the crab *Menaethius monoceros*, and the amphipods *Parhyale hawaiiensi* and *Cymadusa imbroglia*¹⁸³. Furthermore, these cues are conserved geographically, as crude extracts of *L. majuscula* collected from Moreton Bay, Australia, also strongly stimulate feeding in *S. striatus* from Guam^{184,185}. Only a few studies have demonstrated the palatability of secondary metabolites since it is difficult to identify the molecule, or the association of molecules, among the whole metabolome that is responsible for feeding preferences.

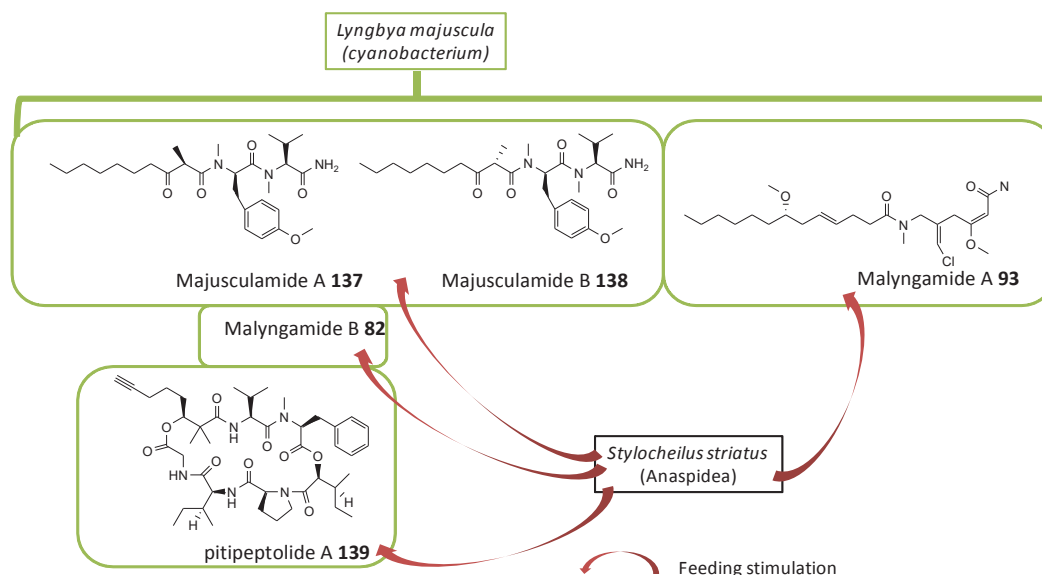


Figure 2. 30. Cyanobacterial secondary metabolites as determinants of feeding preferences for *Stylocheilus striatus*

2.4.2. Secondary metabolites and chemoreception

For marine gastropods, olfaction is an essential sense for medium and long distance reception of signals and cues and is mediated through different sensory organs. In aquatic

eupulmonates (formerly Pulmonata), the osphradium is the main chemosensory organ, although the cephalic tentacles also play a role in orientation towards olfactory cues¹⁸⁶. Several organs are implicated in caenogastropod (formerly prosobranch) chemoreception, including the cephalic and metapodial tentacles, the anterior margin of the foot, the siphon tip, osphradia and the bursicles. Similarly, other heterobranch molluscs (formerly Opisthobranchia) are able to detect chemical cues mainly using their rhinophores and tentacles, and the anterior edge of the oral veil and the osphradium are also implicated to a lesser degree^{23,186–190}. These chemosensory organs play a crucial role in intra-specific communication by detecting pheromones. For example, *Aplysia* sea hares detect water-borne proteins from conspecifics, such as attractin, enticin, temptin and seductin^{191–193}, sex pheromones involved in mate attraction, as well as from eggs which extend the duration of egg-laying. As the first interaction between a predator and its prey involves the detection of chemical cues, chemoreceptors also play an essential role in foraging^{23,189}. The presence of chemoreception in marine gastropods, notably sacoglossans, has been proven from the presence of head lifting behavior^{23,194,195}. Sea slugs show a specific behavior in the presence of chemical cues from their prey; they lift their head and the anterior part of their body in the direction of the stimulus¹⁹⁴. Chemoreception has also been described in nudibranchs¹⁸⁷ and rhinophores are active even at the larval stage, helping larvae during settlement by selecting a habitat based on the presence of suitable food¹⁹⁶. Here, we review the role of prey secondary metabolites in metamorphosis and settlement and in foraging of gastropods using chemoreception.

2.4.2.1. Secondary metabolites as inducers of metamorphosis and settlement of gastropod larvae

Larvae of various benthic species are released into the pelagic zone for the duration of their larval period as either filter-feeding planktotrophs or non-feeding lecithotrophs¹⁹⁷. Larval dispersal enables benthic species to colonize distant areas that cannot be reached by adult movement alone. Larval recruitment can be induced by physical, biological or chemical cues, but in specialist associations, chemical cues released by prey are often identified as the main factor responsible for the settlement of predatory larvae^{198,199}. Most heterobranch larvae need an exogenous cue to induce metamorphosis and settlement¹⁹⁹.

Among shell-less sacoglossans, *Alderia modesta* is considered a specialist on the yellow-green alga *Vaucheria longicaulis*²⁰⁰. This mollusc lives in temperate estuaries and exhibits a rare polymorphism producing both planktotrophic (feeding) and lecithotrophic (non-feeding) larvae²⁰¹. A percentage of the lecithotrophic larvae are able to metamorphose spontaneously, while the other part needs an exogenous cue - compounds released by *V. longicaulis* in the water. Other algal chemical cues do not induce metamorphosis in larval *Alderia modesta*. The chemical signature appears to be composed of low molecular weight carbohydrates such as mannitol or glucose and unknown high molecular weight carbohydrates suggesting that polar compounds can also play a role in marine chemical ecology^{200,202}. The percentages of lecithotrophic larvae that spontaneously metamorphose

and those requiring algal induction are variable. The percentage of spontaneous metamorphosis significantly decreases when adult molluscs are starved over 24h prior to oviposition in order to enhance the dispersal potential of larvae. This example indicates the presence of a variable dispersal strategy²⁰¹.

Settlement of the specialist nudibranch *Phestilla sibogae* has sparked the interest of many researchers as the nudibranch only feeds on *Porites compressa*^{198,203,204}. The sea slug produces planktotrophic larvae that settle and metamorphose in response to a small polar compound released by the coral prey²⁰⁵. Other nudibranch larvae in the *Phestilla* genus that feed on different corals as adults also respond to chemical cues of their prey inducing settlement and metamorphosis. *Phestilla minor* consumes *Porites lutea* and *P. annae*, and metamorphoses in the presence of *P. lutea*, *P. annae* and *P. cylindrical*, while the metamorphosis of *Phestilla* sp. 2, that preferentially feeds on corals in the genus *Goniopora*, is induced in the presence of *G. fruticosa*, *G. minora* and *G. lobata*^{198,204}.

Hermisenda crassicornis is a nudibranch with planktotrophic larvae which can either spontaneously metamorphose or metamorphoses in response to natural inducers released by the hydroid *Tubularia crocea*^{206,207}. The hydroid inducer is water soluble, but is not the only compound that can induce metamorphosis in the sea slug. GABA (γ -aminobutyric acid), choline, serotonin, glutamate or ions such as K^+ and Cs^+ (at low concentration) also induce a high proportion of metamorphosis. In another example, the aposematic nudibranch *Hypselodoris infucata*, a specialist feeder of the sponge *Dysidea* sp., produces planktotrophic larvae that metamorphose and settle in response to chemical-cues released by *Dysidea* sp.²⁰⁸. However, this phenomena also occurs in the presence of three other sponges *Halichondria coerulea*, *Sigmodocia* sp., and *Tedania macrodactyl* which are sympatric with *Dysidea* sp.. Natural inducers may also be produced by mutual microorganisms thriving on the primary biofilm. Larvae of *H. infucata* use non-specific cues to settle on, or close to, specific prey indicating that selection may occur later during the juvenile or adult stages. The larvae of another specialist nudibranch, the sponge feeding *Rostanga pulchra* are able to delay metamorphosis for at least three weeks after becoming competent, and only the presence of a specific prey species, *Ophlitaspongia pennata*, induces metamorphosis and settlement²⁰⁹. Furthermore, the nudibranch *Onchidoris bilamellata* feeds exclusively upon barnacles and its settlement is only triggered in water conditioned with this prey²¹⁰. Lecithotrophic larvae of the nudibranch *Adalaria proxima* metamorphose in the presence of chemicals released by its preferred bryozoan prey *Electra pilosa* and the inducer may be a peptide with low molecular weight (<500 kDa)^{211,212}.

Aplysia californica, a generalist consumer of *Plocamium cartilagineum* and *Laurencia pacifica*, also requires chemical cues to trigger its settlement²¹³. Metamorphosis of *A. californica* larvae occurs in the presence of chemical cues from several algae including *Rhodomenia californica*, *Corallina officinalis*, *Plocamium cartilagineum*, *Laurencia pacifica*, *Callophyllis violacea*, *Dictyopteris undulata*, *Pachydictyon coriaceum*, *Pterocladia capillacea*,

Centroceras clavulatum and *Chondria californica*. However, only juveniles that settle on *P. cartilagineum* and *L. pacifica* actually consume the alga which induced their metamorphosis, the juveniles that settled on the other eight algal species attempt to find another food source.

The settlement of four other sea hares suggests that generalist consumers may also show a preference for a prey species during settlement²¹⁴. *Aplysia juliana* preferentially settles on *Ulva fuscata* and *U. reticula* and post-larval growth is high. On the other hand, metamorphosis is lower on *Enteromorpha* sp. as is post-larval growth. The settlement of *Aplysia dactylomela* is induced by chemical cues from a range of different genera of red algae such as *Chondrococcus*, *Gelidium*, *Laurencia*, *Martensia*, *Polysiphonia*, and *Spyridia*. However, the percentage of metamorphosis is highest for *Laurencia* sp., the mollusc preferentially consumes this species of algae and which provides the highest growth rate. *Stylocheilus striatus* preferentially settles and feeds on the cyanobacterium *Lyngbya majuscula*, yet the red algae, *Acanthophora spicifera*, *Spyridia filamentosa* and *Laurencia* sp., also induce its settlement, but which result in lower post-larval growth²¹⁴. Finally, larvae of *Dolabella auricularia* settle and metamorphose in the presence of the red algae *Laurencia*, *Amansia*, and *Spyridia*, the brown alga *Sargassum* sp. and an unidentified mat-forming cyanobacterium. However, post-metamorphic *D. auricularia* grow faster on the cyanobacterium, and *Spyridia filamentosa* is the preferred food for older juveniles.

The abalone *Haliotis iris* is a shelled generalist herbivore feeding on various algal foods such as the crustose coralline algae *Phymatolithon repandum*²¹⁵. The biofilm on the surface of the alga formed by cyanobacteria and diatoms barely induces larval metamorphosis, whereas the alga without biofilm induces metamorphosis in nearly 100% of the larvae. Another example in a shelled mollusc, is the queen conch *Strombus gigas*, a generalist consumer feeding on various algae, but whose nursery ground substrata is dominated by *Laurencia poitei* and *Thalassia testudinum*^{216,217}. Metamorphosis and settlement are induced by *L. poitei* and the epiphyte *Fosliella* sp. found on the detrital blade of *T. testudinum*. The compounds inducing settlement are water soluble with a low molecular size (<1000 Da).

Crepidula fornicata is an invasive shelled gastropod mollusc exhibiting gregarious behavior and whose settlement is triggered by chemical signals released into the water by conspecifics which may be assimilated with gregarious behavior^{218,219}. Furthermore, metamorphosis also occurs in response to the presence of the halogenated compound dibromomethane **140**, which is released by red algae in the Corallinaceae family (Figure 2. 31).

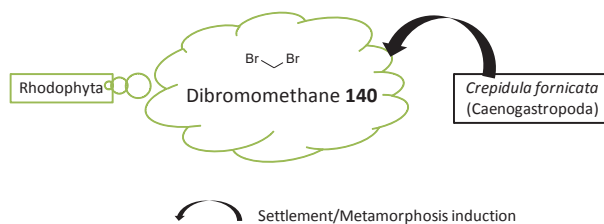


Figure 2. 31. Settlement and metamorphosis of *Crepidula fornicata* induced by the algal secondary metabolites dibromomethane 140

In conclusion, metamorphosis and settlement by many marine gastropod species, either generalists or specialists, are driven by chemical cues, although the inducing compounds have rarely been identified. However, in many cases, prey chemicals implicated in settlement are water soluble with variable molecular weights, whereas nonpolar molecules do not seem to trigger such phenomena²¹¹. The duration of the period during which larval settlement can take place varies among species and larvae with a small competence period are associated with a wide range of chemical cues that induce settlement, whilst larvae with a long competence period can afford to be more specific²¹⁶. However, settlement in some gastropods, such as sea hares, with a relatively long larval stage, occurs on various algal species that are not normally consumed. This would suggest that diet selection occurs later, and that the use of chemoreception for foraging plays a significant role in both the juvenile and adult stages. The trend emerging from these studies seems to indicate that settlement may be more efficient for shelled molluscs with low mobility in response to chemical cues from their preferred prey. However, shell-less, more mobile species colonize and metamorphose on a broad range of species and then may select their preferred food as juveniles or adults.

2.4.2.2. Secondary metabolites as inducers of foraging in juvenile and adult gastropods

The role of sensory organs in foraging during juvenile and adult stages is essential, especially for mobile, shell-less gastropods. However, only a few studies have demonstrated that marine molluscs use olfaction to find food or identified the chemical compounds that elicit foraging behavior. Among them, sacoglossans use olfaction and exhibit a specific behavior (headlifting) in response to food stimuli. *Elysia subornata* prefers to feed upon *Caulerpa ashmeadii*, but also feeds on other algae of the genus *Caulerpa*¹⁹⁴. The sea slug is able to detect large polypeptides with a molecular weight of 2000-3500 Da released by algae of the genus *Caulerpa*. Five other species of sacoglossans: *Oxynoe azuropunctata*, *Elysia eoelinae*, *E.*

papillosa, *E. tuca*, and *Ercolania fuscata* also respond to food stimuli^{23,195}. Indeed, the filtered homogenates, containing proteins, of the preferred algal food induce headlifting behaviors in their respective sea slugs. In addition, most species show a response in the presence of *Caulerpa* homogenates and this may be because this alga is the ancestral food of sacoglossans.

Previously, we discussed the specific interaction between *Halimeda incrassata* and *Elysia tuca* with the transmission of halimedatetraacetate **7** and chloroplasts from the alga to the sea slug³⁵. *H. incrassata* is a chemically defended and calcified seaweed that produces 4-hydroxybenzoic acid **141** and halimedatetraacetate **7**. Despite the fact that reproduction in *Halimeda* remains rare and ephemeral (~36h), the abundance of sea slugs on alga during the reproductive stage, when it is not mechanically defended with calcified thalli, is 12-18 times higher than on a vegetative individual. *E. tuca* intercepts chemical cues released by the seaweed and distinguishes between vegetative and reproductive individuals. Reproductive *H. incrassata* produces the deterrent compound halimedatetraacetate **7** in high concentrations in order to compensate for the lack of a mechanical defense. The sea slug therefore tracks halimedatetraacetate **7** to locate uncalcified algae in preference to 4-hydroxybenzoic acid **141** from vegetative algae (Figure 2. 32).

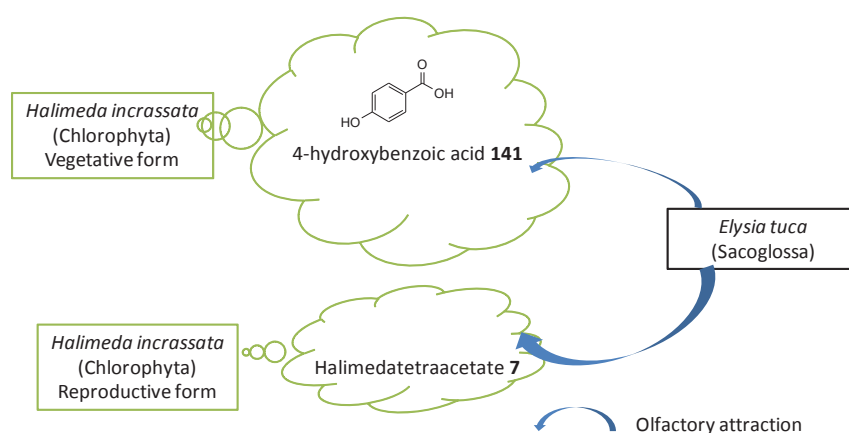


Figure 2. 32. *Elysia tuca* tracks either the algal metabolites halimedatetraacetate **7** or 4-hydroxybenzoic acid **141** to locate its prey

Previously, we described the transmission of tambjamines in an ecosystem formed by the bryozoan *Sessibugula translucens*, two nudibranch consumers *Tambja abdere* and *T. eliora* and their nudibranch predator *Roboastra tigris*⁷³ (Figure 2. 13). *T. abdere* locates *S. translucens* by detecting the presence of tambjamines A **44** and B **45** ($> 10^{-10}$ M) in the water. However, a higher concentration of these two compounds ($> 10^{-8}$ M) deters *T. abdere*. The tambjamines may therefore also act as alarm pheromones produced in response to an attack by the predator *R. tigris* on a conspecific. Such an attack triggers the secretion of mucus containing high concentrations of tambjamines (Figure 2. 33). *T. abdere* is therefore able to detect and differentiate between two concentrations of tambjamines that differ by only orders of magnitude in their concentration. Thus, tambjamines A **44** and B **45** are used as both chemical cues enabling *T. abdere* to find its prey *S. translucens*, as well as chemical signals of intraspecific alarm pheromones in the case of a predatory attack.

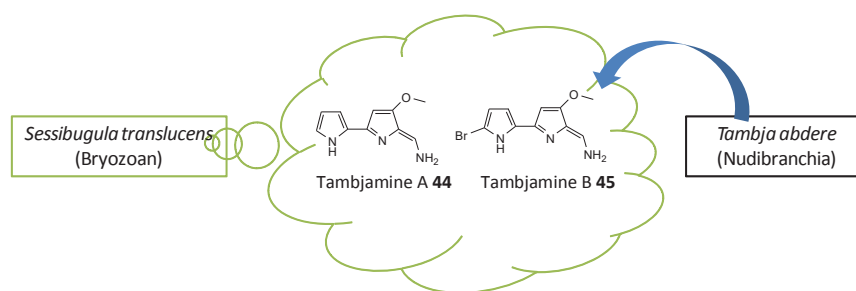


Figure 2. 33. *Tambja abdere* tracks the bryozoan secondary metabolites tambjamins A 44 and B 45 to locate its prey and flee when the concentration is higher

The remaining examples demonstrate the use of olfaction to find food, but without identifying the chemical compounds involved. The sea anemone *Anthopleura elegantissima* constitutes a source of food for the aeolid nudibranch *Aeolidia papillosa*, and it is detected via its chemical cues²²⁰. However, no compound has been identified and the chemical cue could originate from either the sea anemone or from their endosymbiotic algae. Another example involves the specific interaction between *Phestilla sibogae* and *Porites* spp. for which we previously discussed cues used in settlement. The nudibranch is also able to detect chemical cues from *Porites* in water as an adult, but no compound has been identified¹⁸⁷. The hydroid *Tubularia crocea* is a food source for the nudibranch *Hermisenda crassicornis* which detects its prey remotely using chemoreception²²¹. Furthermore, *Tylodina perversa* is also able to track chemical cues from the sponges *Aplysina aerophoba* and *A. cavernicola*, as they both contain the same brominated compound family, yet the mollusc only consumes *A. aerophoba*¹⁰⁶. Contrary to *A. cavernicola* that lives in shady environments, the preferred sponge, *A. aerophoba*, thrives in high solar conditions which enables cyanobacteria to proliferate on its surface. It is these cyanobacteria that *T. perversa* actually consumes, as well as some of the sponge, therefore it appears that *T. perversa* uses chemical cues from the sponge to locate its preferred food. Feeding preferences are actually influenced by the presence or absence of these photosynthetic organisms, as *T. perversa* prefers to feed upon sponges with high concentrations of cyanobacteria¹⁰⁸.

Pleurobranchaea californica uses chemical cues to detect previously encountered unpalatable prey²²². During the first encounter between *P. californica* and the aposematic nudibranch *Flabellina iodine*, the former attacks the latter, but the toxicity of the prey causes the predator to release it. During subsequent encounters, *P. californica* detects the presence of nudibranch chemical cues and alters its behavior so as not to physically encounter the toxic sea slug, an example of adaptive learned avoidance.

Finally, the scavenging gastropod *Nassarius festivus* spends most of its time resting in sand and uses its siphon to detect chemical cues from carrion and then uses chemoreception to find its prey²²³. However, it has been proven that ocean acidification can influence this behavior. Indeed, a pH of 7.0 has a strongly negative effect on foraging performance, such as reducing travel speed during foraging, foraging success and consumption rate while also increasing feeding time.

2.4.3. Secondary metabolites as inducers of mucus trail following

Mucus secretion is used by molluscs for several purposes including defense, sliding, as well as prey and conspecific recognition. Indeed, shelled and shell-less marine gastropods such as periwinkles^{224–231}, abalone²³², pulmonates^{233,234} and heterobranchs^{235,236} are known to use contact chemosensory to detect a conspecific's mucus trail. Most of the mucus trail is polarized enabling the mollusc to follow the trail "in the right direction" which maximizes the chances of encountering a conspecific^{225,236}. Some marine gastropods are even able to determine the sex of the congener²²⁷. This phenomenon enables the mollusc to find a conspecific by following the slime/mucus trail in order to mate. However, only one example of trail following with mucus containing diet-derived compounds has been demonstrated. Indeed, the above-mentioned nudibranch *Roboastra tigris* is able to detect and follow the mucus secreted by its prey *Tambja abdere* and *T. eliora*⁷³. Diet-derived tambjamines A-D **44-47** are present at low concentration in mucus and could be responsible for trail following.

2.5. Conclusion

Chemicals are essential for structuring marine gastropod-prey interactions. Marine gastropod molluscs benefit from the chemical defenses of their prey, steal these defenses, and sometimes even biotransform them to enhance their own defenses, use them as intraspecific chemical signals, or as tracking cues to locate their food. Shell-less species, such as nudibranchs, are able to sequester diet-derived defense compounds and create their own chemical shield against predators by concentrating metabolites in external body tissues. Additionally, the excretion of toxic diet-derived compounds via mucus or ink remains an efficient defense mechanism used by sacoglossans and sea hares against predators. The biotransformation of diet-derived compounds, driven by a well-organized enzymatic structure, may either further increase the strength of chemical defenses as found in some nudibranchs, or decrease toxicity such as in sea hares or shelled species. Indeed, biotransformation in shelled species is always carried out for the purpose of detoxification, whereas it has a dual role in shell-less species. It is therefore not unlikely that the ancient detoxification pathways of some molluscs have evolved into biotransformation pathways that aim to improve the defenses of the consumer. Chemical cues and signals are omnipresent in gastropod-prey interactions whether for foraging, settlement or as intraspecific pheromones, but only a few studies have identified the molecules involved in these processes. Therefore, identifying such chemicals constitutes an interesting challenge in coming years. Moreover, understanding such phenomena is essential to further comprehend the impact that our changing environment, such as global warming and ocean acidification, may have on structuring not only marine gastropod-prey interactions, but entire ecosystems.

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Chapter 3. Isolation of acyclic Laxaphycin B-Type Peptides: A Case Study and Clues to Their Biosynthesis

This chapter corresponds to a part of the following publication:

Bornancin, L.; Boyaud, F.; Mahiout, Z.; Bonnard, I.; Mills, S.C.; Banaigs, B.; Inguibert, N. Isolation and Synthesis of Laxaphycin B-Type Peptides: A Case Study and Clues to Their Biosynthesis. *Mar. Drugs* **2015**, *13*, 7285-7300.

Abstract

The laxaphycin's B family constitutes a group of five related cyclic lipopeptides isolated from diverse cyanobacteria from all around the world. This group shares a typical structure of 12 amino acids from the *L* and *D* series, some of them hydroxylated at the *beta* position, and all containing a rare beta-amino decanoic acid. Nevertheless, they can be differentiated due to slight variations in the composition of their amino acids, but the configuration of their *alpha* carbon remains conserved. Here, we provide the characterization of new laxaphycin B-type peptides. We isolate minor acyclic laxaphycins B, which are considered clues to their biosynthesis.

3.1. Introduction

Among marine organisms, filamentous cyanobacteria occupy a special place and/or are of great interest for chemists because they produce a wide range of bioactive molecules, mainly cyclic lipopeptides¹⁻³. Interestingly, they produce this class of secondary peptide metabolites via a non-ribosomal pathway that is responsible, for example, for the modification of natural amino acids into *D*-, *N*-methyl, β -hydroxylated, or dehydrated amino acids. These non-ribosomal peptide synthases (NRPS) are often associated with polyketide synthases (PKS) that allow fatty amino acids to be inserted within the peptide sequence^{4,5}. The concomitant effects of these two multi-domain enzymes contribute to the vast diversity of structure observed in these secondary cyclopeptide metabolites⁶. Laxaphycins are cyclic lipopeptides synthesized through a hybrid PKS/NRPS biosynthetic pathway by different marine or freshwater cyanobacteria. They contain amino acids of alternate stereochemistry (*L* or *D*) and feature a rare fatty β -amino acid with a linear chain of up to 12 carbons⁷. Several studies have reported structural variants and likely biosynthetic derivatives of laxaphycins that can be separated into two groups, the laxaphycin A-type peptides, which are cyclic undecapeptides, and the laxaphycin B-type peptides, which are cyclic dodecapeptides. Laxaphycin A-type and laxaphycin B-type peptides are generally found in the same

cyanobacteria. *Anabaena laxa*⁸, *A. torulosa*⁹, *Lyngbya confervoides*¹⁰, *Trichormus* sp.¹¹, and *cf. Oscillatoria* sp.¹² express laxaphycins B, B2, B3, and D, lobocyclamides B and C, and trichormamides B and C. Furthermore a *Lyngbya* sp. strain produces lyngbyacyclamides A and B¹³ (Figure 3. 1). Horizontal gene transfer between cyanobacteria has been suggested as an explanation for the presence of all these closely related compounds in diverse species¹⁴.

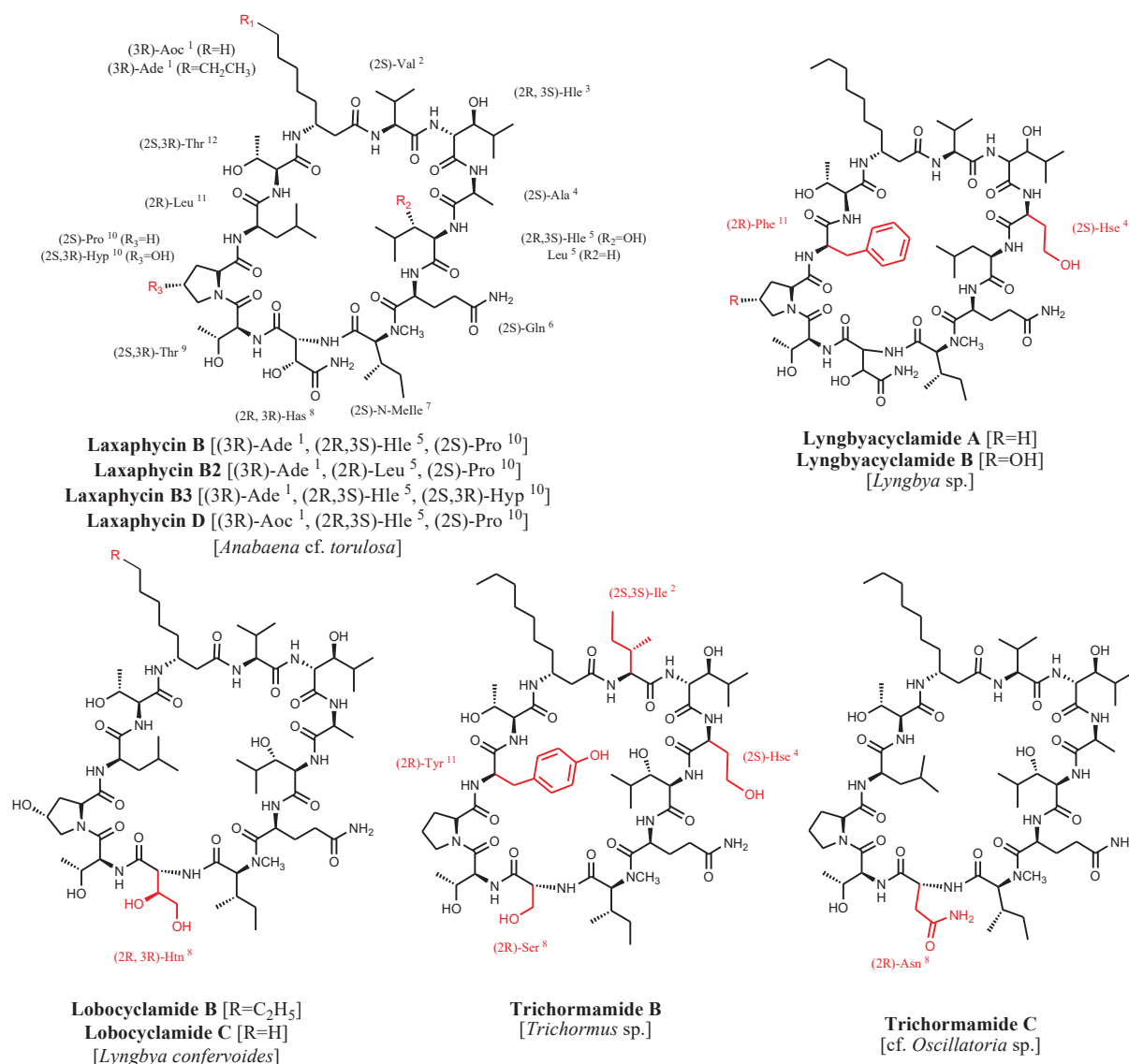


Figure 3. 1. Laxaphycins B, B2, B3, and D and their analogs lyngbyacyclamides A–B, lobocyclamides B–C and trichormamides B–C. Differences between laxaphycins and their homologs are highlighted in red.

In the present study we were interested in the study of laxaphycin peptides from *A. cf. torulosa*, compounds that have already been found to reduce damage by consumers¹⁵. Here we describe the structure of two new acyclic laxaB-type peptides. The presence of these acyclic dodecapeptides, named acyclolaxaphycin B (**3**) and acyclolaxaphycin B3 (**4**), together with the other laxaphycins in the extract of *A. cf. torulosa*, provide valuable information for the biosynthesis of laxaphycins.

3.2. Results and Discussion

Collection and extraction of *A. cf torulosa* and initial separation of the organic extract were described in a previous paper⁹. Further examination of the more polar flash chromatography fractions obtained from the organic extract, by C18 RP HPLC yielded two HPLC pure peaks, acyclolaxaphycin B (short form: acyclolaxaB) and acyclolaxaphycin B3 (short form: acyclolaxaB3). AcyclolaxaB (2 mg) and acyclolaxaB3 (3 mg) were obtained as colorless amorphous solids and responded positively to a ninhydrin test suggesting a non-blocked *N*-terminus. LC-MS analysis of pure compounds with electrospray positive ionization revealed two different peptides whose *m/z* values are 18 units higher than both laxaphycins B (1) and B3 (2).

3.2.1 Structure elucidation of Acyclolaxaphycins B (3) and B3 (4)

3.2.1.1. Acyclolaxaphycin B (3)

High-resolution electrospray ionization mass spectrometry (HRESIMS) analysis yielded an $[M + H]^+$ pseudomolecular ion at *m/z* 1413.8595 for a molecular formula of C₆₅H₁₁₆N₁₄O₂₀ that was supported by NMR spectroscopic analysis. A comparison with laxaphycin B (C₆₅H₁₁₄N₁₄O₁₉) revealed that this corresponds to a gain of H₂O.

In the ¹H-NMR spectrum of acyclolaxaB (3), recorded at 500 MHz in DMSO-*d*₆, the close structural relationship between the two peptides was clear; the spectrum exhibited, in the NH proton region, signals typical for CONH₂ protons corresponding to Gln (2 bs, δ_H6.79, and δ_H7.14) and Asn (2 bs, δ_H7.26, and δ_H7.30) similar to those observed for laxaB (Figure S3. 1). Only one significant difference was found in the NH proton region: nine NH doublets and one large singlet (2H) were visible in acyclolaxaB ¹H-NMR spectrum, instead of the 10 NH doublets observed between 7.4 and 8.4 ppm for laxaB.

Almost all ¹H and ¹³C resonances of acyclolaxaB (Table 3. 1) could be assigned using extensive 2D NMR analysis including COSY, TOCSY, HSQC, HSQC-TOCSY, and ROESY (Figures S3. 2–S3. 5). Initially, spin systems in TOCSY spectrum were identified starting from the signals of the backbone amide protons in the region 8.5 to 6.5 ppm. From the characteristic chemical shift and comparison with laxaB, eight amino acids could be identified as Hle (2×), Gln, Val, Leu, Thr (2×), and Has. A β-Ade residue system was identified starting from a doublet at 7.53 ppm and possessing an AA'BB' spin system (2.27 and 2.38 ppm) with additional signals at 4.05, 1.34–1.40, then 1.20–1.23 ppm. One spin system lacking an amide proton was identified as N-Melle due to the correlations of its H_α and H_β at 4.71 and 1.91 ppm, respectively. One last amino acid, attributed to Ala residue, was identified starting from a broad singlet (two protons) at 8.04 ppm, to H_α (δ_H4.03, 1H, overlapped bs) and H_β (δ_H1.36, 3H, d).

Table 3. 1. ¹H and ¹³C NMR data for laxaphycins B and B3 and acyclolaxaphycins B and B3 in DMSO-*d*₆.

	Laxaphycin B		Acyclolaxaphycin B		Laxaphycin B3		Acyclolaxaphycin B3	
	¹³ C	¹ H	¹³ C	¹ H	¹³ C	¹ H	¹³ C	¹ H
β Ade¹								
NH	-	7.58	-	7.53	-	7.52	-	7.53
CαH ₂	40.28	2.33/2.40	40.52	2.27/2.38	-	2.30/2.44	40.42	2.28/2.40
CβH	45.93	4.11	46.27	4.05	45.92	4.08	46.13	4.05
CγH ₂	33.45	1.29/1.40	33.62	1.34/1.40	33.41	1.40	33.49	1.33/1.40
CδH ₂	28.67 *	1.24	28.77	1.23	28.69 *	1.24	28.69	1.21
CεH ₂	28.47 *	1.20	28.61	1.20	28.47 *	1.20	28.53	1.21
CζH ₂	25.18 *	1.20	25.28	1.21	25.22 *	1.20	25.20	1.22
CηH ₂	31.11 *	1.20	31.22	1.21	31.10 *	1.20	31.15	1.20
CθH ₂	21.92 *	1.20	22.04	1.20	21.92 *	1.20	21.97	1.24
CιH ₃	13.79	0.84	13.91	0.85	13.79	0.82	13.81	0.83
CO	171.14	-	170.30	-	171.30	-	170.15	-
Val²								
NH	-	8.18	-	7.89	-	8.10	-	7.89
CαH	59.03	4.09	57.64	4.30	58.89	4.12	57.50	4.31
CβH ₂	29.33	1.97	30.59	2.02	29.37	1.98	30.51	2.02
CγH ₃	18.80	0.91	18.85	0.93	18.56	0.88	18.89	0.93
Cγ'H ₃	18.87	0.85	18.95	0.81	18.85	0.84	18.78	0.81
CO	171.05	-	171.27	-	171.30	-	171.15	-
Hle³								
NH	-	7.94	-	7.69	-	7.90	-	7.70
CαH	55.23	4.34	54.30	4.44	55.15	4.37	54.21	4.44
CβH	76.37	3.49	76.06	3.53	76.48	3.50	76.13	3.53
OH	-	4.94	-	—	-	4.90	-	—
CγH	30.54	1.58	30.68	1.51	30.57	1.60	30.84	1.52
CδH ₃	19.22 *	0.89	19.19	0.91	18.76 *	0.89	19.23	0.91
Cδ'H ₃	18.56	0.76	18.74	0.76	18.43	0.76	18.67	0.76
CO	171.35	-	172.40	-	-	-	172.34	-
Ala⁴								
NH/NH ₂	-	7.86	-	8.04	-	7.87	-	8.05
CαH	49.28	4.22	48.30	4.03	49.30	4.22	48.20	4.04
CβH ₃	17.55	1.31	17.43	1.36	17.65	1.32	17.38	1.36
CO	172.33	-	170.02	-	172.47	-	169.87	-
Hle⁵								
NH	-	7.69	-	8.34	-	7.61	-	8.37
CαH	55.52	4.28	55.40	4.44	55.64	4.28	55.27	4.46
CβH	75.80	3.49	76.21	3.53	75.78	3.48	75.94	3.53
OH	-	5.03	-	—	-	5.05	-	—
CγH	29.90	1.56	30.65	1.51	29.84	1.58	30.73	1.51
CδH ₃	18.65 *	0.89	17.58	0.82	18.69 *	0.88	19.14	0.83
Cδ'H ₃	18.56	0.76	19.28	0.81	-	0.74	17.52	0.83
CO	170.50	-	169.74	-	170.60	-	169.64	-
Gln⁶								
NH	-	7.77	-	8.02	-	7.56	-	8.04
CαH	49.16	4.63	48.94	4.69	49.40	4.58	48.78	4.70
CβH ₂	26.39	1.75/1.97	26.90	1.76/1.93	-	1.64/2.00	26.86	1.77/1.94
CγH ₂	30.72	2.04/2.10	30.63	2.12	-	2.15/2.23	30.70	2.13
CON	174.60	-	174.38	-	174.74	-	174.31	-
NH ₂	-	6.85/7.22	-	6.79/7.14	-	6.79/7.17	-	6.80/7.16
CO	172.49	-	172.45	-	172.64	-	172.27	-
N-Melle⁷								
NCH ₃	30.03	2.97	30.24	2.97	30.15	3.01	30.20	2.98
CαH	59.85	4.72	59.94	4.71	59.87	4.73	59.79	4.73
CβH	31.56	1.90	31.50	1.91	31.80	1.90	31.41	1.92
CγH ₂	23.88	0.89/1.29	23.98	0.87/1.28	-	0.74/1.27	23.92	0.87/1.27

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C γ H ₃	15.08	0.76	15.25	0.78	14.99	0.74	15.21	0.79
C δ H ₃	10.33	0.78	10.48	0.79	10.31	0.75	10.41	0.78
CO	170.02	-	169.66	-	170.10	-	169.47	-
Has ⁸								
NH	-	7.64	-	7.41	-	7.66	-	7.41
C α H	55.52	4.63	55.22	4.67	55.53	4.63	55.13	4.71
C β H	70.44	4.31	71.04	4.36	70.33	4.35	70.99	4.37
OH	-	5.79	-	5.78	-	5.70	-	-
CON	173.37	-	173.20	-	173.37	-	173.20	-
NH ₂	-	7.27	-	7.26/7.30	-	7.17	-	7.27/7.32
CO	169.16	-	168.92	-	169.12	-	168.75	-
Thr ⁹								
NH	-	7.33	-	7.63	-	7.12	-	7.63
C α H	55.61	4.49	55.25	4.57	55.83	4.46	55.56	4.56
C β H	66.23	3.93	66.54	3.98	66.43	3.90	66.50	3.97
OH	-	4.94	-	-	-	4.89	-	-
C γ H ₃	18.87	1.05	18.64	1.05	18.85 *	1.03	18.59	1.05
CO	168.58	-	168.87 *	-	168.70 *	-	169.04 *	-
Pro ¹⁰ /Hyp ¹⁰								
C α H	59.60	4.33	59.90	4.37	58.62	4.43	58.87	4.44
C β H ₂	29.08	1.82/2.04	28.77	1.83/2.03	37.73	1.84/2.01	37.45	1.89/2.05
C γ H ₂	24.00	1.80/1.90	24.16	1.83/1.90	68.50	4.32	68.48	4.31
OH	-	-	-	-	-	5.08	-	-
C δ H ₂	47.16	3.68	47.49	3.64/3.75	55.48	3.58/3.72	55.60	3.60/3.76
CO	171.21	-	171.42	-	171.47 **	-	171.33	-
Leu ¹¹								
NH	-	7.89	-	7.77	-	7.86	-	7.84
C α H	51.36	4.31	51.44	4.30	51.31	4.35	51.36	4.29
C β H ₂	40.82	1.47	40.44	1.47	41.24	1.47	40.51	1.46
C γ H	24.06	1.53	24.09	1.58	24.12	1.52	24.06	1.58
C δ H ₃	22.71	0.87	22.96	0.86	22.75	0.86	22.83	0.86
C δ' H ₃	21.76	0.82	21.42	0.84	21.72	0.80	21.43	0.83
CO	171.67	-	171.83	-	171.41 **	-	171.33	-
Thr ¹²								
NH	-	7.74	-	7.57	-	7.68	-	7.59
C α H	57.85	4.11	58.13	4.10	58.17	4.10	58.12	4.10
C β H	66.19	4.00	66.52	3.97	66.35	3.97	66.50	3.97
OH	-	4.78	-	-	-	4.80	-	-
C γ H ₃	19.46	0.99	19.55	0.99	19.48	0.99	19.45	1.00
CO	168.67	-	168.87 *	-	168.67 *	-	168.99 *	-

*** Thr⁹ and Thr¹² Chemical shifts may be interchanged.

Sequence-specific assignments were determined from the HMBC correlations (Figure S3. 6) between carbonyl carbons (residue *i*) and NH or NCH₃ protons (residue *i*+1). These data suggested the presence of two fragments consisting of Ala-Hle-Gln-N-Melle-Has-Thr (fragment 1) and Pro-Leu-Thr- β -Ade-Val-Hle (fragment 2). These two partial sequences were confirmed by ROESY correlations between H α or H β (residue *i*) and NH or NCH₃ (residue *i*+1). Fragments 1 and 2 were assembled by two inter-residue ROESY correlations between H α (δ_{H} 4.57) and H β (δ_{H} 3.98) of Thr⁹ and H δ (δ_{H} 3.64/3.75) of Pro¹⁰, establishing the complete sequence as Ala-Hle-Gln-N-Melle-Has-Thr-Pro-Leu-Thr- β -Ade-Val-Hle (Figure 3. 2). MS/MS data for **11** were consistent with the proposed amino acid sequence with the *y* ions at *m/z* 1213.50 (*y*10), 1085.58 (*y*9), 828.42 (*y*7), and 727.42 (*y*6) and the *b* ions at *m/z* 1266.75 (*b*11), 1167.58 (*b*10), and 456.25 (*b*4).

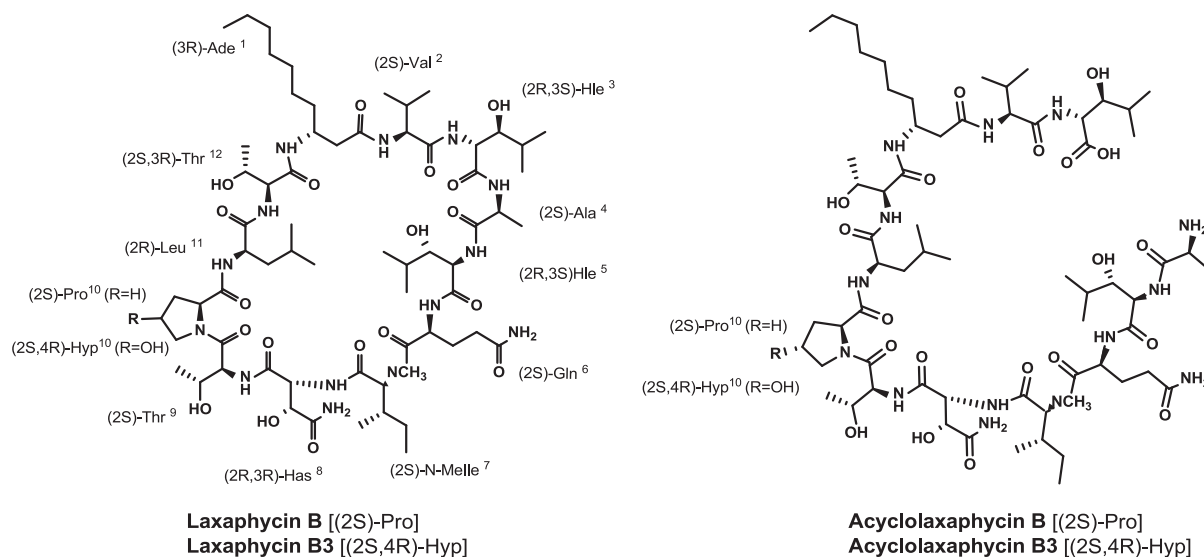


Figure 3. 2. Structures of laxaphycins B (1) and B3 (2), and acyclolaxaphycins B (3) and B3 (4).

3.2.1.2. Acyclolaxaphycin B3 (4)

Preliminary spectral data examination, including ^1H and ^{13}C -NMR spectroscopy, showed that the new compound was an analog of laxaB3 (**2**) and acyclolaxaB (**3**) (Figure S3. 7). HRESIMS analysis yielded a $[\text{M} + \text{H}]^+$ pseudomolecular ion at m/z 1429.8482 for a molecular formula of $\text{C}_{65}\text{H}_{116}\text{N}_{14}\text{O}_{21}$. In comparison to laxaB3 ($\text{C}_{65}\text{H}_{114}\text{N}_{14}\text{O}_{20}$), this corresponds to a gain of H_2O and to acyclolaxaB gain of an oxygen atom.

A similar pattern of fragmentation for both compounds **3** and **4** was observed. Comparison of MS/MS spectra showed the same b4 fragment at m/z 456.25, the b11 (m/z 1282.76), y6 (m/z 743.49), y7 (m/z 844.54), y9 (m/z 1101.68), and y10 (m/z 1229.72) ions being shifted to a higher mass by 16 amu. In the HRESIMS/MS spectra of **4**, b9 (m/z 1014.55060, $\text{C}_{44}\text{H}_{76}\text{N}_{11}\text{O}_{16}^+$, $\Delta\text{obs}/\text{calc} = 0.004$) and b11 (m/z 1282.76672, $\text{C}_{59}\text{H}_{104}\text{N}_{13}\text{O}_{18}^+$, $\Delta\text{obs}/\text{calc} = 0.005$) fragments were observed. The y6 and b9 fragments in compound **4** shifted by 16 amu compared to **3**, suggesting that the variable residue could be in position 10, 11, or 12, corresponding to the Pro, Leu, or Thr residues, respectively.

The NMR spectral analysis (Figures S3. 7–S3. 12) of acyclolaxaB3 showed remarkable similarities with acyclolaxaB (**3**) and established the variable residue as Pro/Hyp (Figure 3. 2). The significant difference was the presence of an additional hydroxyl group on proline [Hy at 4.31 ppm vs. two Hy at 1.83 and 1.90 ppm for compound **3**; Cy at 68.48 ppm vs. 24.16 ppm for compound **3**; C β and C δ were also deblinded by the presence of the hydroxyl function ($\Delta\delta$ 8.68 and 8.11 ppm, respectively)]. HMBC and ROESY correlations established the complete sequence as Ala-Hle-Gln-N-Melle-Has-Thr-Hyp-Leu-Thr- β -Ade-Val-Hle for compound **4**, and the gross structure of the new compound, acyclolaxaphycin B3, differed from acyclolaxaphycin B with a replacement of Pro by Hyp.

3.2.2. Acyclolaxaphycins B (3) and B3 (4): Clues to Their Biosynthesis

Acyclolaxaphycins B (3) and B3 (4) are acyclic analogs of laxaphycins B (1) and B3 (2), respectively. They are two novel acyclic structural variants of a core structure (B-type laxaphycins) composed of about 10 cyanobacterial β -amino fatty acid cyclic dodecapeptides, laxaphycins B, B2, B3, and D, lnyngbyacyclamides A–B, lobocyclamides B–C, and trichormamides B–C (Figure 3. 1) with conserved amino acid residues. The chemical structure of all these compounds were similar, maintaining a 12-membered ring and sharing the (3*R*)- β -amino fatty acid (β -Aoc or β -Ade), (2*R*,3*S*)-Hle, (2*S*)-Gln, (2*S*)-N-Melle, (2*S*,3*R*)-Thr, and (2*S*,3*R*)-Thr in the positions 1, 3, 6, 7, 9, and 12. In positions 2, 4, 5, 8, 10, and 11, the amino acid residues can vary, but their configuration at each position is strongly conserved.

An important subset of the β -hydroxylation of various amino acid residues observed for non-ribosomal synthesized peptides is catalyzed by cytochrome P450 monooxygenases [32–34]. The same biological machinery is certainly responsible for the β -hydroxylation of leucine and asparagine in laxaphycins and as this reaction is stereospecific, the stereochemistry of hydroxy-leucines, in position 3 and 5, and hydroxy-asparagine, in position 8, must be conserved.

Furthermore, both new peptides showed very similar NMR chemical shifts to laxaphycins B and B3 for the peptidic chain as well as for the side chains, indicating a conservation of the stereochemistry between cyclic and acyclic analogs. An indication of this homology could be seen in the comparison of ^1H and ^{13}C resonances of acyclolaxaphycins B and B3 with the parent compounds laxaphycins B and B3 ($\text{H}\alpha$, $\text{H}\beta$, $\text{C}\alpha$, and $\text{C}\beta$ acyclolaxaphycins B and B3 resonances subtracted from the equivalent ones of laxaphycin B and B3, respectively). With the exception of the structurally modified parts of the molecule, residues 4 and 5 on the NH terminal side and residues 2 and 3 on the COOH terminal side, the maximum difference ($\Delta\delta$) observed was less than 0.1 ppm in ^1H and 0.8 ppm in ^{13}C .

Therefore the complete structure of the two new compounds can be reasonably proposed as: (2*S*)-Ala—(2*R*,3*S*)-Hle—(2*S*)-Gln—(2*S*,3*S*)-N-Melle—(2*R*,3*R*)-Has—(2*S*,3*R*)-Thr—(2*S*)-Pro—(2*R*)-Leu—(2*S*,3*R*)-Thr—(3*R*)- β -Ade—(2*S*)-Val—(2*R*,3*S*)-Hle for acyclolaxaphycin B (3) and (2*S*)-Ala—(2*R*,3*S*)-Hle—(2*S*)-Gln—(2*S*,3*S*)-N-Melle—(2*R*,3*R*)-Has—(2*S*,3*R*)-Thr—(2*S*,4*R*)-4-Hyp—(2*R*)-Leu—(2*S*,3*R*)-Thr—(3*R*)- β -Ade—(2*S*)-Val—(2*R*,3*S*)-Hle for acyclolaxaphycin B3 (4).

A putative operon encoding the biosynthetic pathway for β -amino fatty acid lipopeptides, the puwainaphycins, was identified in the cyanobacterium *Cylindrospermum alatosporum*; the peptide biosynthesis process is initiated by the activation of a fatty acid residue via fatty acyl-AMP ligase (FAAL) and continued by a multidomain non-ribosomal peptide synthase/polyketide synthetase¹⁶. The last module incorporates a thioesterase domain in its terminal part that cleaves the finished puwainaphycin chain from the peptidyl

carrier protein, thus promoting its cyclization between the NH₂ of β-amino fatty acid and the COOH of a proline residue. The characterization of the two novel acyclic laxaphycin variants **3** and **4** with alanine as NH terminal and Hle as COOH terminal seemed to indicate that in the case of B-type laxaphycins, the biosynthesis process starts with NRPS modules instead of FAAL and acyl carrier protein (ACP) ligase, with the ring closure being performed through a cyclization reaction between the amino group of the alanine residue and the carbonyl of the hydroxyleucine residue. However, one cannot exclude that acyclolaxaphycins B and B3 are enzymatic degradation products formed during cyanobacteria blooms. Enzymatic degradation is often used in resistance mechanisms in the microbial world or in competitive interspecific interactions. Enzymes that degrade or modify natural products provide protection by decreasing toxicity or by regulating the signaling functions of metabolites. Recently, Hoefler *et al.* have observed hydrolysis of cyclic lipopeptides surfactins by bacterial competition using imaging mass spectrometry¹⁷. However, the ring opening of the cyclic surfactins occurred at the ester functional group, which is not the case in laxaphycin peptides.

3.3. Experimental Section

3.3.1. Sampling Sites

The cyanobacterium *A. cf. torulosa* was collected by SCUBA diving at a depth of 1–3 m in the Pacific Ocean, Moorea, French Polynesia. The cyanobacterium sample was sealed underwater in a bag with seawater and then freeze-dried.

3.3.2. Isolation Procedure

The freeze-dried sample of cyanobacterium *A. cf. torulosa* (600 g) was extracted at room temperature three times with a mixture of MeOH-CH₂Cl₂ (1:1) and ultrasound. The combined extracts were evaporated under reduced pressure to give a greenish organic extract (38 g). The extract was subjected to flash RP18 silica gel column eluted with H₂O (A), H₂O-CH₃CN (2:8) (B), MeOH (C), and MeOH-CH₂Cl₂ (8:2) (D) to afford four fractions (A, B, C, and D). Then, fraction B (2 g) was subjected to flash RP18 silica gel column eluted with a solvent gradient of H₂O-CH₃CN to produce 12 fractions. Fraction 4 was subjected to HPLC purification (UP-50 DB.25M Uptisphere 5 μ) using 62% H₂O-CH₃CN at a flow rate of 3 mL/min to give acyclolaxaphycin B3 (3 mg, tr = 28.8 min) and acyclolaxaphycin B (2 mg, tr = 31.2 min).

3.3.3. Mass and NMR Spectroscopies

High-resolution ESI mass spectra were obtained using a Thermo Scientific LTQ Orbitrap mass spectrometer using electrospray ionization in positive mode. 1D-NMR and 2D-NMR experiments of compounds **1**, **2**, **3**, and **4** were acquired on a Bruker Avance 500

spectrometer equipped with a cryogenic probe (5 mm), all compounds in DMSO-*d*₆ (500 μL) at 303 K. All chemical shifts were calibrated on the residual solvent peak [DMSO-*d*₆, 2.50 ppm (¹H) and 39.5 ppm (¹³C)]. The chemical shifts, reported in delta (δ) units, and in parts per million (ppm) are referenced relatively to TMS.

3.4. Conclusions

In summary, we have isolated two new linear lipopeptides acyclolaxaphins B and B3 (**3**, **4**) from the tropical marine cyanobacterium *Anabaena cf. torulosa*. The presence of these acyclic laxa B-type compounds together with the cyclized ones in the same extract has, to our knowledge, never been described. The search of other minor acyclic potential biosynthetic precursors will provide valuable information concerning the hybrid PKS/NRPS biosynthetic pathway in this exciting lipophilic cyclic dodecapeptide series.

-Supporting Information

Supplementary data (¹H NMR, ¹³C, TOCSY, HSQC, HSQC-TOCSY, HMBC, and ROESY spectra of **3** and **4**) associated with this chapter are available at the end of this thesis (S.3. 1-S3. 12).

3.5. References

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Chapter 4. Cyclic and Acyclic Laxaphycins: Structure and Biological Evaluation of New Natural Analogs

Abstract

5 new laxaphycins have been isolated and fully characterized from the bloom forming cyanobacteria *Anabaena cf. torulosa* sampled from Moorea, French Polynesia; three acyclic peptides, acyclolaxaphycin A (**1**), [des-Gly¹¹]laxaphycin A (**2**) and [des-(Leu¹⁰-Gly¹¹)]laxaphycin A (**3**), as well as two cyclic peptides, laxaphycins A2 (**4**) and A3 (**5**). The absolute configuration of the amino acid residues were established using advanced Marfey's analysis for compounds **2-5** and the previously described acyclolaxaphycins B (**6**) and B3 (**7**). This is the first report of acyclic analogues within the laxaphycin family and their congeners. The isolation of acyclic laxaphycins provides valuable insight into the biosynthesis of laxaphycins. A biological evaluation of the new compounds, together with the already known laxaphycins A, B, and B3, shows that laxaphycin A- and B-type peptides induce differential cell damage.

4.1. Introduction

Marine organisms constitute a prolific source of secondary metabolites that show a range of bioactivities including antibacterial, antitumoral, antifungal or antimalarial^{1,2}. Among them, filamentous cyanobacteria are recognized as producing a wide range of bioactive molecules, mainly cyclic lipopeptides³⁻⁵. The biosynthesis of such compounds consists of a well organized enzymatic machinery involving the multifunctional enzymes: non-ribosomal peptide synthases (NRPS), polyketide synthases (PKS) and hybrid NRPS/PKS⁶⁻⁸. These enzymes are organized in modules and are responsible for the biosynthesis of peptides with usual and non-proteinogenic amino acids. Indeed, NRPS are responsible for the modification of natural amino acids into *D*-, *N*-methyl, β -hydroxylated, or dehydrated amino acids, whereas PKS, sometimes associated with other enzymes such as FAAL (fatty acyl-AMP ligase), enable the insertion of fatty amino acids into the peptide sequence.

Laxaphycins and congeners are lipopeptides that have been isolated from several cyanobacteria found worldwide and contain non-proteinogenic amino acids such as a β -amino acid with an aliphatic side chain (8 or 10 carbon atoms) and *D*-amino acids. They are divided into two sub-families; laxaphycin A- and B-type peptides with laxaphycin A, a cyclic undecapeptide, and laxaphycin B, a cyclic dodecapeptide, as the representative compounds of each sub-family respectively. The configuration of the *alpha* carbon is conserved across the two sub-families despite differences in the side chains.

The biosynthesis of laxaphycins is carried out by different marine, freshwater or terrestrial cyanobacteria using a hybrid NRPS/PKS biosynthetic pathway. The laxaphycin A sub-family contains 7 peptides with laxaphycin A^{9,10}, hormothamnin A¹¹, laxaphycin E, lobocyclamide A¹², scytocyclamide A¹³, trichormamides A¹⁴ and D¹⁵ produced by *Anabaena cf torulosa*, *Anabaena laxa*, *Hormothamnion enteromorphoides*, *Lyngbya confervoides*, *Scytonema hofmanni*, *Trichormus* sp. and *Oscillatoria* sp respectively (Figure 4. 1). Interestingly, laxaphycin A is produced by both the freshwater strain, *A. laxa* and the marine strain, *A. cf torulosa*. The presence of similar compounds with a comparable biosynthetic pathway in different cyanobacterial strains can be explained by horizontal gene transfer events between cyanobacteria¹⁶.

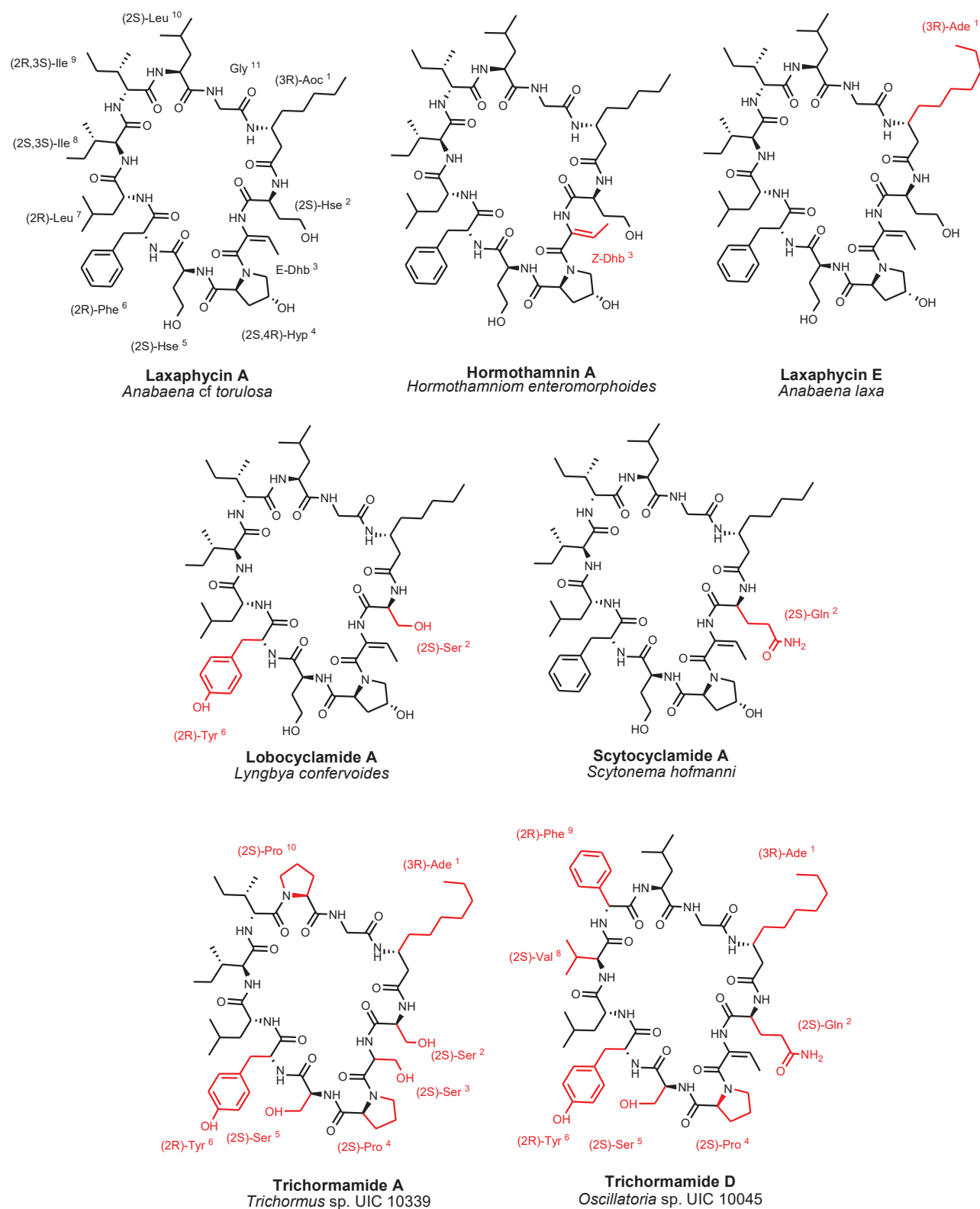


Figure 4. 1. Laxaphycins A and E, and the analogues Hormothamnin A, Lobocyclamide A, Scytocyclamide A and Trichormamides A and D. Amino acid modifications to the reference compound laxaphycin A are highlighted in red.

The structure characterization of acyclolaxaphycins B and B3, acyclic analogues of laxaphycins B and B3 isolated from *A. cf torulosa* have recently been published¹⁷. Here we fully characterize five new laxaphycin A-type peptides, three acyclic analogues named acyclolaxaphycin A (**1**), [des-Gly¹¹]acyclolaxaphycin A (**2**) and [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A (**3**) and two cyclic forms termed [L-Val⁸]laxaphycin A (**4**) and [D-Val⁹]laxaphycin A (**5**). Moreover, the absolute configurations of the previously described acyclolaxaphycins B (**6**) and B3 (**7**) were investigated using Marfey's method. The presence of cyclic and acyclic laxaphycins in the extract of *A. cf torulosa* may provide valuable insight into the biosynthesis of laxaphycin.

4.2. Results and discussion

The cyanobacterium *Anabaena cf torulosa* was sampled during a bloom in the lagoon of Moorea, French Polynesia, sealed underwater in a bag, freeze-dried and extracted. HPLC-DAD-ELSD and LC-MS analysis of the crude extract revealed an unusual chromatographic profile (Figure 4. 2) with an additional polar group of five potentially new peptides with molecular weights of 1043, 1156, 1213 and 1181 (two compounds).

The crude extract was fractionated using flash chromatography and the resulting fractions containing the new peptides were subjected to HPLC purification to yield acyclolaxaphycin A (**1**), [des-Gly¹¹]acyclolaxaphycin A (**2**), [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A (**3**), [L-Val⁸]laxaphycin A (**4**) and [D-Val⁹]laxaphycin A (**4**). All compounds were obtained as colorless amorphous solids and compounds 1, 2 and 3 responded positively to a ninhydrin test suggesting a non-blocked *N*-terminus.

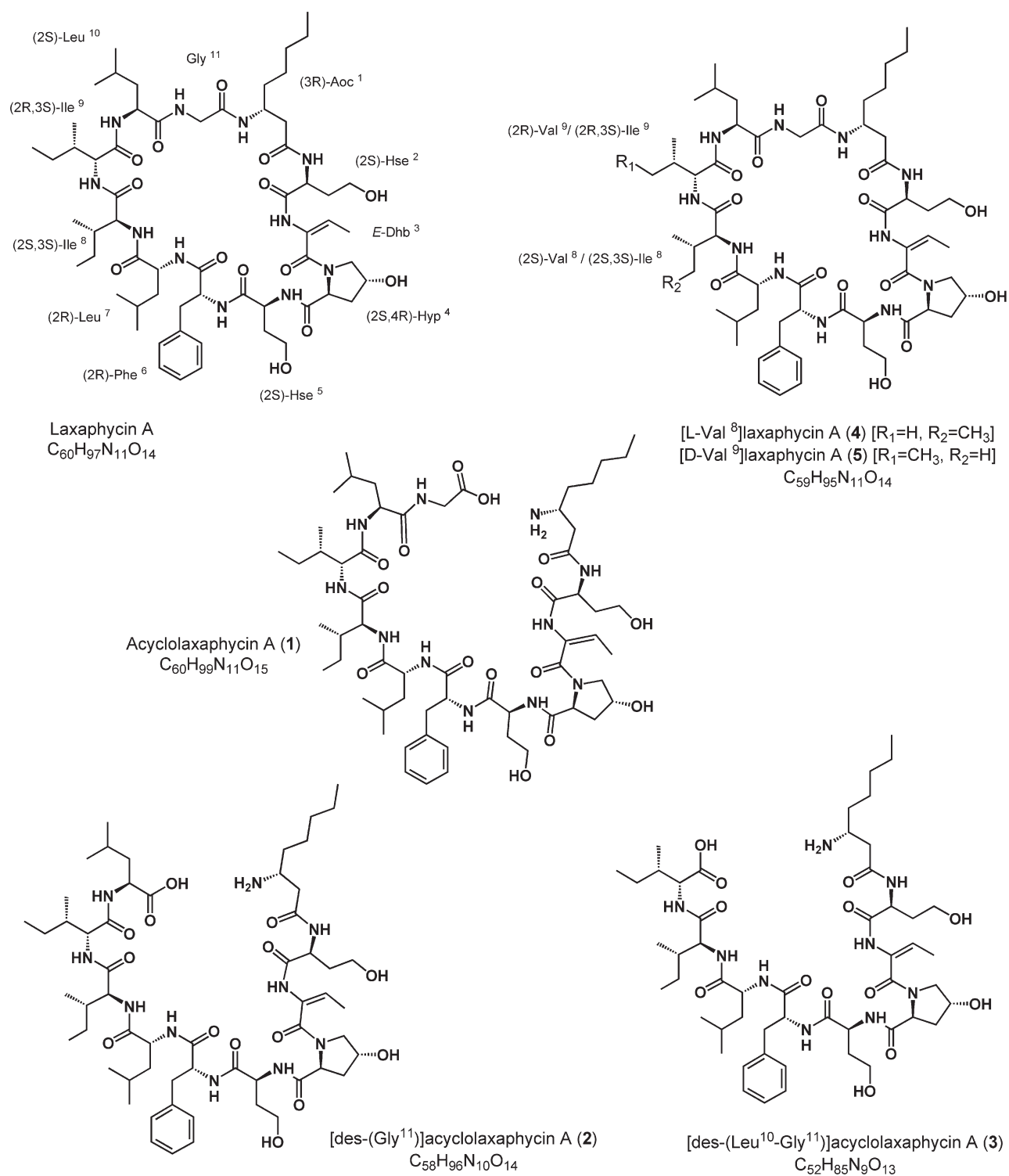


Figure 4. 2. The structures of compounds 1-5 in comparison with laxaphycin A.

4.2.1. Structure elucidation of acyclolaxaphycin A (1), [des-Gly¹¹]acyclolaxaphycin A (2), [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A (3):

The positive high-resolution electrospray ionization mass spectrometry (HRESIMS) spectrum of compound 1 gave a $[M + H]^+$ pseudomolecular ion at m/z 1214.7380, indicating a molecular formula of C₆₀H₉₉N₁₁O₁₅. The molecular formula of compound 2 was determined to be C₅₈H₉₆N₁₀O₁₄ (m/z 1157.7231 $[M + H]^+$) while the HRESIMS spectrum of compound 3 revealed a $[M + H]^+$ pseudomolecular ion at m/z 1044.6385, corresponding to a molecular formula of C₅₂H₈₅N₉O₁₃.

A comparison between the molecular formulas of compound 1 and laxaphycin A (C₆₀H₉₇N₁₁O₁₄) suggests that there is an additional H₂O in compound 1. The difference between compounds 2 and 1 corresponded to a loss of a Gly residue (C₂H₃NO), while the difference between compounds 3 and 2 was a loss of C₆H₁₁NO which could be attributed to a Leu residue. The signal distribution pattern observed in ¹H-NMR spectrum of compounds 1, 2 and 3 is characteristic of lipopeptides with amide NH signals resonating at δ_H 7.30-8.50, C α H signals at δ_H 3.5-4.7, aliphatic CH₂ at δ_H 1.1-1.3 and CH₃ signals at δ_H 0.7-0.9. The presence in each of these 3 spectra of diagnostic signals corresponding to the presence of Dhb indicated that these 3 peptides were related to laxaphycin A.

Acyclolaxaphycin A (1)

The ¹H-NMR spectrum of Acyclolaxaphycin A (1) (Figure S4. 1) revealed a strong structural similarity with laxaphycin. The spectrum featured typical signals of aromatic protons corresponding to Phe (δ_H 7.15-7.30), as well as NH, H β and H γ corresponding to Dhb residues (δ_H 9.93, 5.55 and 1.66, respectively). In the NH proton region, only eight NH doublets and one singlet were observed instead of the nine doublets and one singlet observed for laxaphycin A.

Almost all ¹H and ¹³C resonances of acyclolaxaphycin A could be assigned using extensive 2D-NMR analyses including COSY, TOCSY, HSQC, HSQC-TOCSY, and ROESY (Table 4. 1) (Figures S4. 1- S4.7). Firstly, analysis of the TOCSY spectra enabled us to establish the structure of nine amino acids: Leu (x2), Ile (x2), Hse (x2), Phe, Gly and Dhb. The structure of Aoc was determined using COSY and TOCSY spectra that assigned characteristic values of H α (δ_H 2.45), H β (δ_H 3.30) and aliphatic protons at 1.50, 1.30, 1.26 and 1.22 ppm. Furthermore, HSQC-TOCSY and HMBC correlations from methylene signals (δ_H 1.22, 1.26 and 1.30) highly overlapping with three carbons (δ_C 21.71, 24.39 and 30.92 respectively) completed the structure of Aoc. H₂NAoc was not observed in the conditions used in the experiment. A spin system lacking an amide proton was identified as Hyp due to the correlations between H α (δ_H 4.43) and H β (δ_H 1.93/2.09), H β and H γ (δ_H 4.24), as well as those between H γ and H δ (δ_H 3.39/3.49). Due to the amount of compound 1 available (1.5 mg), the lack of correlations of ³J_{CH} or ⁴J_{CH} in HMBC spectra between carbonyl carbons (residue i) and NH or NCH₃ protons

(residue $i+1$) prevent us from determining sequence-specific assignments. Nevertheless, ROESY correlations (Figure 4. 6) between H_{α} and eventually H_{β} (residue i) and NH (residue $i+1$), with additional correlations between $H\delta$ Phe/ H_{α} Hse, H_{γ} Hse/ H_{γ} Dhb, and H_{γ} Dhb/ $H\delta$ Hyp, suggest the presence of two fragments consisting of Aoc-Hse-Dhb-Hyp-Hse-Phe (fragment 1) and Leu-Ile-Ile-Leu-Gly (fragment 2).

The initial protonation of the acyclic peptide and subsequent fragmentation by loss of amino acid residues of acyclolaxaphycin A by ESIMS/MS revealed the presence of b and y ion fragments. NMR analysis found the presence of b ions at m/z 1139.71 (b10), 1026.63 (b9), 913.54 (b8), 800.4 (b7), 687.38 (b6), 540.26 (b5), 326.21 (b3) and 243.17 (b2) and y ions at m/z 889.54 (y8) and 675.45 (y6) which connect fragments 1 and 2. A second series of fragments (b' and y') was found due to the increased basicity of amide nitrogen atoms of the N-alkylated 4-hydroxyproline (Hyp⁴) protonation and cleavage of these amide bonds. The presence of b' ions at m/z 814.51 ($b'7$), 701.43 ($b'6$), 588.34 ($b'5$), 475.26 ($b'4$) and 362.17 ($b'3$) confirm the partial sequence Hyp-Hse-Phe-Leu-Ile-Ile-Leu-Gly (Figure 4. 3).

The overall mass fragmentation (Figure S4. 8) analysis established the complete sequence as Aoc-Hse-Dhb-Hyp-Hse-Phe-Leu-Ile-Ile-Leu-Gly supporting the proposed structure of **1** that we named acyclolaxaphycin A (Figure 4. 2). The gross structure of acyclolaxaphycin A differs from laxaphycin A due to a ring opening between residues 1 and 11.

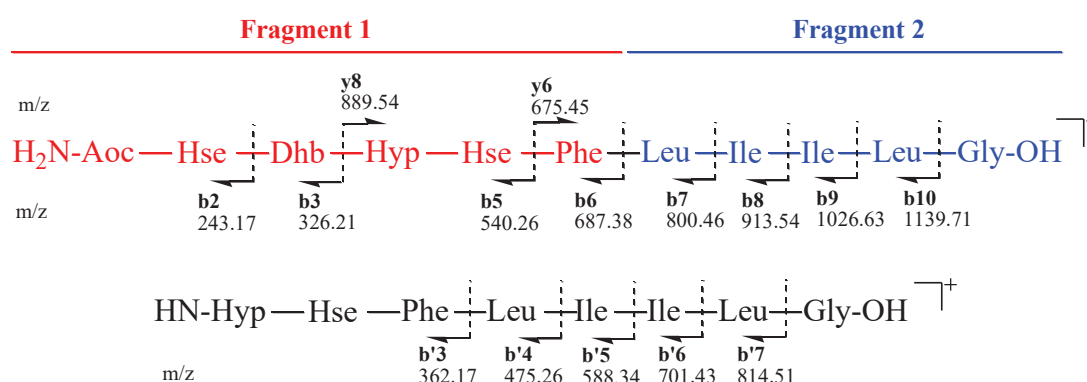


Figure 4. 3. ESIMS/MS fragmentation of acyclolaxaphycin A (1)

[des-(Gly¹¹)]acyclolaxaphycin A (2)

Examination of preliminary spectral data, including ESIMS/MS, ¹H, and ¹³C-NMR (Figures S4. 9-S4. 10) spectroscopy, showed that the new metabolite was a lower homologue of compound 1. The NMR spectral analysis of compound 2 showed remarkable similarities with acyclolaxaphycin A (Table 4. 1) although the ¹H-NMR spectrum revealed the presence of seven doublets in the amide region, one singlet and a broad singlet (2H) instead of eight doublets and one singlet for acyclolaxaphycin A. TOCSY and ROESY (Figures S4. 12 and S4. 15) analyses showed a correlation between the spread NH singlet at δ H 7.77 and H_{α} and H_{β}

of Aoc which establishes that an N-term is present on the Aoc. Other significant differences lie in the lack of a carbonyl signal, as well as a H α signal of Gly, which confirm the lack of Gly in (2). The HMBC cross-peaks between carbonyl carbons (residue *i*) and NH protons (residue *i*+1) suggest the presence of two fragments including β -Aoc-Hse-Dhb (fragment 1) and Hyp-Hse-Phe-Leu-Ile-Ile-Leu (fragment 2). Analysis of ROESY correlations between H α or H β (residue *i*) and NH (residue *i*+1) confirmed the presence of these two partial sequences. Moreover, the two fragments (1 and 2) were assembled by two inter-residue ROESY correlations between H γ (δ H 1.67) of Dhb and H δ (δ H 3.36/3.45) of Hyp resulting in a complete sequence of β -Aoc-Hse-Dhb-Hyp-Hse-Phe-Leu-Ile-Ile-Leu.

MS/MS data (Figure S4. 16) for (2) were consistent with the proposed amino acid sequence and the presence of *y* ions at *m/z* 832.52 (*y*₇) and 618.42 (*y*₅) and *b* ions at *m/z* 1026.63 (*b*₉), 913.54 (*b*₈), 800.46 (*b*₇), 687.38 (*b*₆) and 540.26 (*b*₅) for fragment 1, and 701.43 (*b*'₆), 588.34 (*b*'₅), 475.26 (*b*'₄) and 362.17 (*b*'₃) for fragment 2 (Figure 4. 4).

Mass fragmentation and NMR data established the amino acid sequence to be Aoc-Hse-Dhb-Hyp-Hse-Phe-Leu-Ile-Ile-Leu supporting the proposed structure of 2 that we named [des-(Gly11)]acyclolaxaphycin A (Figure 4. 2). [des-(Gly11)]acyclolaxaphycin A gross structure differs from laxaphycin A due to a ring opening between residues 1 and 11 and the subsequent loss of Gly11.

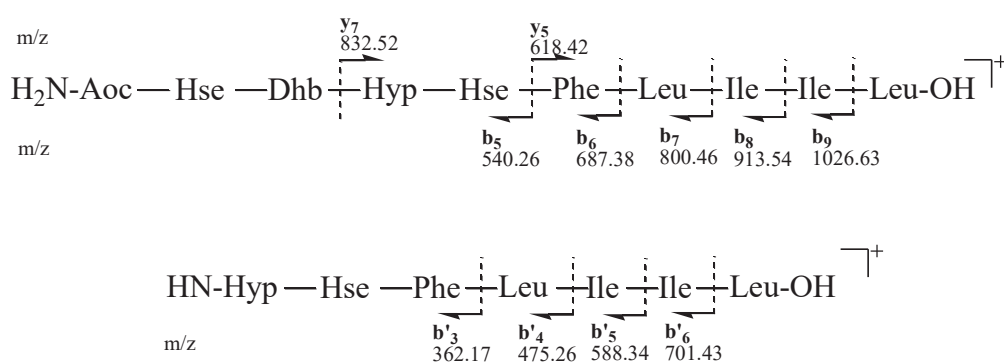


Figure 4. 4. ESIMS/MS fragmentation of [des-(Gly11)]acyclolaxaphycin A (2)

[des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A (3)

The ¹H and ¹³C spectra of compound 3 (Figures S4. 17-S4. 18) are almost identical to those of (2) except for the absence of a NH doublet and a carbonyl, indicating the loss of an amino acid (Table 4. 1). Analysis of TOCSY data (Figure S4. 20) confirmed the lack of a Leu residue as suggested by HRESIMS data. As is the case for [des-(Gly¹¹)]acyclolaxaphycin A, HMBC spectrum provided information on sequence-specific assignments. Indeed, ³J_{CH} correlations between carbonyl carbons (residue *i*) and NH protons (residue *i*+1) suggested the presence of two fragments: β -Aoc-Hse-Dhb (fragment 1) and Hyp-Hse-Phe-Leu-Ile-Ile (fragment 2) and the lacking residue was identified as Leu¹⁰ compared to (2). ROESY

correlations (Figure S4. 23) between $H\alpha$ or $H\beta$ (residue i) and NH (residue $i+1$) confirmed the structure of the partial sequence and correlations between $H\delta$ (δ_H 3.37/3.46) of Hyp and $H\beta$ and $H\gamma$ (δ_H 1.68) of Dhb defined the complete sequence as β -Aoc-Hse-Dhb-Hyp-Hse-Phe-Leu-Ile-Ile.

The sequence was confirmed by ESIMS/MS data analyses (Figure S4. 24), which revealed the presence of y ions at m/z 719.43 (y_6) and b ions at m/z 913.54 (b_8), 800.46 (b_7), 687.38 (b_6), 540.26 (b_5), and 326.21 (b_3) and 701.43 (b'_6), 588.34 (b'_5), 475.26 (b'_4), and 362.17 (b'_3) (Figure 4. 5).

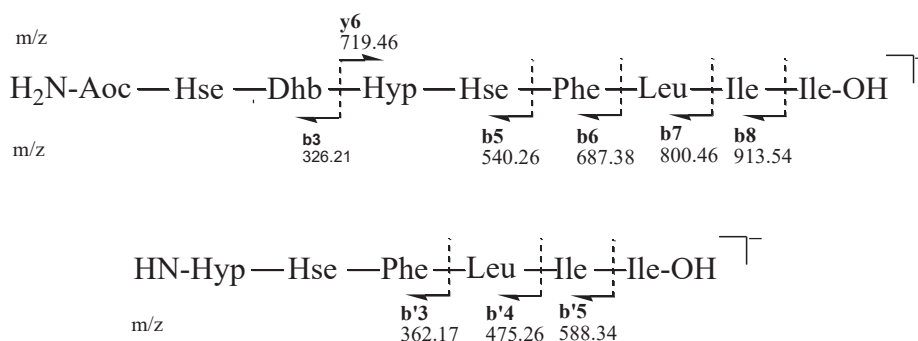


Figure 4. 5. ESIMS/MS fragmentation of [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A (**3**)

Mass fragmentation and NMR data established the amino acid sequence as Aoc-Hse-Dhb-Hyp-Hse-Phe-Leu-Ile-Ile supporting the proposed structure of **3** that we named [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A (Figure 4. 2). The gross structure of [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A differs from compound **2** by a ring opening between residues 1 and 10, and the subsequent loss of Gly¹¹ and Leu¹⁰.

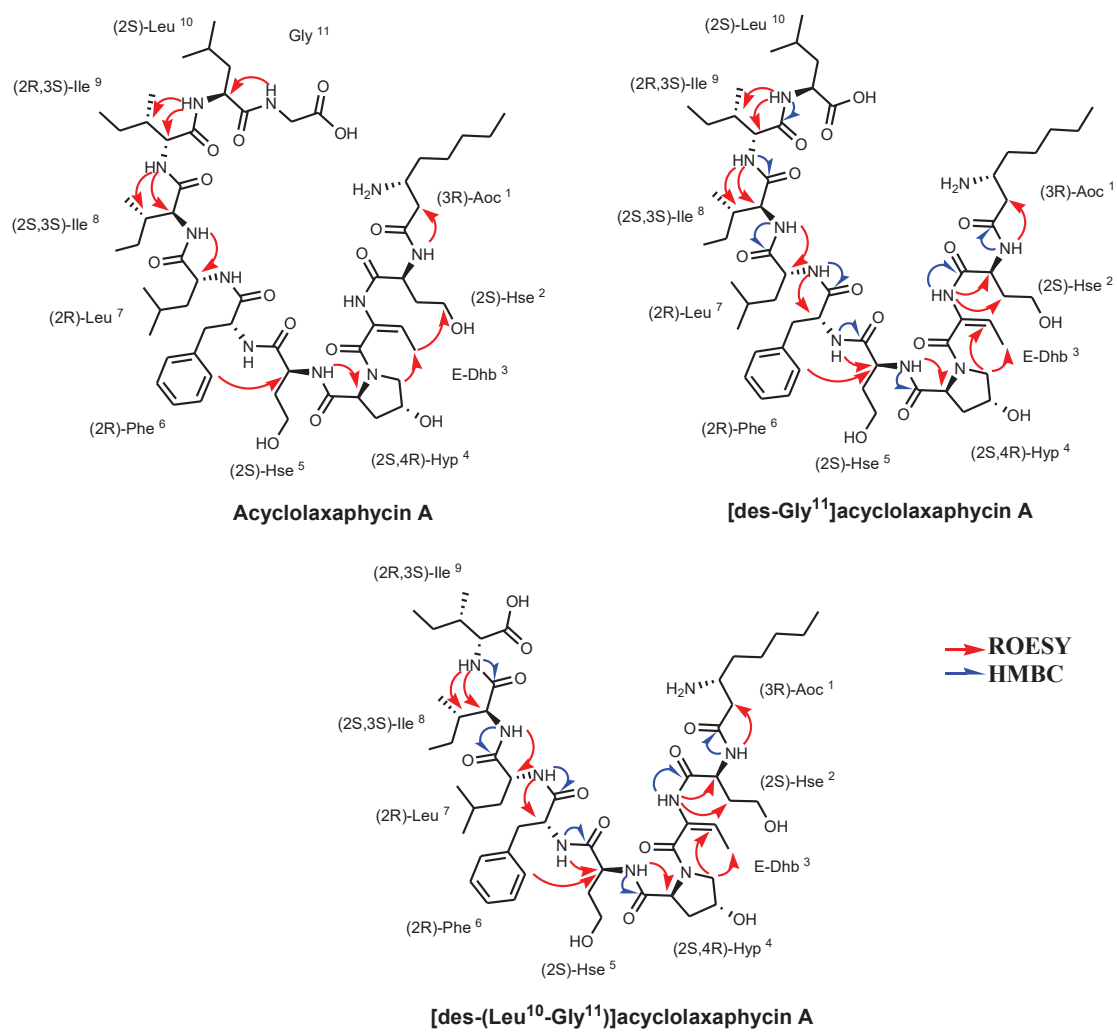


Figure 4. 6. Structures of Acyclolaxaphycin A (1), [des-Gly¹¹]acyclolaxaphycins A (2) and [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycins A (3) with the absolute configuration of each amino acid. ROESY and HMBC correlations are shown with red and blue arrows respectively.

Table 4. 1. NMR Spectroscopic Data for laxaphycin A (318K), Acyclolaxaphycin A (1), [des-Gly11]acyclolaxaphycins A (2) and [des-(Leu10-Gly11)]acyclolaxaphycins A (3) (303 K) in DMSO-d₆

		Laxaphycin A		(1)		(2)		(3)	
		¹³ C	¹ H	¹³ C	¹ H	¹³ C	¹ H	¹³ C	¹ H
		δ (ppm)	δ (ppm)	δ (ppm)	δ (ppm)	δ (ppm)	δ (ppm)	δ (ppm)	δ (ppm)
β Aoc ¹	NH	-	6.82	-	-	-	7.77	-	7.77
	CαH ₂	39.92	1.69/1.97	36.13	2.45	36.31	2.54/2.44	36.53	2.53/2.44
	CβH	44.86	4.27	48.26	3.26	48.03	3.35	48.04	3.35
	CγH ₂	34.76	1.34	31.29	-	31.93	1.50	31.95	1.51
	CδH ₂	28.76	1.23	24.39	1.30	24.07	1.30	24.07	1.30
	CεH ₂	24.98	1.23	21.71	1.26	21.71	1.26	21.71	1.27
	CζH ₂	30.72	1.23	30.92	1.22	30.79	1.23	30.79	1.23
	CηH ₃	13.68	0.84	13.74	0.86	13.68	0.86	13.69	0.86
	CO	169.06	-	166.54	-	169.50	-	169.51	-
Hse ²	NH	-	7.10	-	8.31	-	8.31	-	8.31
	CαH	49.06	4.54	49.76	4.43	49.85	4.40	49.84	4.41
	CβH ₂	33.78	1.76	34.95	1.66/1.81	34.67	1.83/1.69	34.96	1.82/1.67
	CγH ₂	56.97	3.46	53.35	3.28/3.41	57.31	3.41	57.32*	3.41
	OH	-	4.42	-	-	-	-	-	-
	CO	172.89	-	170.25	-	170.00	-	170.05	-
Dhb ³	NH	-	10.75	-	9.93	-	9.76	-	9.77
	Cα	130.79	-	131.47	-	131.48	-	131.47	-
	CβH	118.34	5.57	113.67	5.55	113.96	5.53	114.09	5.53
	CγH ₃	11.95	1.69	12.16	1.66	12.09	1.67	12.15	1.68
	CO	167.25	-	166.71	-	164.72	-	164.74	-
Hyp ⁴	CαH	59.06	4.51	58.09	4.43	57.62	4.46	57.82	4.46
	CβH ₂	37.84	1.92/2.27	37.98	1.93/2.09	37.75	1.92/2.02	37.76	2.02/1.91
	CγH	67.90	4.28	68.17	4.24	68.39	4.24	68.39	4.24
	OH	-	5.03	-	-	-	4.85	-	-
	CδH ₂	56.97	3.34/3.59	55.46	3.39/3.49	55.46	3.36/3.45	55.50	3.37/3.46
	CO	170.09	-	170.74	-	171.10	-	170.60	-
Hse ⁵	NH	-	7.22	-	8.19	-	8.00	-	7.99
	CαH	48.90	4.27	50.03	4.26	50.30	4.19	50.27	4.19
	CβH ₂	33.78	1.88/1.96	34.95	1.61/1.71	34.67	1.58	34.64	1.58
	CγH ₂	56.97	3.31/3.45	57.35	3.26	57.35	3.29/3.43	57.36*	3.29/3.43
	OH	-	-	-	-	-	-	-	-
	CO	171.97	-	171.66	-	171.56	-	171.73	-
Phe ⁶	NH	-	7.79	-	8.47	-	7.92	-	7.86
	CαH	56.05	4.28	54.68	4.52	53.92	4.46	53.81	4.46
	CβH ₂	36.99	2.94/3.04	37.67	2.79/3.12	36.93	2.79/3.05	37.04	2.79/3.04
	Cγ	137.82	-	138.07	-	137.72	-	137.65	-
	CδH ₂	126.11	7.34	129.19	7.26	131.48	7.24	129.08	7.22
	CεH ₂	127.95	7.24	127.82	7.20	129.03	7.23	127.90	7.22
	CζH	128.95	7.18	125.99	7.15	127.90	7.17	126.07	7.16
	CO	171.86	-	171.28	-	170.67	-	171.05**	-
	Leu ⁷	NH	-	7.22	-	9.27	-	7.98	-
CαH	51.55	4.28	56.06	4.27	51.61	4.35	51.47	4.37	
CβH ₂	42.24	1.18/1.34	40.56	1.46	40.83	1.47	40.98	1.47	
CγH	23.94	1.58	24.06*	1.60	24.17	1.60	24.13	1.59	
CδH ₃	22.70	0.80	23.15	0.86	22.86	0.87	22.84	0.87	
Cδ'H ₃	20.31	0.73	22.85	0.86	21.47	0.83	21.57	0.83	
CO	171.54	-	170.93	-	171.92	-	171.70	-	
Ile ⁸	NH	-	6.61	-	7.95	-	7.73	-	7.76
	CαH	55.95	4.63	56.31	4.34	57.00	4.31	56.46	4.41

	CβH	38.40	1.76	36.82	1.76	36.80	1.75	37.32	1.77
	CγH ₂	21.92	1.18	24.15	0.99/1.36	24.00	1.07/1.41	23.73	1.39
	Cγ'H ₃	15.25	0.76	15.04	0.77	15.32	0.81	15.38	0.81
	CδH ₃	11.32	0.75	11.27	0.78	11.02	0.78	11.13	0.80
	CO	172.18	-	172.70	-	170.92	-	171.08**	-
Ile ⁹	NH	-	8.68	-	7.99	-	7.82	-	7.96
	CαH	53.85	4.63	56.92	4.21	54.90	4.44	54.51	4.38
	CβH	36.73	1.97	35.72	1.80	37.00	1.84	36.36	1.88
	CγH ₂	26.08	1.18	25.26	1.07/1.29	25.77	1.10/1.26	25.66	1.13/1.28
	Cγ'H ₃	14.34	0.80	14.80	0.80	14.33	0.78	14.83	0.85
	CδH ₃	11.04	0.84	10.71	0.83	11.42	0.83	11.40	0.84
	CO(OH)	172.35	-	171.38	-	170.87	-	173.11	-
	(OH)	-	-	-	-	-	-	-	12.51
Leu ¹⁰	NH	-	8.34	-	8.47	-	8.05		
	CαH	52.59	4.03	51.20	4.19	50.08	4.24		
	CβH ₂	42.24	1.58/1.59	39.87	1.53	39.86	1.53		
	CγH	23.94	1.56	24.16	1.60	24.77	1.60		
	CδH ₃	21.24	0.83	21.34	0.81	22.75	0.87		
	Cδ'H ₃	22.53	0.89	20.72	0.80	21.11	0.83		
	CO(OH)	172.69	-	172.34	-	173.79	-		
	(OH)	-	-	-	-	-	12.37		
Gly ¹¹	NH	-	8.56	-	7.40				
	CαH	42.24	3.22/3.81	43.54	3.50				
	CO(OH)	166.77	-	173.72	-				
	(OH)	-	-	-	-				

4.2.2. The elucidation of the structures of [L-Val⁸]laxaphycin A (4) and [D-Val⁹]laxaphycin A (5):

The positive high-resolution electrospray ionization mass spectrometry (HRESIMS) spectrum of compound 4 showed the $[M + H]^+$ pseudomolecular ion at m/z 1182.7095 indicating a molecular formula of $C_{59}H_{95}N_{11}O_{14}$. A comparison between the molecular formulas of [L-Val⁸]laxaphycin A and laxaphycin A ($C_{60}H_{97}N_{11}O_{14}$) showed that the new metabolite was a lower analogue (14 amu smaller) of laxaphycin A. The ¹H-NMR spectrum revealed strong structural similarity with laxaphycin A (Figure S4. 26). Analysis of 2D NMR including COSY, TOCSY, HSQC, HSQC-TOCSY, and ROESY (Figures S4. 28-S4. 32) enabled almost all of the ¹H and ¹³C chemical shifts to be determined for (4) (Table 4. 2). Analysis of the TOCSY spectrum established the structures of ten amino acid residues as β-Aoc, Hse (x2), Dhb, Phe, Leu (x2), Gly, Hyp and Ile. HSQC analysis revealed the loss of correlations between Cβ and Hβ, Cγ and Hγ, Cδ and Hδ of Ile⁸ observed in laxaphycin A. In addition, TOCSY, HSQC, HSQC-TOCSY correlations were observed from an amide signal (δ_H 6.53) to a Hα (δ_H 4.67/ δ_C 55.59), a Hβ (δ_H 0.12/ δ_C 32.31) and two methyl groups (δ_H 0.73/ δ_C 19.20 and δ_H 0.64/ δ_C 15.27), features corresponding to a valine spin system. ROESY determined that the same NH was directly connected to HαLeu⁷. NOESY correlations from the NHlle⁹ (δ_H 8.33) to Hα (δ_H 4.67), Hβ (δ_H 2.12) and Hγ (δ_H 0.73) Val, and HMBC cross-peaks between NHlle⁹ (δ_H 8.33) and the adjacent carbonyl of Val (δ_C 172.20) and Hα (δ_H 4.67) Val and the adjacent carbonyl of Leu⁷ (δ_C 171.65) determined that the valine residue is positioned between Ile⁹ and Leu⁷. Finally, HMBC correlations between carbonyl carbons (residue i) and NH or Cα protons

(residue $i+1$) and ROESY correlations between $H\alpha$ (residue i) and NH (residue $i+1$) completed the cyclic structure of compound **4**. Thus, the sequence was established as cyclo[β -Aoc-Hse-Dhb-Hyp-Hse-Phe-Leu-Val-Ile-Leu-Gly]. Mass fragmentation analysis confirmed the sequence assignment of (**4**). Similar to laxaphycin A, the protonation and cleavage of the amide bond preferentially occurred on the amide nitrogen at Pro. Thus the cyclic ion was opened between Dhb³ and Hyp⁴ and formed a linear acylium ion which generated a series of fragments. These observed fragments (Figure S4. 33) were consistent with the proposed amino acid sequence (Figure 4. 7) determined by NMR.

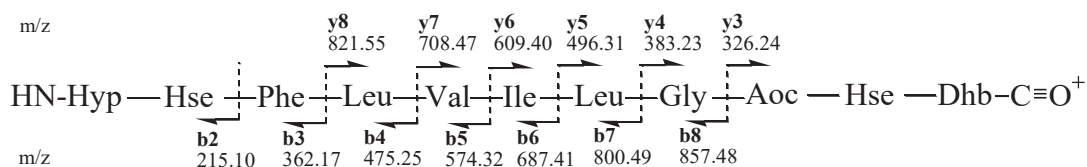


Figure 4. 7. ESIMS/MS fragmentation of [L-Val⁸]laxaphycin A (**4**)

The molecular formula of [D-Val⁹]laxaphycin A (**5**) was determined as C₅₉H₉₅N₁₁O₁₄ by HRESIMS analysis (m/z 1182.7095 [M+H]⁺) and the new metabolite was found to be a lower analogue (14 amu smaller) of laxaphycin A. This result supported the presence of two isomers (compounds **4** and **5**). The ¹H-NMR spectrum (Figure S4. 35) revealed strong structural similarity with [L-Val⁸]laxaphycin A and 2D-NMR including TOCSY, HSQC, HSQC-TOCSY and ROESY (Figures S4. 37-S4. 41) revealed the presence of eleven amino acid residues: Aoc, Hse (x2), Dhb, Phe, Leu (x2), Gly, Hyp, Ile and Val (Table 4. 2). However, the Val residue was located in position 9 since the correlation from C β Ile⁸/H β Ile⁸, observable in the HSQC spectrum of laxaphycin A, was also present in the HSQC spectrum of (**5**), while the correlation C β Ile⁹/H β Ile⁹ was absent. HMBC data confirmed this assumption by revealing correlations between Ile⁸ carbonyl (δ_C 172.12) and NH Val (δ_H 8.33), as well as between Val carbonyl (δ_C 172.25) and Leu¹⁰ NH (δ_H 8.35). Additionally, ROESY correlations were observed between Ile⁸ H α (δ_H 4.61) and H β (δ_H 1.80) to Val NH (δ_H 8.33) and between Val H α (δ_H 4.41) and H β (δ_H 2.22) to Leu¹⁰ NH (δ_H 8.35) and confirmed the position of Val. Using HMBC and ROESY correlations, the complete sequence was defined as cyclo[β -Aoc-Hse-Dhb-Hyp-Hse-Phe-Leu-Ile-Val-Leu-Gly]. The mass fragmentation analysis revealed the preferential opening of the macro ring between Dhb³ and Pro⁴ as in laxaphycin A and [L-Val⁸]laxaphycin A. The fragments (Figure S4. 33) were in complete agreement with the structure proposed above (Figure 4. 8).

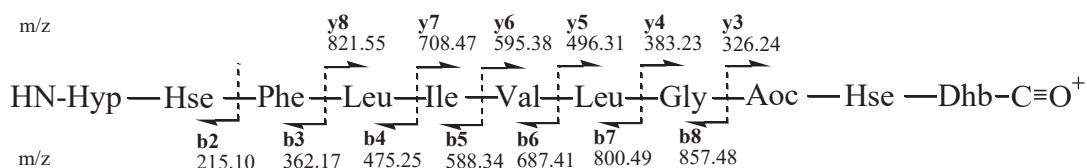


Figure 4. 8. ESIMS/MS fragmentation of [D-Val⁹]laxaphycin A (**5**)

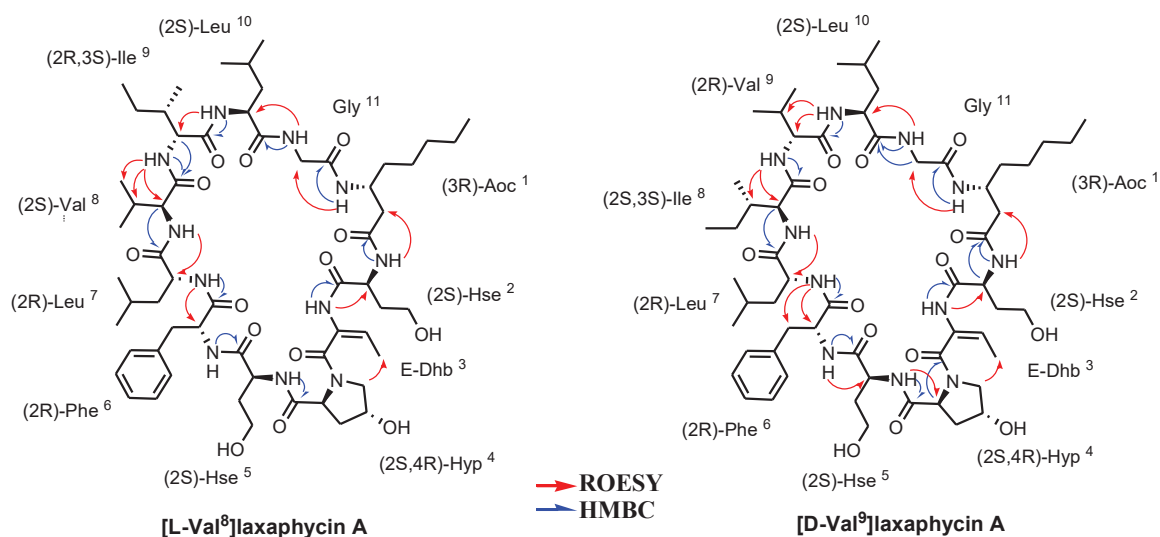


Figure 4. 9. [L-Val⁸]laxaphycin A (4) and [D-Val⁹]laxaphycin A (5) with the absolute configuration of each amino acid, ROESY (red arrows) and HMBC (blue arrows) correlations

Table 4. 2. NMR Spectroscopic Data for laxaphycin A (318K), [L-Val⁸]laxaphycin A (4) and [D-Val⁹]laxaphycin A (5) (303 K) in DMSO-d₆

	Laxaphycin A		[L-Val ⁸]laxaphycin A (4)		[D-Val ⁹]laxaphycin A (5)		
	¹³ C δ (ppm)	¹ H δ (ppm)	¹³ C δ (ppm)	¹ H δ (ppm)	¹³ C δ (ppm)	¹ H δ (ppm)	
β Aoc ¹	NH	-	6.82	-	6.84	-	6.88
	CαH ₂	39.92	1.69/1.97	40.09	1.66/1.91	44.93	1.74/1.97
	CβH	44.86	4.27	44.66	4.25	40.11	4.21
	CγH ₂	34.76	1.34	34.93	1.34	34.70	1.34
	CδH ₂	28.76	1.23	25.10	1.24	25.10	1.23
	CεH ₂	24.98	1.23	22.08	1.24	22.04	1.23
	CζH ₂	30.72	1.23	30.77	1.22	30.82	1.23
	CηH ₃	13.68	0.84	13.85	0.85	13.81	0.84
CO	169.06	-	169.00	-	169.18	-	
Hse ²	NH	-	7.10	-	7.15	-	7.14
	CαH	49.06	4.54	49.08	4.55	49.17	4.54
	CβH ₂	33.78	1.76	33.67	1.76	33.85	1.76
	CγH ₂	56.97	3.46	56.96	3.31/3.42	57.09	3.45
	OH	-	4.42	-	-	-	-
	CO	172.89	-	173.49	-	172.87	-
Dhb ³	NH	-	10.75	-	10.80	-	10.75
	Cα	130.79	-	130.11	-	130.84	-
	CβH	118.34	5.57	118.82	5.59	118.25	5.57
	CγH ₃	11.95	1.69	12.15	1.69	12.14	1.68
	CO	167.25	-	167.59	-	167.26	-
Hyp ⁴	CαH	59.06	4.51	59.33	4.51	59.14	4.51
	CβH ₂	37.84	1.92/2.27	37.83	1.87/2.29	38.02	1.87/2.26
	CγH	67.90	4.28	68.04	4.29	68.01	4.27
	OH	-	5.03	-	-	-	5.15
	CδH ₂	56.97	3.34/3.59	57.14	3.32/3.61	57.02	3.31/3.59
	CO	170.09	-	170.20	-	170.22	-
Hse ⁵	NH	-	7.22	-	7.29	-	7.29

	C α H	48.90	4.27	48.83	4.28	49.01	4.25
	C β H ₂	33.78	1.88/1.96	33.78	1.85/1.99	33.95	1.83/1.99
	C γ H ₂	56.97	3.31/3.45	56.89	3.28/3.43	56.78	3.29/3.43
	OH	-	-	-	-	-	-
	CO	171.97	-	178.08	-	172.03	-
Phe ⁶	NH	-	7.79	-	7.86	-	7.80
	C α H	56.05	4.28	56.52	4.26	56.14	4.28
	C β H ₂	36.99	2.94/3.04	37.00	2.95/3.06	37.02	2.95/3.01
	C γ	137.82	-	137.93	-	137.88	-
	C δ H ₂	126.11	7.34	129.04	7.36	129.07	7.35
	C ϵ H ₂	127.95	7.24	128.13	7.25	128.06	7.24
	C ζ H	128.95	7.18	126.28	7.19	126.23	7.18
	CO	171.86	-	171.91	-	171.90	-
Leu ⁷	NH	-	7.22	-	7.20	-	7.32
	C α H	51.55	4.28	51.67	4.24	51.58	4.29
	C β H ₂	42.24	1.18/1.34	34.92	1.37	39.41	1.15/1.28
	C γ H	23.94	1.58	24.02	1.56	23.97	1.58
	C δ H ₃	22.70	0.80	22.85	0.80	22.85	0.80
	C δ' H ₃	20.31	0.73	20.28	0.72	20.44	0.73
	CO	171.54	-	171.65	-	171.65	-
Ile ⁸ /Val ⁸	NH	-	6.61	-	6.53	-	6.71
	C α H	55.95	4.63	55.59	4.67	56.02	4.61
	C β H	38.40	1.76	32.31	2.12	38.52	1.80
	C γ H ₂	21.92	1.18	19.2	0.73	22.41	1.20
	C γ' H ₃	15.25	0.76	15.27	0.64	15.37	0.77
	C δ H ₃	11.32	0.75	-	-	11.46	0.73
	CO	172.18	-	172.20	-	172.12	-
Ile ⁹ /Val ⁹	NH	-	8.68	-	8.33	-	8.33
	C α H	53.85	4.63	53.54	4.68	56.78	4.41
	C β H	36.73	1.97	37.07	2.02	30.44	2.22
	C γ H ₂	26.08	1.18	26.26	1.15	19.17	0.83
	C γ' H ₃	14.34	0.80	14.35	0.80	16.62	0.83
	C δ H ₃	11.04	0.84	11.31	0.85	-	-
	CO	172.35	-	172.66	-	172.25	-
Leu ¹⁰	NH	-	8.34	-	8.42	-	8.35
	C α H	52.59	4.03	52.97	4.00	52.60	4.04
	C β H ₂	42.24	1.58/1.59	39.35	1.37/1.54	39.39	1.40/1.51
	C γ H	23.94	1.56	23.95	1.58	24.02	1.58
	C δ H ₃	21.24	0.83	22.59	0.89	22.63	0.89
	C δ' H ₃	22.53	0.89	21.69	0.84	21.34	0.83
	CO	172.69	-	172.94	-	172.87	-
Gly ¹¹	NH	-	8.56	-	8.70	-	8.59
	C α H	42.24	3.22/3.81	42.27	3.22/3.78	42.30	3.30/3.78
	CO	166.77	-	166.71	-	166.92	-

4.2.3. Absolute configuration of compounds 2-5, acyclolaxaphycin B (6) and acyclolaxaphycin B3 (7)

The absolute configuration of each amino acid residue in compounds 2-7 was established using the advanced Marfey's method after hydrolysis^{18,19} (Figures S4. 25, S4. 34, S4. 43 and S4. 44).

The LC-MS comparison between the Marfey's derivatives of the acid hydrolysate of [des-(Gly¹¹)]-acyclolaxaphycin A (2) assigned the 2*S* configuration of Hse (x2), the 2*R* configuration of Phe and 3*R* configuration of Aoc (Supporting Information). Leucine and

Isoleucine standard stereoisomers were derived using Marfey's method, analyzed with LC-MS and retention times were compared with Marfey's derivative of compound 2. This indicated the presence of (2*R*)-Leu, (2*S*,3*S*)-Ile, (2*R*,3*S*)-Ile and (2*S*)-Leu as found in laxaphycin A. Hydroxyproline constitutes an exception of the Marfey's rule because the D-FDLA-(2*S*)-Hyp derivative elutes before the L-FDLA-(2*S*)-Hyp derivative⁹. Thus the absolute configuration of the α carbons of Hyp was assigned as 2*S* and the same retention time observed for the Hyp derivative of laxaphycin A and compound 2 enabled the γ configuration to be assigned as 4*R*. The geometric configuration of Dhb was determined from ROESY correlations. Strong ROESY cross-peaks between the NH (δ_{H} 9.76) and the olefinic proton (δ_{H} 5.53) and between the H_{γ} (δ_{H} 1.67) of Dhb and the H_{δ} (δ_{H} 3.36/3.45) of Hyp were observed, assigning the geometric configuration of the double bond as *E*. Therefore, we established the complete structure of [des-(Gly¹¹)]-acyclolaxaphycin A (**2**) as (3*R*)-Aoc-(2*S*)-Hse-(*E*)-Dhb-(2*S*,4*R*)-Hyp-(2*S*)-Hse-(2*R*)-Phe-(2*R*)-Leu-(2*S*,3*S*)-Ile-(2*R*,3*S*)-Ile-(2*S*)-Leu.

[des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A (**3**): the absolute configuration of each amino acid residue was assigned as (3*R*)-Aoc, (2*S*)-HSe (x2), (2*S*,4*R*)-Hyp, (2*R*)-Phe (2*R*)-Leu, (2*S*,3*S*)-Ile and (2*R*,3*S*)-Ile (Supporting Information). The geometric configuration of Dhb was defined as *E* with ROESY correlations between the NH (δ_{H} 9.77) and the olefinic proton (δ_{H} 5.53) and between H_{γ} (δ_{H} 1.68) of Dhb and H_{δ} (δ_{H} 3.37/3.46) of Hyp. The complete structure could be defined as (3*R*)-Aoc-(2*S*)-Hse-(*E*)-Dhb-(2*S*,4*R*)-Hyp-(2*S*)-Hse-(2*R*)-Phe-(2*R*)-Leu-(2*S*,3*S*)-Ile-(2*R*,3*S*)-Ile.

[L-Val⁸]laxaphycin A (**4**): the absolute configuration of each amino acid residue was defined with Marfey's method (Supporting Information). The absolute configuration of Val was determined as 2*S* and the geometric configuration of Dhb was assigned as *E* with ROESY correlations between NH (δ_{H} 10.80) and H_{β} (δ_{H} 5.59) of Dhb and between H_{γ} (δ_{H} 1.69) of Dhb and H_{δ} (δ_{H} 3.32/3.61) of Hyp. The complete structure of (**4**) was defined as cyclo-[(3*R*)-Aoc-(2*S*)-Hse-(*E*)-Dhb-(2*S*,4*R*)-Hyp-(2*S*)-Hse-(2*R*)-Phe-(2*R*)-Leu-(2*S*)-Val-(2*R*,3*S*)-Ile-(2*S*)-Leu-Gly-].

[D-Val⁹]laxaphycin A (**5**): the ROESY correlations between NH (δ_{H} 10.75) and H_{β} (δ_{H} 5.57) of Dhb and between H_{γ} (δ_{H} 1.68) of Dhb and H_{δ} (δ_{H} 3.31/3.59) of Hyp determined that the geometric configuration of Dhb was *E*. In contrast with [L-Val⁸]laxaphycin A (**4**), the Val residue was assigned as (2*R*)-Val, but this result is consistent with the stereochemistry of the C_{α} backbone of the ring, the (2*R*)-Val replacing the (2*R*, 3*S*)-Ile. Thus the structure was cyclo-[(3*R*)-Aoc-(2*S*)-Hse-(*E*)-Dhb-(2*S*,4*R*)-Hyp-(2*S*)-Hse-(2*R*)-Phe-(2*R*)-Leu-(2*S*,3*S*)-Ile-(2*R*)-Val-(2*S*)-Leu-Gly-].

Acyclolaxaphycin A (**1**): due to the small amount of compound obtained, the stereochemistry of acyclolaxaphycin A was not elucidated, but the configuration of the C_{α} overall backbone seems to be maintained in laxaphycin A analogs. Indeed, the configurational analysis with Marfey's procedure gave the same results for laxaphycin A and

compounds 2-5. Thus the absolute configuration was not established for compound 1, but we speculate that the complete structure of acyclolaxaphycin A (**1**) is (3*R*)-Aoc-(2*S*)-Hse-(*E*)-Dhb-(2*S*,4*R*)-Hyp-(2*S*)-Hse-(2*R*)-Phe-(2*R*)-Leu-(2*S*,3*S*)-Ile-(2*R*,3*S*)-Ile-(2*S*)-Leu.

Interestingly, the NMR chemical shifts of laxaphycin A and acyclolaxaphycin A are relatively close. These results were unexpected for a cyclic peptide and its acyclic equivalent. We hypothesise that the secondary structures of both molecules are similar although we are unable to confirm this from intra-molecular NOESY correlations.

Marfey's method was also used to determine the absolute configuration of acyclolaxaphycin B (**6**) and acyclolaxaphycin B3 (**7**) (Supporting Information) previously described¹⁷. The chromatographic comparison between the Marfey's derivatives of the acid hydrolysate of acyclophycin B established the 2*S* configuration of Val, Ala, Gln, Pro, N-Melle, the 2*R* configuration of Leu, as well as the 3*R* configuration of Ade. The Marfey's analysis of the four stereoisomers of standard threonine revealed the (2*S*,3*R*) configuration of both threonines present in acyclolaxaphycin B (Supporting Information). The Marfey's method also revealed the 2*R* configuration of the two 3-hydroxyleucines. The absolute configuration of C β of both 3-hydroxyleucines (2*R*,3*S*) was established through NOESY correlations between the Hy and the NH observed²⁰ (Supporting Information). As previously described⁹, the elution order of the 3-hydroxyasparagine (HAsp), which results from the acid hydrolysis of Has, is another exception of the Marfey's rule. Indeed, the D-FDLA-(2*R*)-HAsp derivative elutes after the L-FDLA-(2*R*)-HAsp derivative. Thus, we established that the C α configuration of the Has residue was 2*R*. The configuration of the C β of Has was established to be 3*R* by a comparison with laxaphycin B Marfey's derivatives. Therefore, the complete structure of acyclolaxaphycin B was established as (2*S*)-Ala-(2*R*,3*S*)-Hle-(2*S*)-Gln-(2*S*)-N-Melle-(2*R*,3*R*)-Has-(2*S*)-Thr-(2*S*)-Pro-(2*R*)-Leu-(2*S*,3*R*)-Thr-(3*R*)-Ade-(2*S*)-Val-(2*R*,3*S*)-Hle.

As regards the acyclolaxaphycin B3, the configuration of Val, Ala, Gln, N-Melle, Leu, Ade, Has, Thr (x2) and Hle (x2) were found to be the same as for acyclophycin B. The absolute configuration of the C α of the Hyp residue appeared to be (2*S*) and a comparison with laxaphycin B3 derivative enabled the C γ configuration to be assigned to 4*R*, establishing the complete structure as (2*S*)-Ala-(2*R*,3*S*)-Hle-(2*S*)-Gln-(2*S*)-N-Melle-(2*R*,3*R*)-Has-(2*S*,3*R*)-Thr-(2*S*,4*R*)-Hyp-(2*R*)-Leu-(2*S*)-Thr-(3*R*)-Ade-(2*S*)-Val-(2*R*,3*S*)-Hle.

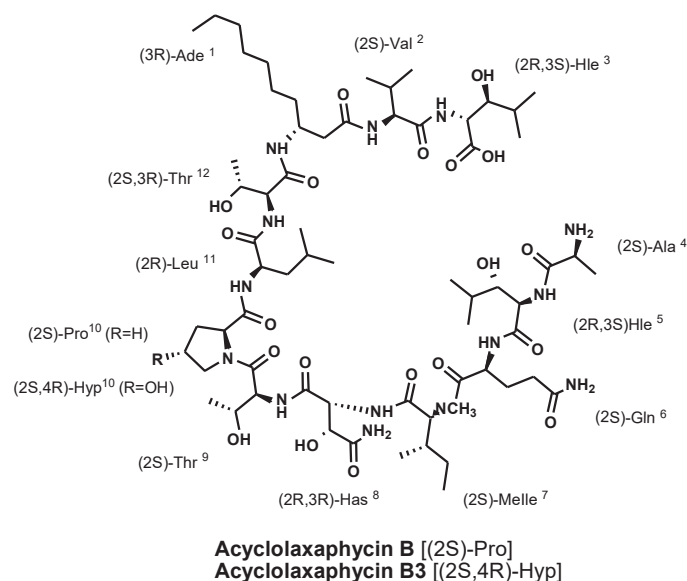


Figure 4. 10. Structures of acyclolaxaphycins B (6) and B3 (7) with the absolute configuration of each amino acid

4.2.5. Biosynthesis within the laxaphycin A sub-family.

[L-Val⁸]laxaphycin A and [D-Val⁹]laxaphycin A are two variants of laxaphycin A with the presence of a Val residue in position 8 or 9 instead of an Ile residue. These two new compounds, together with laxaphycin A, are structurally related to hormothamnin A, laxaphycin E, lobocyclamide A, scytocyclamide A, trichormamides A and D which are produced by different cyanobacteria. All of them have the particularity to possess eleven amino acids and share the (3*R*)- β -amino fatty acid (Aoc or Ade), (2*R*)-Leu and Gly in positions 1, 7 and 11. Furthermore, a Dhb residue is present in position 3 of all compounds of the family except for trichormamide A, which possesses a Ser residue.

In position 4, trichormamides A and D share a Pro instead of a Hyp, commonly found in other compounds. Lobocyclamide A, trichormamide A and D present a Tyr residue in position 6 while a Phe is present in all other compounds. It is important to emphasize that the amino acid residues can vary, but their configurations at each position are conserved. Compared to laxaphycin A, [L-Val⁸]laxaphycin A and [D-Val⁹]laxaphycin A share the same absolute configuration for each asymmetric carbon. Indeed, the absolute configuration of the carbon α remains unchanged for the residue in positions 8 or 9, when a Val is substituted for an Ile. Acyclolaxaphycins A (1), (2) and (3) are three acyclic analogs of laxaphycin A obtained by a ring opening between Gly¹¹ and Aoc¹ (compound 1) and the successive loss of one (compound 2) or two residues (compound 3), with the stereochemistry of all amino acids being retained from laxaphycin A to (1), (2) and (3).

Recently, the complete biosynthetic pathway for puwainaphycins, lipopeptides containing β amino fatty acid, was reported for the first time in cyanobacteria²¹. The mechanism includes the activation of a fatty acid by adenylation carried out by FAALs (fatty

acyl-AMP ligases), ligation to the first ACP (Acyl carrier protein) module, then an elongation of the chain by a PKS enzyme and an amination of the fatty acid by a hybrid PKS/NRPS enzyme which also binds the Val residue. Amino acids were subsequently assembled by NRPSs and a final NRPS enzyme, comprising a thioesterase module, operated the macrocyclization of the peptide between the NH₂ of the β amino fatty acid and the COOH of a proline residue. Although no study has been published on the biosynthetic pathway of the laxaphycin A-type peptide, characterization of the minor acyclic acyclolaxaphycins (**1**), (**2**) and (**3**) could indicate a mechanism similar to the biosynthesis of puwainaphycins. Indeed, the modification of the fatty acid, carried out by FAAL and PKS/NRPS enzymes and leading to the β-aminooctanoic acid, could constitute the first step, followed by amino acid assembly starting from Hse² to Gly¹¹ and the final cyclization occurring between the NH₂ of the β-Aoc and the COOH of the Gly residue. We recently published the characterization of acyclolaxaphycins B (**6**) and B3 (**7**), two acyclic laxaphycins B-type and argued that the biosynthesis process of such compounds could start with the NRPS module instead of FAAL and ACP ligase, and finish with the cyclization between the NH₂ of the Ala residue and the COOH of the OH-Leu residue. Thus, isolation of the minor acyclic laxaphycin could highlight different uses of the FAAL/PKS/NRPS machinery and reveal two putative biosynthetic pathways leading to laxaphycin A or B-type peptides.

However, as we previously argued for acyclolaxaphycins B and B3, it is not unlikely that acyclolaxaphycins A, [des-(Gly¹¹)]-acyclolaxaphycin A (**2**) and [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A (**3**) ensued from an enzymatic degradation as a resistance mechanism in a competitive interspecific interaction. A recent study showed the hydrolysis, operating on an ester bond, of the bacterial lipodepsipeptide surfactin was carried out by a filamentous bacterium²². A similar enzymatic hydrolysis may occur for laxaphycins A, B and B3, though an ester bond is present making them more robust and less inclined to hydrolysis. Moreover, the presence of acyclolaxaphycins (**1**), (**2**) and (**3**), three putative biosynthesis intermediates at different stages, lend credibility to the biosynthesis hypothesis developed above.

4.3. Experimental section

4.3.1. Biological material

The cyanobacterium, *A. cf torulosa*, was collected by SCUBA diving at a depth of 1-3m in the Pacific Ocean at Moorea, French Polynesia (S 17°29'22'', W149°54'17''). The cyanobacterium sample was sealed underwater in a bag with seawater and then freeze-dried.

4.3.2. Extraction and isolation

Freeze-dried biomass of *A. cf torulosa* (600g) was extracted at room temperature 3 times with a mixture of MeOH-CH₂Cl₂ (1:1) and an ultrasound was performed over 10 minutes. The evaporation of the combined extracts under reduced pressure led to a greenish organic extract (38 g) that was subjected to flash RP18 silica gel column eluted with H₂O (A), H₂O-CH₃CN (2:8) (B), MeOH (C) and MeOH-CH₂Cl₂ (8:2) (D) successively resulting in 4 fractions (A, B, C and D). Afterwards, fraction B was subjected to flash RP18 column eluted with a solvent gradient of H₂O-CH₃CN resulting in 12 fractions. Fraction 5 was subjected to reverse-phase HPLC purification (Interchim, UP-50 DB.25M Uptisphere, 250x10 mm, 5µm) using an isocratic elution with 68% H₂O-CH₃CN at a flow rate of 3 mL/min to give compounds 3 (4 mg, rt=22.7 min) and 1 (1.5 mg, rt=31.3 min). Fraction 8 gave compound 2 (5 mg, rt=22.4 min) with 58% H₂O-CH₃CN, while fraction 9 led to compounds 4 (6.5 mg, rt=24.7 min) and 5 (6.5 mg, rt=25.9 min).

4.3.3. LC-MS and HPLC-ELSD analyses

LC-MS analyses were carried out using a Thermo Fisher Scientific LC-MS device, Accela HPLC coupled to a LCQ Fleet equipped with an electrospray ionization source and a 3D ion-trap analyzer. HPLC-ELSD analyses were performed with a Waters Alliance HPLC system coupled to an ELS detector. The analyses were performed on a reversed-phase column (Thermo Hypersil Gold C-18, 150 x 2.1 mm, 3µm) employing a gradient of 10% to 100% CH₃CN over 40 min followed by 25 min at 100% CH₃CN (all solvents buffered with 0.1% formic acid) with a flow rate of 0.3 mL/min.

4.3.4. Mass and NMR Spectroscopies

High-resolution ESI mass spectra were obtained on a Thermo Scientific LTQ Orbitrap mass spectrometer using electrospray ionization in positive mode. 1D-NMR and 2D-NMR experiments were acquired on a Bruker Avance 500 spectrometer equipped with a cryogenic probe (5 mm), all compounds were dissolved in DMSO-*d*₆ (500 µL) at 303 K. All chemical shifts were calibrated on the residual solvent peak (DMSO-*d*₆, 2.50 ppm (¹H) and 39.5 ppm (¹³C)). The chemical shifts, reported in delta (δ) units, and in parts per million (ppm) are referenced relatively to TMS.

4.3.5. Advanced Marfey's analyses

The Marfey's analyses were carried out on compounds 2, 3, 4, 5, 6, 7, laxaphycins A, B and B3. Approximately 0.3 mg of each compound were hydrolyzed with 1 mL of 6 N HCl for 20 h at 110 °C in sealed glass vials. The cooled hydrolysate mixtures were evaporated to dryness and traces of HCl were removed from the reaction mixtures by repeated evaporation. Each hydrolysate mixture was dissolved in H₂O (100 µL). 110 µL of acetone, 20 µL of 1 N NaHCO₃, and 20 µL of 1% L or D/L FDLA (1-fluoro-2,4-dinitrophenyl-5-L-

leucinamide) in acetone were added to each 50 μL aliquot. The mixtures were then heated to 40 $^{\circ}\text{C}$ for 1 h. The cooled solutions were neutralized with 1 N HCl (20 μL), and then dried in vacuo. The residues were dissolved in 1:1 $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ and then analyzed by LC-MS. LC-MS analyses were performed on a reversed-phase column (Thermo Hypersil Gold C-18, 150 x 2.1 mm, 3 μm) with two linear gradients: (1) from 20% CH_3CN -80% 0.01 M formic acid to 60% CH_3CN -40% 0.01 M formic acid at 0.3 mL/min over 70 min and (2) from 10% CH_3CN -90% 0.01 M formic acid to 50% CH_3CN -50% 0.01 M formic acid at 0.3 mL/min over 70 min, then to 80% CH_3CN -20% over 10 min. The configuration of the α carbon for each residue can be assigned in accordance with the elution order of the D- and L-FDLA derivatives^{18,19}: amino acids for which the D-FDLA analogue elutes first have a D configuration, while those for which the L-FDLA analogue elutes first have a L configuration. Detailed reports of retention times of each amino acid can be found in Supporting Information. Furthermore the hydrolysates were compared to those of laxaphycins A and B.

4.4. Conclusion

In summary, we have obtained three new acyclic lipopeptides, termed acyclolaxaphycin A (**1**), [des-(Gly¹¹)]acyclolaxaphycin A (**2**) and [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A (**3**) from the cyanobacterium *Anabaena cf. torulosa*. From the same species, we also isolated two new cyclic lipopeptides, laxaphycins A2 (**4**) and A3 (**5**). The two cyclic compounds appear to be close analogues of the known laxaphycin A, previously isolated from the same species of cyanobacterium. To the best of our knowledge, the presence of cyclic lipopeptides with their acyclic equivalents has never been described and raises several assumptions. We cannot exclude that the acyclic derivatives ensue from an enzymatic degradation, but compounds **1**, **2** and **3** may also be potential biosynthetic precursors of laxaphycin A. We previously described the structure of acyclolaxaphycins B (**6**) and B3 (**7**), putative biosynthetic intermediates of laxaphycins B and B3 which have all been isolated from *A. cf. torulosa*. Thus the isolation of minor acyclic analogues of lipopeptides may shed light on the hybrid PKS/NRPS biosynthetic machinery involved in the biosynthesis of such compounds. Moreover, we highlight differences in the biosynthesis and the enzymatic architecture between laxaphycins A-type and B-type peptides.

Associated content

-Supporting Information

Supplementary data (¹H NMR, ¹³C, TOCSY, HSQC, HSQC-TOCSY, HMBC, and ROESY spectra of **1,2,3,4** and **5** and advanced Marfey's analysis of **2,3,4, 5, 6** and **7**) associated with this chapter are available at the end of this thesis (S.4. 1-S4. 44).

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Chapter 5. Secondary Metabolites from Marine Cyanobacteria Inducing Behaviors along a Trophic Cascade

Abstract

In the lagoon of Moorea, we have identified an ecosystem based on two benthic filamentous cyanobacteria *Lyngbya majuscula* and *Anabaena cf torulosa*, three herbivores, the anaspidean molluscs *Stylocheilus striatus* and *Stylocheilus longicauda*, and the cephalaspidea *Bulla orientalis*, and two carnivores, the nudibranch *Gymnodoris ceylonica* and the crab *Thalamita coerulipes*. The herbivores *S. striatus* and *B. orientalis* are found feeding upon *L. majuscula*, *G. ceylonica* is also present on the cyanobacteria and feed upon *S. striatus* while *T. coerulipes* is an opportunist predator feeding upon the three molluscs. While the sea hare *S. striatus* has been previously described as a specialist herbivore of *L. majuscula*, we observed it feeding upon *A. cf torulosa* as well as *S. longicauda* lacking the predation of *G. ceylonica* and *T. coerulipes*. In chapters 3 and 4 we completed the characterization of the *A. cf torulosa*'s secondary metabolites (8 new lipopeptides), whereas the *L. majuscula*'s secondary metabolites (3 new heptalipopeptides, to be published) were already known. The overall objective of this chapter is to determine the role of cyanobacterial secondary metabolites in structuring this ecosystem.

We demonstrate here first that foraging is chemically mediated since *B. orientalis* and *S. striatus* are able to track their cyanobacteria of origin. In addition, foraging is specifically stimulated by the organic portion of the cyanobacterial metabolome. Feeding choices highlight the generalist behavior of these two herbivores. Further chemical investigations reveal the sequestration of *L. majuscula* and *A. cf torulosa*'s cyclic lipopeptides and amino alcohols in the three herbivores. Concerning the three herbivores feeding on *A. cf torulosa* we demonstrate that laxaphycin B-type compounds are biotransformed into acyclic analogs. The sequestration of *L. majuscula*'s peptides occurs mainly in the hepatopancreas (digestive gland) of *S. striatus*. The question remains if the peptides are the chemical cues attracting the primary predators of the cyanobacteria (*Stylocheilus striatus*, *S. longicauda*, *Bulla orientalis*) and if the same putative chemical cues attract or repel the secondary predator *G. ceylonica* feeding on *S. striatus*.

5.1. Introduction

Chemical mediation is an important factor governing communities and ecosystems¹⁻³. Secondary metabolites play a major role in complex intraspecific and interspecific interactions at different trophic levels. Some compounds are toxic and have been selected by prey as an effective defense strategy against potential herbivores and predators. In turn, consumers have developed toxin resistance strategies enabling them to feed with no detrimental effects, as well as to enjoy the shelter provided by their food source against their own competitors and potential predators. Some of these resistant consumers sequester prey secondary metabolites and acquire chemical defenses associated with either cryptic coloration⁴ or aposematic coloration⁵.

Chemical mediation is important in the aquatic environment especially for species lacking efficient vision and hearing. Among them, marine molluscs are known to feed upon chemically defended prey such as algae, sponges, bryozoans, ascidians or cyanobacteria⁶⁻⁸. The anaspidean mollusc *Stylocheilus striatus* is considered as a specialist herbivore of the cyanobacterium *Lyngbya majuscula*⁹⁻¹² that is known to produce a wide range of secondary metabolites, mainly lipopeptides¹³. The cryptic sea hare sequesters many of these compounds in its digestive gland and is able to biotransform some of them^{9,14}. The aposematic nudibranch *Gymnodoris ceylonica*, often associated with *L. majuscula* and *S. striatus*, is a voracious predator of the sea hare¹⁵. In the lagoon of Moorea, French Polynesia, *S. striatus* and the cephalaspidean mollusc *B. orientalis* are found to feed upon *L. majuscula* while *G. ceylonica* and the crab *Thalamita coerulipes*, which consume all three molluscs, are also present on the cyanobacterium. *L. majuscula* is a prolific source of secondary metabolites and specimen collected in Moorea are known to express tiahuramides, a cyclic lipodepsipeptide family (results obtained in the laboratory, to be published). However, we found also *S. striatus* feeding upon *Anabaena cf torulosa*, another cyanobacterium, accompanied by *Stylocheilus longicauda*. *A. cf torulosa* is known to produce laxaphycins, a cyclic lipopeptide family (ref¹⁶ and chapter 3). Interestingly, the herbivores seem to be less exposed to the predation since *G. ceylonica* and *T. coerulipes* are not present on *A. cf torulosa*.

In the present study, we combined ecological assays and chemical analyses to determine **(i) whether *S. striatus* and *B. orientalis* showed adaptative preference to their host using olfactory and feeding choice experiments**. We wondered about the feeding specialization of the herbivores, especially *S. striatus* which was considered as a specialist but was found on different host and *B. orientalis*, only found on *L. majuscula*. In most case, feeding specialization appeared to be chemically mediated. A second issue we want to investigate **(ii) the sequestration of cyanobacterial secondary metabolites and their role in determining the length of the trophic web**.

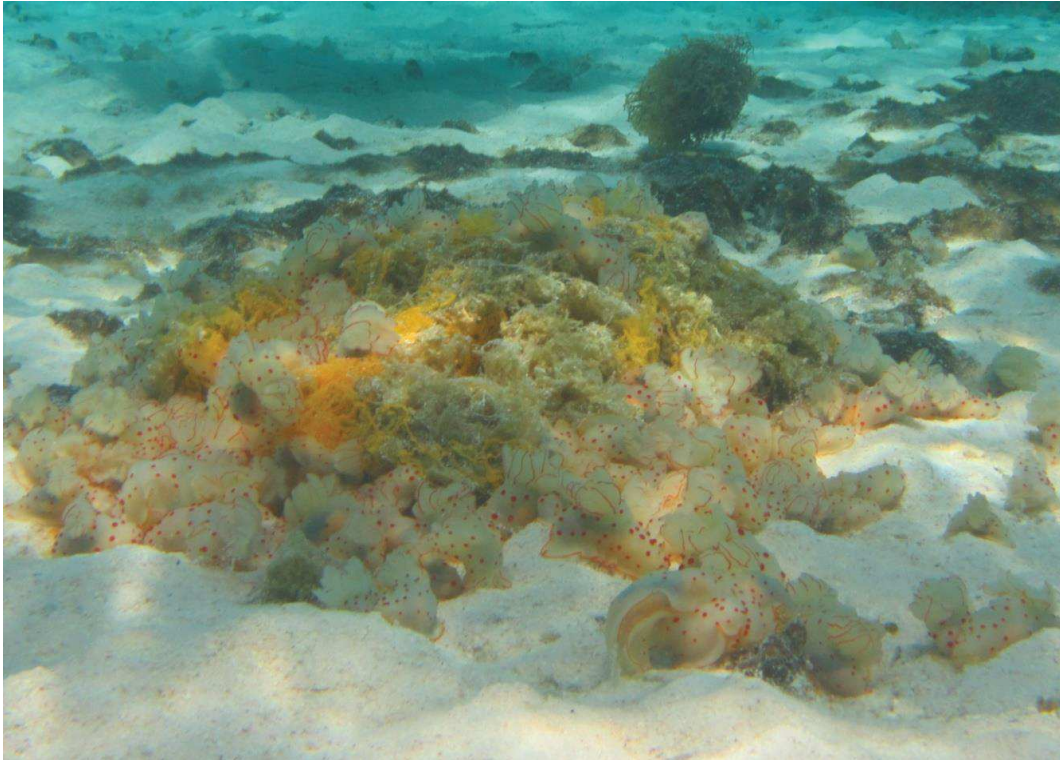


Figure 5. 1. *Gymnodoris ceylonica* swarming on *Lyngbya majuscula* and eating *Stylocheilus striatus*



Figure 5. 2. Three *G. ceylonica*. The one at the bottom is eating a *S. striatus*. Orange ribbons are nudibranch eggs.

5.2. Results

5.2.1. Cyanobacterial chemicals and herbivores 's foraging behavior; assay with conditioned seawaters

Field observations indicated the presence of *B. orientalis* exclusively on *L. majuscula* while *S. striatus* was observed on both *L. majuscula* and *A. cf torulosa*. To investigate the role of olfaction in host seeking, *S. striatus* reared on *L. majuscula*, *A. cf torulosa* and naïve, as well as *B. orientalis* reared on *L. majuscula* were subjected to T-maze pair-wise trials, modeled on those of Painter et al¹⁷ and adapted with a flow rate and smaller design dimension. The experiments have been done with seawater conditioned with control seawater (C), *L. majuscula* (Lm) and *A. cf torulosa* (At) and have been conducted as follow: 1) C vs At, 2) C vs Lm, 3) At vs Lm. All herbivores use chemical detection for orientation, but as a function of their previous diet.

- *S. striatus* reared on *L. majuscula* preferentially chose the water flow containing *L. majuscula* when provided [Lm vs C (Fig. 3b) or Lm vs At (Fig. 3c)], and chose the water flow containing *A. cf torulosa* when Lm is not provided in the choice [At vs C (Fig. 3a)],

- *S. striatus* reared on *A. cf torulosa* preferentially chose the water flow containing *A. cf torulosa* when provided [At vs C (fig. 3a) or At vs Lm (fig. 3c)], and did not show any preference between the two water flows when At is not provided in the choice [Lm vs C (fig. 3b)],

- Naïve *S. striatus*, reared on foods not presented during the choice experiment, did not show any preference between the two water flows containing either one of the two cyanobacterial foods and a control [Lm vs C (Fig. 3b) or At vs C (Fig. 3a)], or both cyanobacterial foods [At vs Lm (Fig. 3c)],

- *B. orientalis* reared on *L. majuscula*, preferentially chose the water flow containing *L. majuscula* when provided [Lm vs C (Fig. 3b) or Lm vs At (Fig. 3c)], and did not show any preference between the two water flows when Lm is not provided in the choice [At vs C (fig. 3a)].

In general, it seems that herbivores reared on one cyanobacteria preferentially track the species of cyanobacteria familiar to them, and, excepting *S. striatus* naïve to *A. cf torulosa*, it is less evident whether herbivores are able to recognize the chemical compounds of an unknown cyanobacteria.

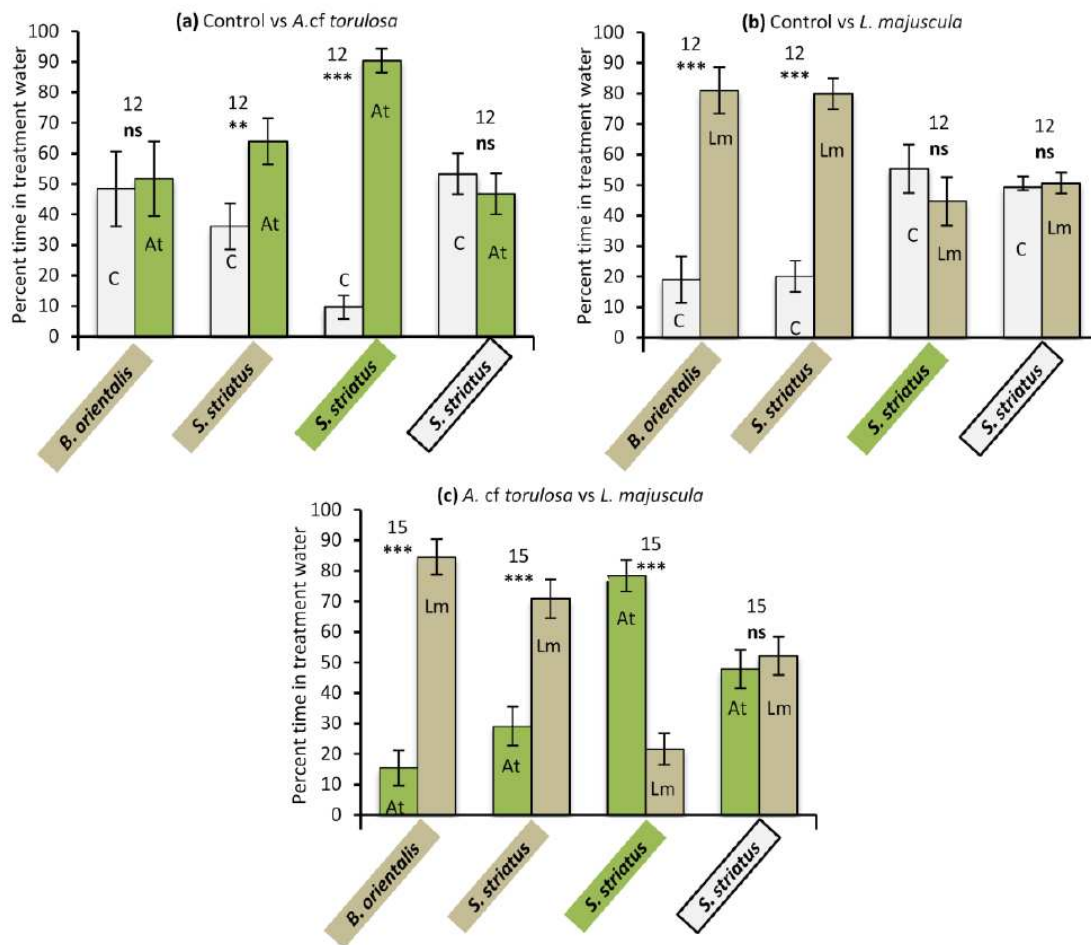


Figure 5. 3. The influence of cyanobacterial chemical cues on the orientation of *S. striatus* and *B. orientalis* reared on *L. majuscula*, *S. striatus* reared on *A. cf. torulosa* and naive *S. striatus* (not reared on either cyanobacterial species) to two water flows between either (a) seawater control (C) vs seawater conditioned with *A. cf. torulosa* (At), (b) seawater control (C) vs seawater conditioned with *L. majuscula* (Lm) and (c) seawater conditioned with *A. cf. torulosa* (At) vs seawater conditioned with *L. majuscula* (Lm). Data indicate the percent of time spent in each water flow (total time = 10 mins). The means, standard errors, p-value (**= $p < 0.01$, ***= $p < 0.001$ and ns= $p > 0.05$) and number of individuals tested (above bars) are provided for each pair-wise t-test.

5.2.2. Cyanobacterial chemicals and herbivores 's foraging behavior; assay with cotton balls soaked with chemical extracts

The T-maze choice experiments confirmed that orientation is chemically mediated based on compounds released by the cyanobacteria into the water column. Further, colonization experiments were carried out in order to target the molecules responsible for this attraction and that play the role of chemical cues in our model system. Due to the lack of *A. cf. torulosa* in the lagoon during the period of the experiments, the trials were only carried out with *S. striatus* reared on *L. majuscula*. Cotton balls were either soaked in water conditioned with *L. majuscula* and control seawater or coated with different cyanobacterial extracts: organic extract (apolar and midpolar compounds), hydroalcoholic extract (polar compounds) or whole extract (organic and hydroalcoholic extracts). A similar pattern as those used in T-maze choice was applied with 1) C vs Lm, 2) C vs At and 3) At vs Lm. Similar

to the T-maze choice trials, *S. striatus* reared on *L. majuscula* was able to locate chemical cues released by the cyanobacteria.

- *S. striatus* systematically tracked the cotton ball soaked with one of the three extracts of At [At vs C (fig. 4.a)],

- *S. striatus* tracked the cotton ball soaked with the hydroalcoholic or the whole extract of Lm [Lm vs C (fig. 4b)], and did not show any preference between the two cotton balls soaked with one of these two extracts from At or Lm [Lm vs At (fig. 4c)],

- *S. striatus* systematically tracked the cotton ball soaked with the organic extract of Lm [Lm vs C (fig. 4b) and Lm vs At (fig. 4c)].

Thus, *S. striatus* uses chemical cues present in organic extracts to track *L. majuscula* which is coherent with T-maze experiments. The next step is to further investigate the organic content of both cyanobacteria from our model system to research the compound or the compounds blend underlying the observed attraction.

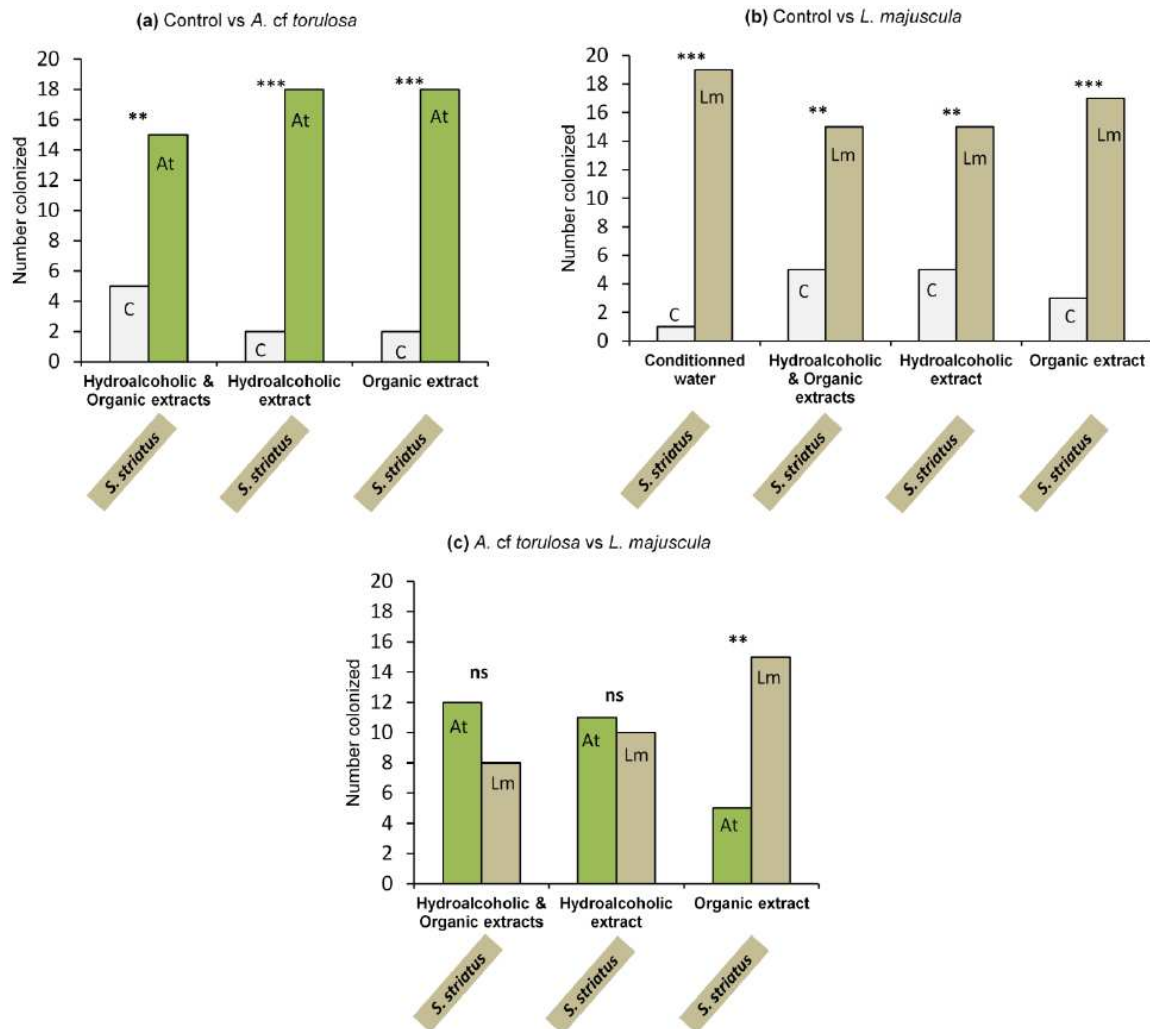


Figure 5.4. The influence of cyanobacterial chemical cues and extracts on the orientation of *S. striatus* reared on *L. majuscula* to two cotton balls between either (a) control (□) vs *A. cf. torulosa* (■), (b) control (□) vs *L. majuscula* (■) and (c) *A. cf. torulosa* (■) vs *L. majuscula* (■). Data indicate the number of cotton balls colonized. The p-value (***)= $p < 0.001$, (**)= $p < 0.01$ and (*)= $p < 0.05$) is provided for each experiment.

5.2.3. Cyanobacterial chemicals and herbivores 's feeding preferences

Sea hares preferentially track the cyanobacterial species on which they have been reared, but does this preference translate into a specialized feeding relationship? Using pair-wise feeding assays with artificial diets, the palatability of cyanobacterial crude extracts was assessed by all herbivorous found in *L. majuscula* and *A. cf torulosa*. An experimental design of pair-wise T-maze trials was used for feeding assays with 1) C vs At, 2) C vs Lm and 3) At vs Lm.

- *A. cf torulosa* crude extract strongly stimulated feeding in *S. striatus* regardless its origin while *B. orientalis* did not show any preference for *A. cf torulosa*. [At vs C (fig. 5a)],
- *L. majuscula* crude extract highly stimulated feeding by *S. striatus* reared on *A. cf torulosa* but no significant preference were observed in *S. striatus* naïve or reared on *L. majuscula*, and in *B. orientalis* [Lm vs C (fig. 5b)],
- when exposed to a choice between two foods (Lm or At crude extracts), molluscs reared on *L. majuscula* preferred to consume food with *A. cf torulosa* crude extract while *S. striatus* reared on *A. cf torulosa* preferred to feed upon *L. majuscula* crude extract. *S. striatus* reared naïve did not show any significance preference.

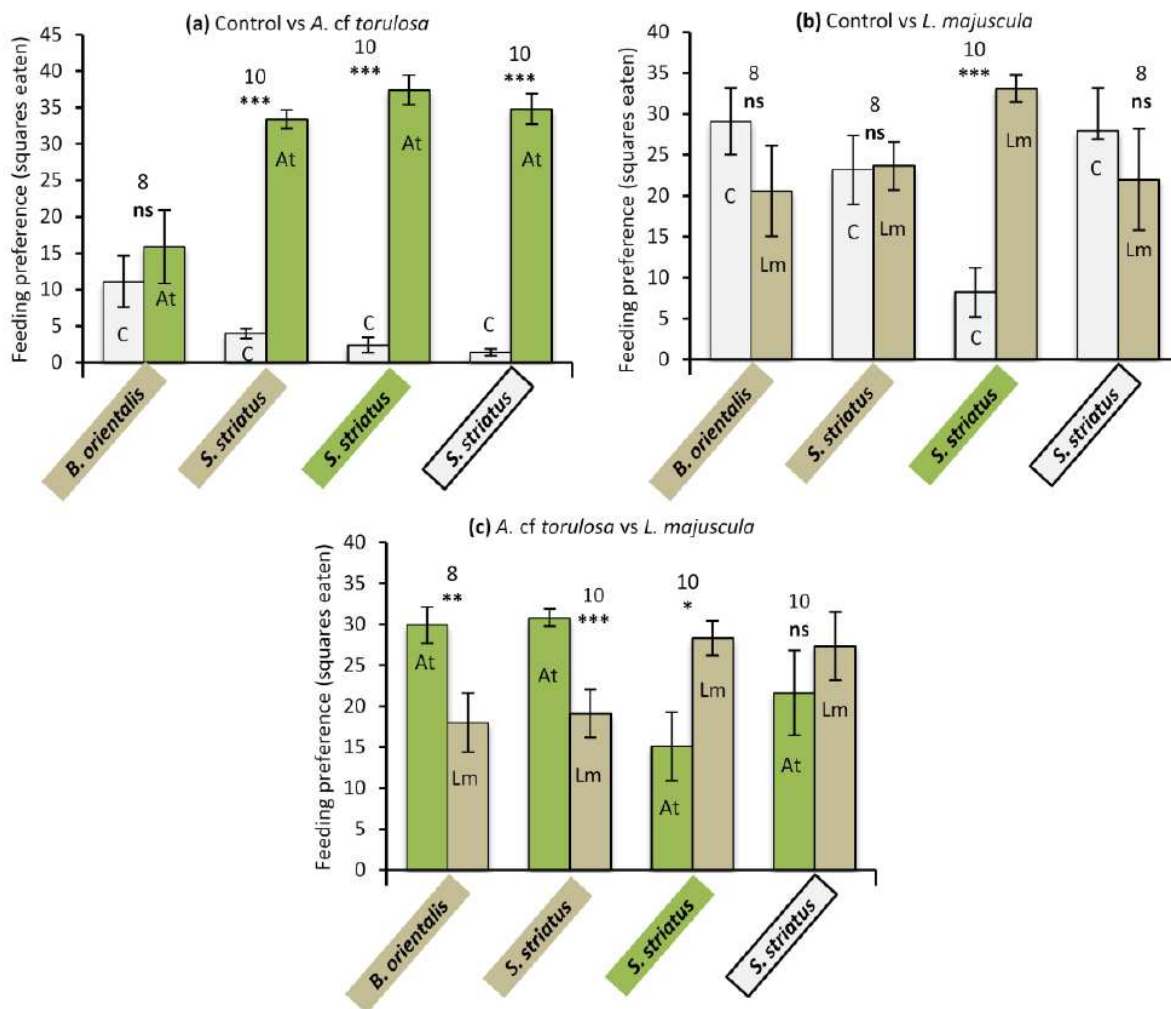


Figure 5. Effect of cyanobacterial secondary metabolites on feeding choices of *S. striatus* and *B. orientalis* reared on *L. majuscula*, *S. striatus* reared on *A. cf torulosa* and naïve *S. striatus* (not reared on either cyanobacterial species) when presented with a choice of two artificial diets between (a) control (C) and *A. cf torulosa* (At), (b) control (C) and *L. majuscula* (Lm), and (c) *A. cf torulosa* (At) and *L. majuscula* (Lm). Data indicate the number of squares of treated and control food strips consumed. Mean, standard errors, p-value (***=p<0.001, **=p<0.01 and *=p<0.05) and number of individuals tested are plotted for each pair wise test.

5.2.4. Chemical compounds in primary producers and their sequestration along the trophic web

All cyanobacteria, all organisms within the trophic web, mollusc eggs, and ink secreted by the sea hares were chemically analyzed with LC-MS and HPLC-DAD-ELSD.

All *L. majuscula* organic extract analyses revealed the presence of depsipeptides tiahuramides A-C and trungapeptins A-C, as well as serinols 4a and 4b that have previously been described^{18,19}. The cyclic lipopeptides laxaphycins A, B and B3 were detected as major compounds in the organic extracts of the cyanobacterium *A. cf torulosa*¹⁶.

In order to determine which compounds are released by cyanobacteria into their surroundings, the seawaters conditioned with primary producers that were used in T-maze experiments were chemically analyzed. Seawater was filtered on SPE-C18 cartridges to

retain the organic components. LC-MS and HPLC-DAD-ELSD analyses of the organic eluates revealed the presence of laxaphycins A, B and B3 in seawater containing At (Fig. 6b), while tiahuramides A-C were detected at low concentration in seawater containing Lm (Fig. 6a).

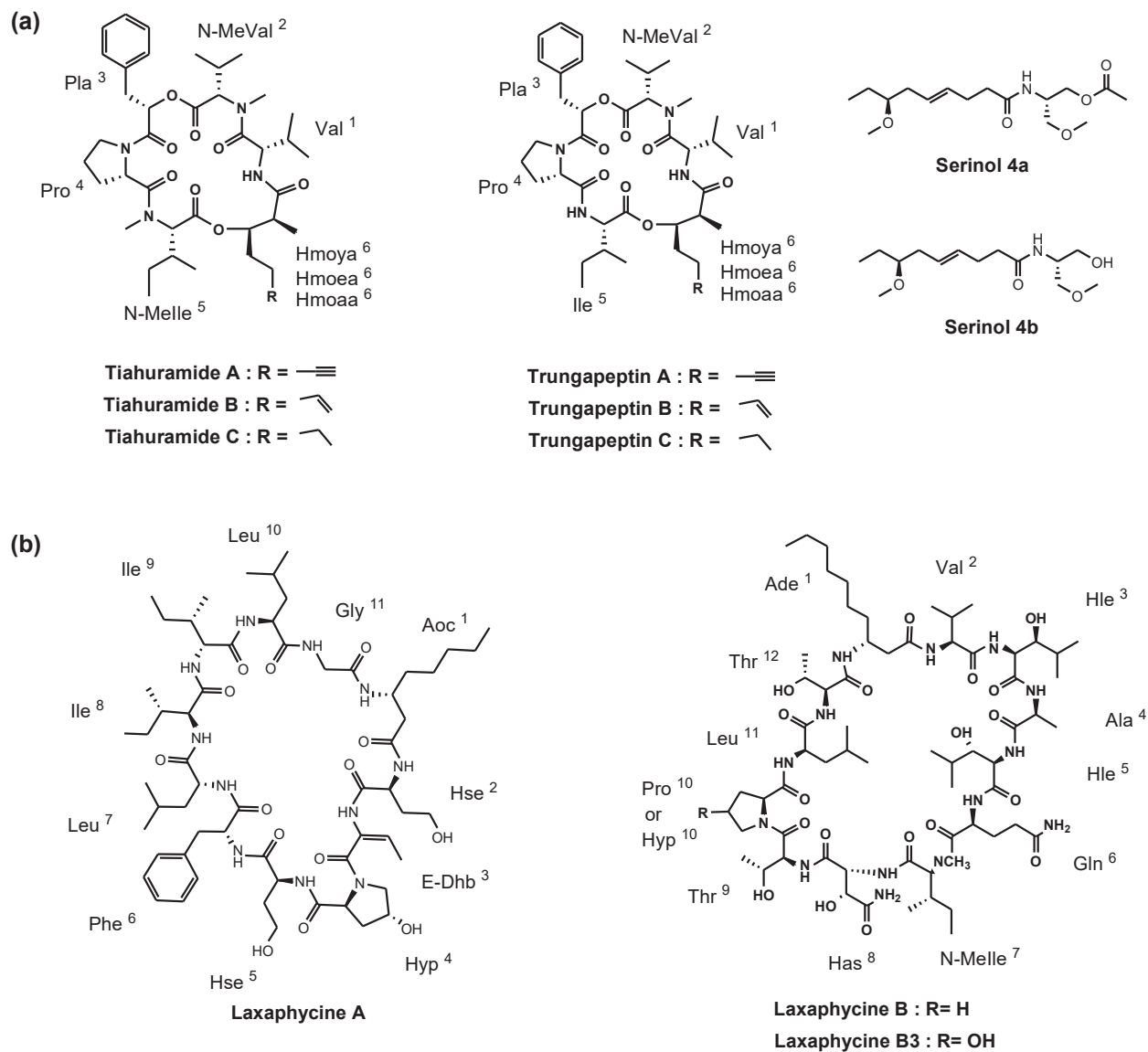


Figure 5. 6. Molecular structures of secondary metabolites produced by *Lyngbya majuscula* (a) and *Anabaena cf torulosa* (b) collected in Moorea, French Polynesia

As regards the sequestration of diet-derived compounds in the organisms feeding directly on *L. majuscula*, chromatographic analysis of the organic extracts from *S. striatus* and *B. orientalis* revealed the presence of tiahuramides A-C, trungapeptins A-C and serinol 4a and 4b (Fig. 7). However, none of these compounds were detected in either adult *G. ceylonica*, adult *T. coerulipes*, or in the eggs of *S. striatus*, *B. orientalis* and *G. ceylonica*.

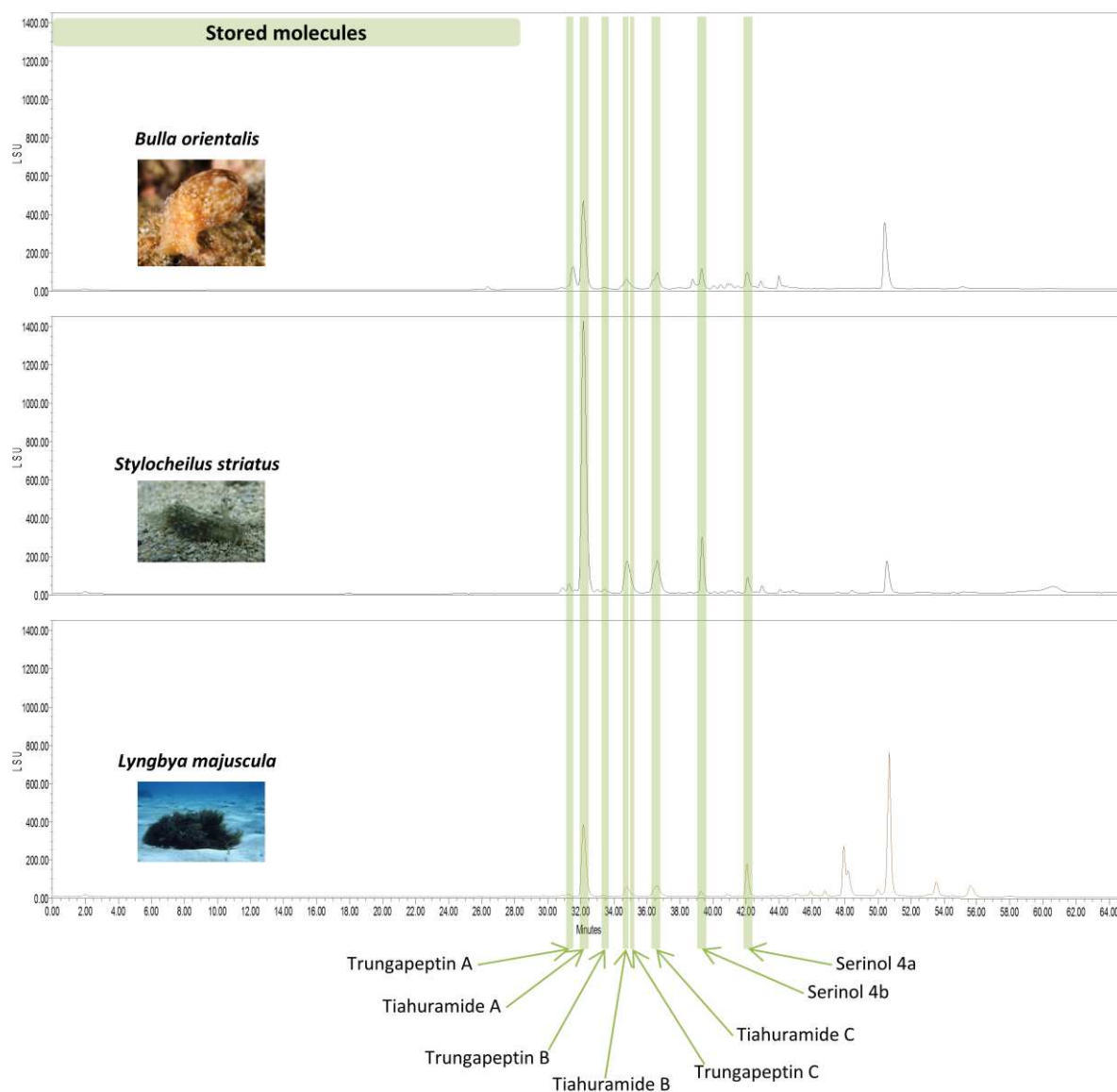


Figure 5. 7. HPLC-ELSD chromatograms of the crude extracts of *Lyngbya majuscula* and of its main herbivores (*Stylocheilus striatus* and *Bulla orientalis*). Chromatographic conditions are detailed in the experimental section. The compounds were identified by RT and m/z comparisons with previously purified compounds.

A chemical investigation of the organic extracts of the herbivores *S. striatus* and *S. longicauda* feeding on *A. cf torulosa* determined that they sequestered laxaphycin A (Fig. 8) but not laxaphycins B and B3 (Fig. 8). However, four new compounds, absent in the cyanobacterium *A. cf torulosa*, were also found in the herbivores (Fig. 8). The egg extracts of both herbivores feeding on *A. cf torulosa* did not show the presence of any cyanobacterial secondary metabolites.

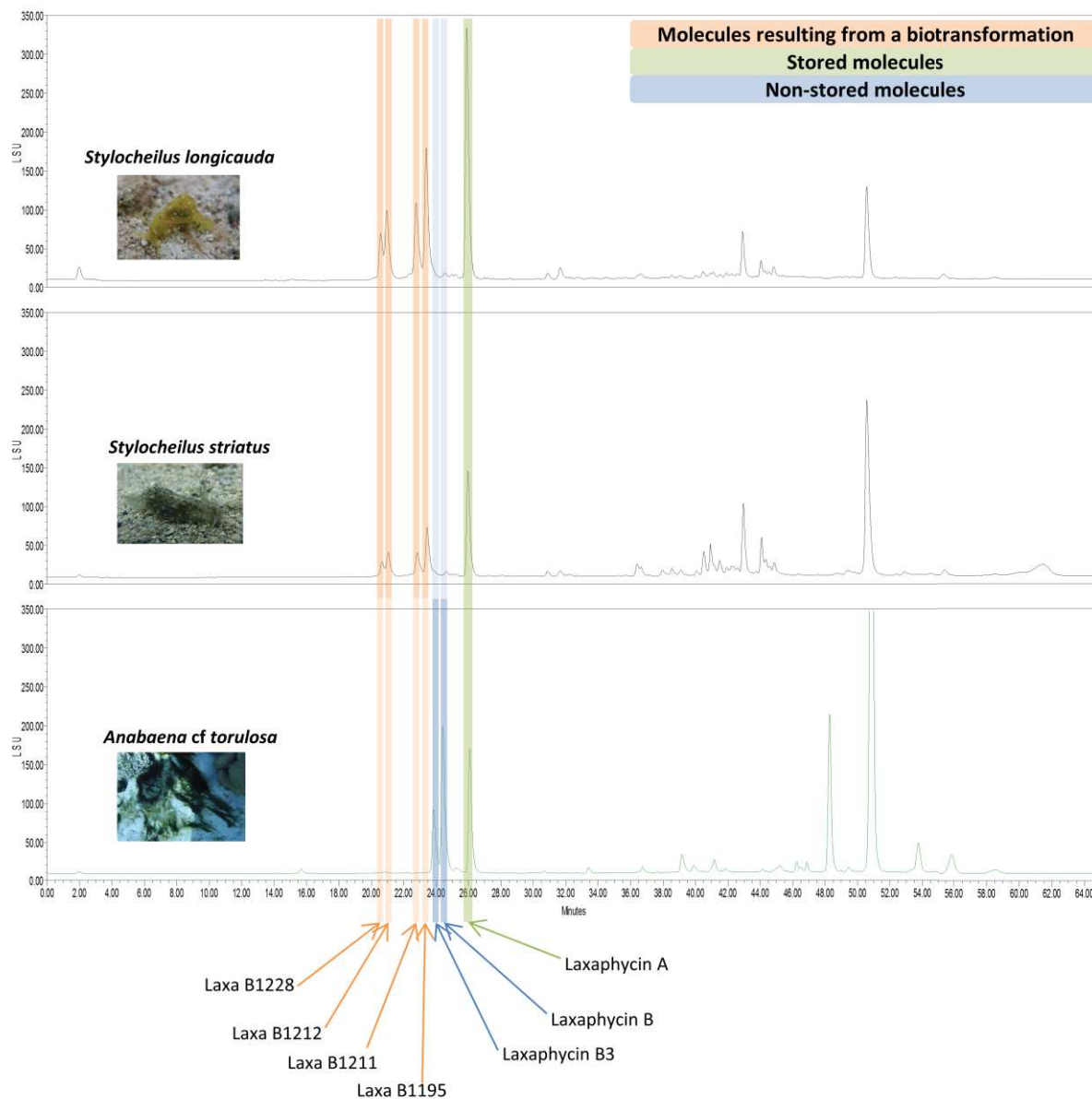


Figure 5. 8. HPLC-ELSD chromatograms of the extracts of *Anabaena cf torulosa* and the herbivores feeding on it (*Stylocheilus striatus* and *Stylocheilus longicauda*). Chromatographic conditions are detailed in the experimental section. Laxaphycins A, B and B3 were identified by RT and m/z comparisons with previously purified compounds.

5.2.5. Location of sequestered cyanobacterial secondary metabolites in *S. striatus*

The location of sequestered compounds was investigated in *S. striatus* reared on *L. majuscula* in order to understand their putative role for the sea hare. Four sea hares were dissected into head and foot, mantle, hepatopancreas (digestive gland), intestine, buccal bulb, female gland and gizzard (Figure 9).



Figure 5. 9. Dissection of *S. striatus*: (1) view of the different organs in their initial position and (2) expanded form of the organs

The diet-derived compounds appeared to be much more concentrated in the hepatopancreas (digestive gland) compared to *L. majuscula* and less concentrated in the intestine and buccal bulb (Fig. 10). Indeed, tiahuramides and trungapeptins are approximately 200 times more concentrated in digestive gland than in the cyanobacterium while it is only 10 to 30 times more concentrated in intestine and buccal bulb. The lower concentrations found in the intestine and buccal bulb may be explained by undigested or partial digestion in the two organs. Interestingly, the ratio of compounds is not the same in the sea hare and in the cyanobacteria since serinol 4b is more bioaccumulated than the other compounds (Bioaccumulation factor ≈ 1500). The cyanobacterial secondary metabolites were not detected in mantle, head and foot, gizzard or female gland. Thus, the sequestration occurring in the hepatopancreas (digestive gland) was consistent with previous studies demonstrating the sequestration of monoterpenes, terpenoids, and alkaloids in the same organ.

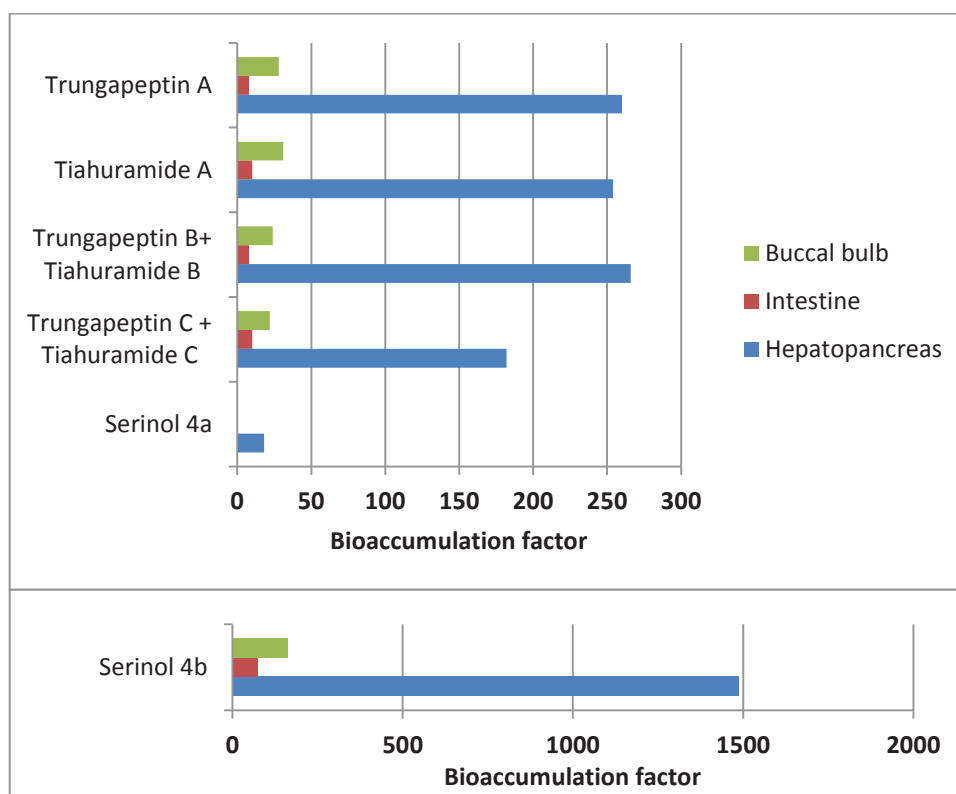


Figure 5. 10. Bioaccumulation of cyanobacterial compounds in *S. striatus* 's hepatopancreas, intestine and buccal bulb. Data indicate the bioaccumulation factor (details of the calculation are given in Materials and Methods section)

5.2.6. Characterization of compounds biotransformed by *S. striatus*

Stylocheilus striatus were collected on *A. cf. torulosa* in the lagoon of Moorea, French Polynesia, sealed underwater in a bag, freeze-dried and extracted. The crude extract was fractionated using flash chromatography and the resulting fractions containing new peptides was subjected to HPLC purification to yield laxaphycin B1195 (6.5 mg), laxaphycin B1211 (4.5 mg), laxaphycin B1212 (2 mg) and laxaphycin B1228 (6 mg). All the compounds were obtained as a white, amorphous powder and laxaphycins B1212 and B1228 responded positively to a ninhydrin test suggesting a non-blocked *N*-terminus¹.

¹ Note: The NMR experiments of the four compounds (laxaphycins B1195, B1211, B1212 and B1228) were carried out by the JEOL Company. Unfortunately, they lost the compounds in attempting to return them to us. Thus, we could not carry out HRMS analyses, other NMR experiments (on laxaphycins B1212 and B1228) or investigate their biological activities.

5.2.6.1. Structure elucidation of laxaphycin B1195

The positive high-resolution electrospray ionization mass spectrometry (HRESIMS) spectrum of laxaphycin B1195 (Fig. 10) showed the $[M + H]^+$ pseudomolecular ion at m/z 1196.7. All the NMR experiments were conducted in DMSO-*d*₆. The signal distribution pattern observed in the ¹H-NMR spectrum of laxaphycin B1195 was characteristic of lipopeptides displaying amide NH signals (δ_H 7.40-7.90), C α H signals (δ_H 3.5-4.7), aliphatic CH₂ (δ_H 1.1-1.3) and CH₃ signals (δ_H 0.7-0.9). It is noteworthy that two conformers of laxaphycin B1195 in solution (integration ratio 3:1 in DMSO-*d*₆) were observed according to the doubling of some signals in the ¹H-NMR spectrum. In the NH proton region, 8 doublets and 2 singlets were observed. The values of chemical shifts (table 1) were reported using 2D-NMR spectra including COSY, TOCSY, HSQC, HSQC-TOCSY, and ROESY. Analysis of TOCSY correlations revealed the presence of 10 amino acid residues: *N*-methylisoleucine (N-Melle), 3-hydroxyasparagine (Has), two threonines (Thr), proline (Pro), leucine (Leu), β -aminodecanoic acid (β -Ade), valine (Val) and 3-hydroxyleucine (Hle). In comparison with laxaphycin B, ¹H-NMR revealed the absence of two NH singlets corresponding to NH₂ belonging to a glutamine lateral chain, and analyses of HSQC spectrum revealed the absence of typical correlations within alanine (between H β δ_H 1.31 and C β δ_C 17.55) and within one of the two hydroxyleucines (between H β δ_H 3.49 and C β δ_C 75.8-76.4) suggesting the lack of these two residues in laxaphycin B1195. The remaining non-identified spin system revealed correlations between NH proton at 7.77 ppm and protons at 2.09/2.22 and 2.03/2.20 ppm. The presence of pyroglutamate (Glp) was deduced by HMBC correlations (Fig. 10) from the amide proton (δ_H 7.77) to C α (δ_C 53.17), C β (δ_C 23.97), C γ (δ_C 29.19) and C δ (δ_C 177.31), from H α (δ_H 4.50) to C δ (δ_C 177.31), from H β (δ_H 2.09/2.22) to C α (δ_C 53.17), C γ (δ_C 29.19) and C δ (δ_C 177.31) and from H γ (δ_H 2.03/2.20) to C β (δ_C 23.97) and C δ (δ_C 177.31) as well as by a ROESY correlation (Fig. 10) between the amide proton (δ_H 7.77) and the H α (δ_H 4.50). HMBC spectrum provided information on sequence-specific assignments. Indeed, the cross-peaks between carbonyl carbons (residue *i*) and NH, NCH₃ protons or H α (residue *i*+1) suggested the presence of two partial sequences including Glp-N-Melle-Has-Thr (fragment 1) and Pro-Leu-Thr- β -Ade-Val-Hle (fragment 2). Analyses of the ROESY spectra revealed a correlation between H α (δ_H 4.55) of Thr⁴ and H δ (δ_H 3.63/3.76) of Pro⁵, assembling the fragments 1 and 2 and establishing the complete sequence as Glp-N-Melle-Has-Thr-Pro-Leu-Thr- β -Ade-Val-Hle. The lack of HMBC or ROESY correlations between Glp and Hle revealed that the peptide is linear (Fig. 10).

Table 5. 1. NMR spectroscopic data for laxaphycin B1195 and laxaphycin B1211 (303 K) in DMSO-*d*₆

	Laxaphycin B1195		Laxaphycin B1211	
	¹³ C δ (ppm)	¹ H δ (ppm)	¹³ C δ (ppm)	¹ H δ (ppm)
Glp¹	NH		NH	7.79
	CαH	53.17	CαH	4.50
	CβH ₂	23.97	CβH ₂	2.09/2.22
	Cγ H ₂	29.19	Cγ H ₂	2.04/2.22
	CONH	173.31	CONH	177.95
	CO	172.97	CO	173.00
N-MeLe²	NCH ₃	29.62	NCH ₃	2.88
	CαH	59.84	CαH	4.73
	CβH	31.13	CβH	1.92
	CγH ₂	24.06	CγH ₂	1.29/1.90
	Cγ' H ₃	15.24	Cγ' H ₃	0.78
	CδH ₃	10.42	CδH ₃	0.80
	CO	169.87	CO	169.87
Has³	NH		NH	7.52
	CαH	55.42	CαH	4.62
	CβH	71.00	CβH	4.36
	OH		OH	
	CONH ₂	173.39	CONH ₂	173.43
	NH ₂		NH ₂	7.29/7.35
CO	169.32	CO	168.77	
Thr⁴	NH		NH	7.43
	CαH	55.17	CαH	4.56
	CβH	66.55	CβH	3.95
	OH		OH	
	CγH ₃	18.49	CγH ₃	1.01
	CO	169.38	CO	169.13
Pro⁵ ou Hyp⁵	CαH	60.01	CαH	4.40
	CβH ₂	29.22	CβH ₂	1.89/2.05
	CγH(2)	24.16	CγH	4.27
	OH		OH	
	CδH ₂	47.49	CδH ₂	3.57/3.76
	CO	170.35	CO	171.45
Leu⁶	NH		NH	7.97
	CαH	51.51	CαH	4.26
	CβH ₂	40.50	CβH ₂	1.45
	CγH	24.16	CγH	1.58
	CδH ₃	23.01	CδH ₃	0.85
	Cδ' H ₃	21.41	Cδ' H ₃	0.83
	CO	172.01	CO	172.30
Thr⁷	NH		NH	7.72
	CαH	58.37	CαH	4.07
	CβH	66.43	CβH	3.95
	OH		OH	
	CγH ₃	19.67	CγH ₃	0.99
	CO	169.59	CO	170.08
β-Ade⁸	NH		NH	7.57
	CαH ₂	40.47	CαH ₂	2.36
	CβH	46.33	CβH	4.02
	CγH ₂	33.51	CγH ₂	1.34/1.40
	CδH ₂	28.85	CδH ₂	1.21
	CεH ₂	28.71	CεH ₂	1.21
	CζH ₂	25.44	CζH ₂	1.21
	Cη H ₂	31.30	Cη H ₂	1.22

	C θ H ₂	22.13	1.23	C θ H ₂	22.13	1.24
	C ι H ₃	13.99	0.83	C ι H ₃	14.00	0.83
	CO	171.85		CO	170.41	
Val ⁹	NH		7.87	NH		7.89
	C α H	57.73	4.26	C α H	57.73	4.25
	C β H ₂	30.40	2.01	C β H ₂	30.32	2.02
	C γ H ₃	19.30	0.81	C γ H ₃	19.30	0.82
	C γ' H ₃	17.68	0.81	C γ' H ₃	17.74	0.81
	CO	171.00		CO	170.95	
Hle ²⁰	NH		7.60	NH		7.59
	C α H	54.69	4.35	C α	54.86	4.35
	C β H	76.15	3.51	C β H	76.16	3.51
	OH		5.90	OH		
	C γ H	30.82	1.51	C γ H	30.83	1.52
	C δ H ₃	19.19	0.89	C δ H ₃	19.17	0.89
	C δ' H ₃	19.06	0.75	C δ' H ₃	19.10	0.76
	CO	172.49		CO	172.03	

5.2.6.2. Structure elucidation of laxaphycin B1211

The molecular formula of laxaphycin B1211 (Fig. 10) was determined as C₅₆H₉₇N₁₁O₁₈ by HRESIMS analysis (m/z 1212.7 [M+H]⁺). Comparison with laxaphycin B1195 suggested a gain of oxygen comparable to the difference between laxaphycin B and laxaphycin B3, which suggests the presence of a Hyp residue instead of a Pro. The NMR spectral analysis of laxaphycin B1211 showed strong similarities with laxaphycin B1195. As is the case with laxaphycin B1195, an analysis of ¹H-NMR spectrum revealed the presence of 8 doublets and 2 singlets in the NH proton region. Analysis of 2D-NMR including COSY, TOCSY, HSQC, HSQC-TOCSY, and ROESY determined almost all ¹H and ¹³C chemical shifts of laxaphycin B1211. By comparison with laxaphycins B3 and B1195, TOCSY spectrum allowed us to identify ten amino acid as pyroglutamate (Glp), N-methylisoleucine (N-Melle), 3-hydroxyasparagine (Has), two threonines (Thr), 4-hydroxyproline (Hyp), leucine (Leu), β -aminodecanoic acid (β -Ade), valine (Val) and 3-hydroxyleucine (Hle). HMBC spectrum confirmed the presence of pyroglutamate by displaying correlations from the amide proton (δ_H 7.79) to C α (δ_C 53.16), C β (δ_C 24.07) and C γ (δ_C 29.12), from H β (δ_H 2.09/2.23) to C δ (δ_C 177.95) and from H γ (δ_H 2.04/2.22) to C β (δ_C 24.07) and C δ (δ_C 177.95). Correlations in HMBC between carbonyl carbons (residue *i*) and NH, NCH₃ protons or H α (residue *i*+1) partially enabled us to determine sequence-specific assignments (Fig. 10). Indeed, the partial sequence Glp-N-Melle-Has-Thr (fragment 1) was established with correlations between the H α of N-Melle² (δ_H 7.89) and the carbon carbonyl of Glp¹ (δ_C 173.00), between the NH proton of Has³ (δ_H 7.52) and the carbon carbonyl of N-Melle² (δ_C 169.87) and between the NH proton of Thr⁴ (δ_H 7.43) and the carbonyl carbon of Has³ (δ_C 168.77). Hyp and Ile residues were assembled (fragment 2) by a correlation between the H α of Leu¹ (δ_H 4.26) and the carbonyl carbon of Hyp⁵ (δ_C 171.45). Finally, the fragment Thr- β -Ade-Val (fragment 3) was deduced from correlations between the NH proton of β -Ade⁸ (δ_H 7.57) and the carbonyl carbon of Thr⁷ (δ_C 170.08) and between the NH proton of Val⁹ (δ_H 7.89) and the carbonyl carbon of β -Ade⁸ (δ_C 170.41). Fragments 1, 2 and 3 were connected by inter-residue ROESY correlations (Fig. 10) between the H α of Thr⁴ (δ_H 4.56) and the H δ of Hyp⁵ (δ_H 3.57/3.76) and between the H α

of Leu⁶ ($\delta_{\text{H}}4.26$) and the NH of Thr⁷ ($\delta_{\text{H}}7.57$). No ROESY or HMBC correlations were observed between Hle and Glp confirming that the cyclic is linear while a ROESY correlation between the H α of Val⁹ ($\delta_{\text{H}}4.25$) and the NH of Hle¹⁰ ($\delta_{\text{H}}7.57$) indicated that the C-terminus is carried by the Hle, defining the complete sequence as Glp-N-Melle-Has-Thr-Hyp-Leu-Thr- β -Ade-Val-Hle.

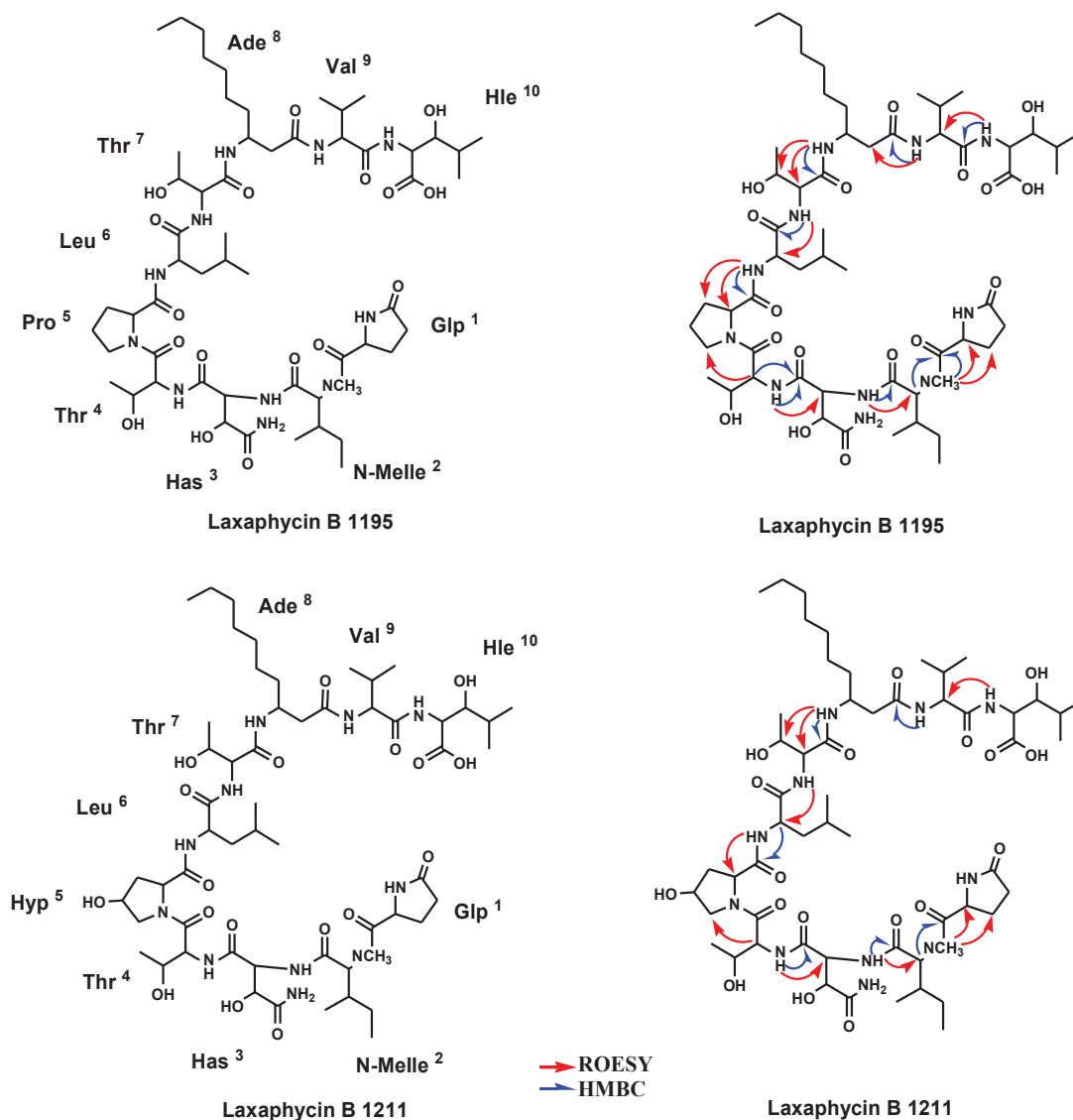


Figure 5. 11. Molecular structures of laxaphycins B 1195 and B 1211 with ROESY (red arrows) and HMBC (blue arrows) correlations.

5.2.6.3. Structure hypothesis for laxaphycins B1212 and B1228

LC-MS analyses revealed a difference of 17 uma between laxaphycins B1195 and B1212 as well as between laxaphycins B1211 and B1228 (Fig. 11) that could be considered to be the loss of NH₃ linked to the cyclization of glutamine into pyroglutamate. The poor resolution in the 1D-NMR and 2D-NMR spectra prevented us from confirming the complete structure of laxaphycins B1212 and B1228. We could speculate the presence of several conformers for both compounds. The presence of glutamine NH₂-terminus and glutamine lateral chain NH₂ may enable the formation of several hydrogen bonds and the presence of several 3D conformers that support the assumption developed above. However, for laxaphycin B1228, the analyses of ¹H-NMR and 2D-NMR including TOCSY and HSQC enabled us to identify Ade, Val and Has, while the presence of Thr, Leu and N-Melle is suggested (Table 2). As regards to laxaphycin B 1212, the small amount of isolated compounds prevented us from identifying any residues.

Table 5. 2. NMR spectroscopic data for laxaphycin B1228 (303 K) in DMSO-d₆

		Laxaphycin B1228	
		¹³ C	¹ H
		δ (ppm)	δ (ppm)
Glu ¹	NH ₂		-
	CαH	-	-
	CβH ₂	-	1.90
	Cγ H ₂	30.57	2.20
	CONH ₂	-	
	NH ₂		-
	CO	-	
N-MeLe ²	NCH ₃	28.72	2.88
	CαH	-	-
	CβH	-	-
	CγH ₂		-
	Cγ'H ₃	15.35	0.76
	CδH ₃	10.32	0.78
	CO	-	
Has ³	NH		7.56
	CαH	55.48	4.69
	CβH	70.88	4.37
	OH		-
	CONH ₂	173.46	
	NH ₂		-
Thr ⁴	NH		-
	CαH	56.14	4.52
	CβH		3.99
	OH		-
	CγH ₃	19.78	0.99
	CO		
Hyp ⁵	CαH	59.17	4.35
	CβH ₂	37.66	1.88/2.04
	CγH	68.24	4.29
	OH		-
	CδH ₂	55.81	3.62/3.76
	CO	-	

Leu ⁶	NH		-
	C α H	51.84	4.23
	C β H ₂	40.24	1.47
	C γ H	24.04	1.57
	C δ H ₃	-	-
	C δ' H ₃	-	-
Thr ⁷	CO	-	-
	NH		-
	C α H	-	-
	C β H	-	-
	OH	-	-
	C γ H ₃	-	-
β -Ade ⁸	CO	-	-
	NH		7.65
	C α H ₂	40.42	2.35
	C β H	46.40	3.98
	C γ H ₂	33.50	1.38
	C δ H ₂	28.85	1.19
	C ϵ H ₂	28.72	1.19
	C ζ H ₂	25.62	1.19/1.23
	C η H ₂	31.28	1.19
	C θ H ₂	22.14	1.22
	C ι H ₃	13.98	0.81
Val ⁹	CO	170.50	-
	NH		7.90
	C α H	55.40	4.19
	C β H ₂	29.67	2.00
	C γ H ₃	19.41	0.75
	C γ' H ₃	17.83	0.78
Hle ¹⁰	CO	170.58	-
	NH		-
	C α H	-	-
	C β H	-	-
	OH	-	-
	C γ H	-	-
	C δ H ₃	-	-
	C δ' H ₃	-	-
CO	-	-	

HPLC-ELSD analysis highlighted the conversion of laxaphycins B1212 and B1228 into laxaphycins B1195 and B1211 over a time span of 18 days. The conversion occurred spontaneously in CH₃OH at room temperature (20-25 °C) during the observation period, while no conversion was observed at -25 °C. The two compounds, laxaphycins B1212 and B1228, were also subjected to an extraction condition in CH₃OH-CH₂Cl₂ (50:50) with ultrasound over ten minutes (three times) followed by evaporation and did not convert into laxaphycins B1195 and B1211. It is noteworthy that the conversion of laxaphycins B1212 and B1228 did not occur in dry extracts. Thus we can conclude that laxaphycins B1195 and B1211 were present in the sea hare before the extraction.

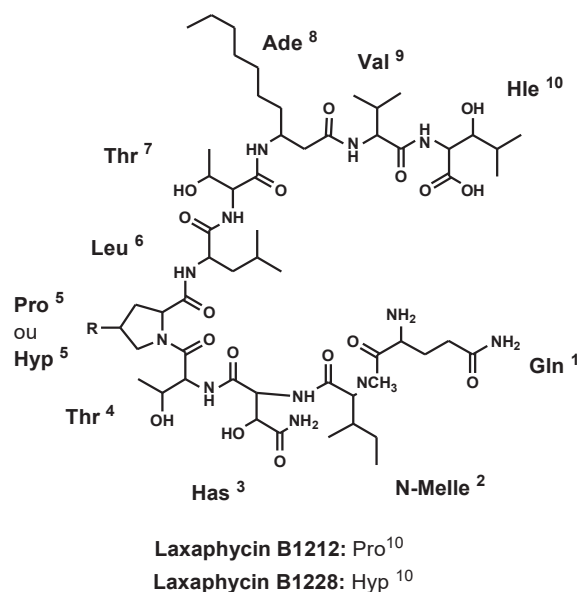


Figure 5. 12. Putative molecular structures of laxaphycin B1212 and laxaphycin B1228

5.2.7. Chemical compounds in ink and opaline mixtures

It is well known that sea hares excrete ink when they are attacked as a defensive behavior. Our examination of ink mixtures excreted by *S. striatus* reared on *L. majuscula* and by *S. striatus* and *S. longicauda* reared on *A. cf. torulosa* revealed that they displayed different colors. *S. striatus* reared on *L. majuscula* excreted a purple ink when attacked, while the latter two secreted a blue ink. These colour differences suggested that the chemical component composition of ink is dependent on diet. Differences in pigment composition were observed by LC-PDA-MS analyses of ink crude extracts and from comparisons of retention times, m/z and UV-Vis spectra: ink from sea hares reared on *L. majuscula* was composed of phycoerythrobilin, while ink from sea hares reared on *A. cf. torulosa* contained phycoerythrobilin and aplysioviolin. These two molecules are derived from a light-harvesting protein present in red algae and cyanobacteria and are known to act as deterrent chemicals against the blue crab *Callinectes sapidus*²³. However, the two molecules were not predominant and unidentified hydrophilic molecules formed the rest of the ink mixture. Further analyses are needed to identify these compounds. No known metabolites (cyclic lipopeptides or others) were found in the ink mixtures.

5.3. Discussion

5.3.1. Adaptative preference of *Stylocheilus striatus* and *Bulla orientalis* to their prey

We pondered about the feeding specialization of the herbivores. Previous studies have established that the sea hare *S. striatus* is a specific predator of the cyanobacterium *L. majuscula*^{9,10,12}. However, our field observations demonstrated that *S. striatus* not only feeds on *L. majuscula* but also on the cyanobacterium *A. cf torulosa*. Further field observations will determine whether *S. striatus* is found on other cyanobacteria or algal species. By contrast, the cephalaspidean *B. orientalis* was found to feed exclusively on *L. majuscula*, while other species of *Bulla* genus are considered as generalist herbivores^{24–26}. We wondered about the role of cyanobacterial secondary metabolites in herbivores foraging and feeding behaviors. The T-maze olfactory results proved that the sea hare *S. striatus* was able to follow the chemical trails leading to the cyanobacterium on which it has been collected: *S. striatus* reared on *L. majuscula* is attracted by the “odor” of *L. majuscula* and *S. striatus* reared on *A. cf torulosa* is attracted by the “odor” of *A. cf torulosa*. *S. striatus* tracks naturally the odor it knows. *S. striatus* reared on *A. cf torulosa* is not attracted by *L. majuscula* and the individuals reared naïve could not distinguish between the chemical cues released by the two cyanobacteria. However, it is noteworthy that *S. striatus* reared on *L. majuscula* followed the chemical trail leading to *A. cf torulosa* in the experiment control vs *A. cf torulosa* (Fig. 3a) even though the herbivore had not encountered the cyanobacteria before. This result suggests that the chemicals cues from *A. cf torulosa* may be more effective than those from *L. majuscula*. As regards to *B. orientalis*, the results were also consistent with field observations since the cephalaspidea also showed a specific consumer behavior by locating its prey, *L. majuscula*, in all experiments with seawater conditioned with *L. majuscula* (Fig. 3b and 3c). The presence of laxaphycins and, to a lesser degree, tiahuramides in the water surrounding the cyanobacteria may indicate their putative role in chemically cueing the herbivores. The colonization experiments were carried out with *S. striatus* reared on *L. majuscula* in order to highlight the role of cyanobacterial lipophilic or hydrophilic compounds in prey tracking. We found that the sea hare was able to use both hydrophilic and lipophilic compounds to locate its food, but that the mollusc used the prey lipophilic blend to distinguish between the two cyanobacteria and to return to their food source of origin. We suggest that the hydrophilic blend are composed of non-specific molecules produced by different cyanobacteria and do not enable consumers to distinguish between food sources. By contrast, the lipophilic blend, notably with the presence of different families of molecules by the two primary producers, may be more specific and enables the mollusc to locate the cyanobacterium on which it was reared. Moreover, the lipophilic blend appeared to be mainly composed by lipopeptides belonging to tiahuramide family in *L. majuscula* and laxaphycin family in *A. cf torulosa* suggesting a putative role in sea hare cyanobacterial tracking.

The feeding choice trials of *S. striatus* and *B. orientalis* enable us to determine the extent of herbivory specialisation of both herbivores that show a varied diet. The feeding choice trial results provide information on the palatability of the chemical contents of cyanobacteria and indicate that the herbivores favored a diet that they are not accustomed to feed upon. These results suggest a generalist foraging behavior for both herbivores and could be supported by the detoxification limit hypothesis (DLH)²⁷. This hypothesis applies for generalist herbivores and suggests that overall feeding rates, as well as overall consumer performance, are enhanced with a mixed diet compared to a single one. The processing of secondary metabolites includes their detoxification (or biotransformation) and excretion that are carried out by several enzymes²⁸⁻³⁰. As there is a limit to the amount of each secondary metabolite that can be detoxified, the ingestion of different secondary metabolites with non-overlapping detoxification pathways could increase consumer fitness. However, the results regarding the trial control vs *A. cf torulosa* were not consistent with this DLH as feeding by *S. striatus* reared on *A. cf torulosa* and reared naive was strongly stimulated by *A. cf torulosa*, whereas the DLH would predict the opposite. In addition, feeding on different foods may simply offer a nutritional complement to herbivores as is the case of the sea hare *Dolabella auricularia* which grows faster on a mixed, rather than on a single diet³¹, but our experiments were carried out using diets of equivalent nutritional value and do not allow us to test this hypothesis.

It is noteworthy that herbivores seem to show different behaviors in T-maze experiments and feeding choice experiments but these behavior differences are not inconsistent. Indeed, the T-maze experiments involve a significant movement to track the cyanobacteria. The mollusc will not risk a substantial energy loss in tracking an unknown cyanobacterium that may be unpalatable, that may not bring sufficient nutritional values and that may not provide it a shelter from potential predators. By contrast, the feeding choice experiments involve less displacements since the food strips remain close each other. Thus, the mollusc does not spend considerable energy foraging and it can easily taste different food including unknown nourishment.

As regards both herbivores tested, it is difficult to be definitive about a specialist or generalist behavior as *S. striatus* appear to consume different food but cyanobacterial secondary metabolites clearly influence its host tracking and feeding choice whereas *B. orientalis*, observed only upon *L. majuscula*, tracks preferentially its host using chemoreception but consume *A. cf torulosa*'s compounds without apparent negative effect.

5.3.2. Sequestration of secondary metabolites and their role in determining the length of the trophic web

After consumption of cyanobacteria, we are interested in the fate of their secondary metabolites in herbivores higher trophic levels. It is noteworthy that Glutathione S-transferases (GSTs), an enzyme family involved in the biotransformation process, are also responsible for sequestering secondary metabolites as a protective mechanism in cell cytosol³²⁻³⁵. *S. striatus* is known to sequester several secondary metabolites produced by *L. majuscula*^{9,10,36}. Here we found that *S. striatus* and *B. orientalis* reared on *L. majuscula* are able to sequester the tiahuramides A-C, the trungapeptines A-C and the serinols 4a and 4b. Nevertheless, no study revealed prey secondary metabolite sequestration by the shelled gastropod *B. orientalis* and species belonging to the *Bulla* genus are known to biosynthesize polypropionates that are used defensively^{25,26}. Interestingly, *S. striatus* reared on *A. cf. torulosa* sequesters laxaphycin A but biotransforms laxaphycin B and B3 into laxa B1212, laxa B1228, laxa B1195 and laxa B1211. Several studies report the biotransformation of prey secondary metabolites by *S. striatus* and other sea hares. Generally, the reaction appears to be an acetylation which biotransforms lynbyatoxin A (LTA) and malyngamide B, produced by *L. majuscula* collected in Moreton Bay, Australia and Guam, into LTA acetate and malyngamide B acetate respectively^{9,37}. Similarly, the anaspidean *Aplysia dactylomela* transforms the brown algal compound 14-ketoepitaondiol as well as the red algal secondary metabolites isolaurenisol and allolaurenisol into 3-ketoepitaondiol, isolaurenisol acetate and allolaurenisol acetate respectively. In our study, the biotransformation is novel since it involves the hydrolysis of the cyclic lipopeptides with the loss of two amino acid residues, hydroxyleucine and alanine, leading to laxa B1212 and laxa B1228 and then cyclization of the N-terminal glutamine to pyroglutamate with the loss of NH₃ leading to laxa B1195, laxa B1211. The hydrolysis may be carried out by phase I peptidase enzymes which are known to cleave the amide bond between contiguous amino acids by initiating a nucleophilic attack on the carbonyl moiety²⁹. Glutaminy-peptide cyclotransferase are enzymes belonging to the aminoacyltransferase family and are able to carry out the conversion of glutamine to pyroglutamate³⁸⁻⁴⁰. Although these kinds of enzymes have only been identified in humans and plants, it is possible that similar enzymes are present in *S. striatus* which could convert laxaphycins B1212 and B1228 into laxaphycins B1195 and B1211. However, we cannot exclude that the cyclization occurs spontaneously without the intervention of any enzyme as is the case of human monoclonal antibodies⁴¹. Furthermore, we found that laxaphycins B1212 and B1228 spontaneously cyclize in MeOH even if the conversion occurred slowly.

We investigated the horizontal and vertical transmissions of the diet-derived secondary metabolites and their location in the body of the *S. striatus* reared on *L. majuscula*. The cyanobacterial compounds were not transmitted to adult carnivorous predators, *G. ceylonica* or *T. coeruleipes*, nor to eggs of *S. striatus*, *B. orientalis* and *G. ceylonica*. The cyanobacterial compounds appeared to be highly concentrated in the hepatopancreas (digestive gland) of *S. striatus* and at a lower concentration in the buccal

bulb and intestine, while being absent in external tissues. These results were consistent with previous studies showing the sequestration of diet-derived compounds in the digestive gland of *S. striatus*^{9,14,36}. It is not unlikely that herbivores have developed a tolerance mechanism to feed upon potentially repellent cyanobacteria in order to take advantage of the shelter provided by the primary producer. However we did not investigate the location of the diet-derived secondary metabolites and the biotransformed compounds in the body of neither *S. striatus* and *S. longicauda* reared on *A. cf torulosa* nor *B. orientalis* reared on *L. majuscula*. Sequestration and biotransformation may be considered a defense strategy if sequestration occurs in external parts of the body or in the ink mixture, but we did not find any diet-derived compounds in *S. striatus* ink or in external body parts. One might wonder why the molluscs sequester the cyanobacterial secondary metabolites and why *S. striatus* biotransform some of them. Are the biotransformed laxaphycins less or more toxic than the original ones? The sequestration could simply occur as a protective mechanism since the GSTs, involved in sequestration, possess high binding activity with some exogenous compounds in order to keep them away from target nuclear proteins^{32–34,42}.

Our field observations revealed that *G. ceylonica*, predator of *S. striatus*, and *T. coerulipes*, generalist predator feeding upon *S. striatus*, *B. orientalis* and *G. ceylonica*, are only present upon *L. majuscula*. We suggest that, contrary to *L. majuscula*, secondary metabolites produced by *A. cf torulosa* could repel *G. ceylonica* and *T. coerulipes*. This hypothesis is supported by a previous study which showed that laxaphycin A strongly deterred feeding by the parrotfish *Scarus schlegeli*, the sea urchin *Diadema savignyi* and the crabs *Leptodius* spp ref. To our knowledge, the repellent activity has not been investigated for tiahuramides, trunapeptins and serinols 4a and 4b so we are unable to compare the deterrence activity of each compound. We hypothesise that the presence of *S. striatus* on *A. cf torulosa* is adaptive, since the sea hare appeared to be protected from predation by *G. ceylonica* and *T. coerulipes*.

The sea hare *S. striatus* and the cephalaspidean *B. orientalis* are able to track the chemical cues released by the cyanobacteria. As regards to *L. majuscula*, Geange and Stier¹⁵ showed that the herbivory pressure of *S. striatus* does not benefit the cyanobacterium but that the presence of *G. ceylonica*, consumer of the sea hare, reduced this herbivory pressure. The fact that *G. ceylonica* is present only on *L. majuscula* and does not consume *S. striatus* feeding upon *A. cf torulosa* indicates that *G. ceylonica* might be attracted by the chemicals released by *L. majuscula* induced during feeding. Indeed, some primary producers are able to increase chemical defense in response to herbivory pressure^{43–46}. Thus, the chemical cues unintentionally released by *L. majuscula* attracting *S. striatus* may also attract *G. ceylonica* or might have evolved as a chemical signal toward the nudibranch.

5.4. Materials and Methods

5.4.1. Organism collection

The cyanobacteria *Anabaena cf torulosa* and *Lyngbya majuscula* as well as all the specimens of molluscs were collected by SCUBA diving at a depth of 1-20 m in Moorea atoll, French Polynesia in Pacific Ocean. Specimens of the sea hare *Stylocheilus striatus* were found on the two cyanobacteria *L. majuscula* and *A. cf torulosa* while the sea hare *Stylocheilus longicauda* was found only on *A. cf torulosa*. The cephalaspidean *Bulla orientalis*, the nudibranch *Gymnodoris ceylonica* and *Thalamita coerulipes* were only found on *L. majuscula*. Organisms were either frozen in the field or were kept in different aquaria in order to increase their body mass: herbivorous animals with the cyanobacterium on which they have been collected and *G. ceylonica* in an aquarium containing *L. majuscula* and *S. striatus*. *S. striatus* reared naïve were collected in tanks at the CRIIBE research station that had entered the system at the settlement stage and fed upon control food lacking secondary metabolites. Ink mixtures of the different *S. striatus* were collected by gently squeezing the sea hares until they released ink. Eggs of *G. ceylonica*, *B. orientalis*, *S. striatus* and *S. longicauda* were collected in the different aquaria.

5.4.2. T-maze choice

Individual herbivores were starved 12 to 24 h before the experiment. Cyanobacteria were added to tanks (25x15x15cm) containing seawater 1 h prior to the experiment to condition the seawater. Three header tanks were prepared containing either 1) fresh oxygenated seawater, or fresh oxygenated seawater with a sample of either 2) *L. majuscula* or 3) *A. torulosa*. The flow rate of the T-maze chamber (Fig. 12) was set to 100 mL/min using flowmeters. At the start of each trial, an individual herbivore was acclimated to the choice chamber for 5 minutes. Each individual was placed at the base of the T-maze behind a grid preventing access to the choice chambers. The dimension of each chamber was 20 cm and the base dimension was 15 cm for all experiments with *Stylocheilus striatus*, while the dimensions were shorter for the experiments with *Bulla orientalis* at 6.5 and 5 cm respectively. At the start of the 10 minutes trial, the grid was removed and the presence of each individual herbivore in each chamber was recorded over time so that we were able to calculate the total time spent in each chamber. We also recorded whether the herbivore reached the end of the either T-junction. The herbivore was then removed and the T-maze cleaned to avoid mucus trail following, emptied and filled up again, but with the water sources switched between the T-junctions. This took approximately 2 minutes in total. The experiment was then repeated as before with both the 5 minutes acclimation period and the 10 minutes of choice. An experiment was considered as null when the mollusc stayed more than 5 minutes in the base. In each experiment, approximately 1 liter of seawater conditioned with cyanobacterium was filtered on SPE cartridges (Phenomenex Strata C18-SPE, 2 g, 12 mL, 55 µm, 70 Å) for further analyses in LC-MS and HPLC-ELSD. 15 individuals

were tested for each assay. Data were analysed using independent samples Mann Whitney tests.

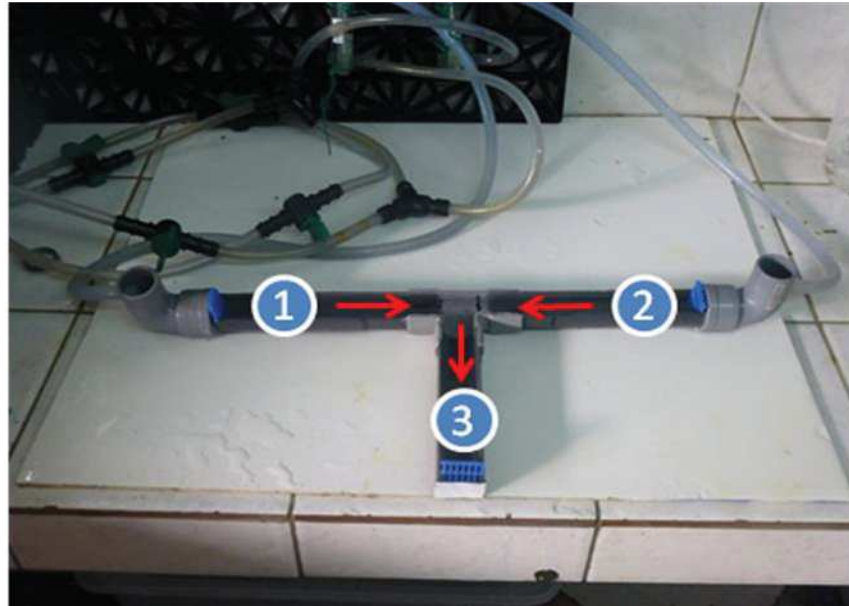


Figure 5. 13. Picture of the T-maze choice chamber. Flow direction is represented by red arrows. 1 and 2 are chambers and 3 is the base of the T-maze.

5.4.3. Colonization experiments

Colonization experiments were carried out following the method described by Rasher et al.⁴⁷. As a first step, cotton wool balls were soaked for 2 min in either 1 L of seawater previously conditioned with a cyanobacterium for 3 h (treated cotton wool balls) or with unconditioned seawater (control). All cotton balls were dried under reduced pressure after soaking. At the start of the colonization experiment, treated and control cotton wool balls were placed randomly in the opposite corners of a small aquarium (25x15x15cm). After 2 min, a *S. striatus* was positioned in the center of each aquarium. The first cotton ball reached by the mollusc within a 5 min period was scored. If *S. striatus* touched the water surface, it was placed back in the middle of the aquarium. A result was considered as null if the mollusc touched the water surface twice or did not colonize any cotton ball within the test period. Secondly, the experiments were performed using treated cotton wool balls coated with cyanobacterial extracts (either hydroalcoholic extract or organic extract or both hydroalcoholic and organic extracts) at natural volumetric concentrations (hydroalcoholic extract 10.9% for *A. cf torulosa* and 4.4% for *L. majuscula*, organic extract 0.2% and 0.7%). To achieve this, hydroalcoholic extracts were coated on cotton wool balls using seawater, while organic extracts were coated using dichloromethane and ethyl acetate (50:50) and solvents were then evaporated under reduced pressure. 20 individuals were tested for each assay. Results of these experiments were achieved by processing binary response data with Fisher's exact test.

5.4.4. Feeding assays

In order to determine the dietary preferences of the four herbivore populations, three artificial diets were created following the method described by Nagle et al.¹² 2.5 g of agar were boiled in 100 mL water. 6 g *Spirulina* lyophilized powder and 2 g *Ulva sp.* lyophilized powder were mixed together and added to the cooled agar solution. For the control diets, solvents were added to the algal mixture and then the solvents were evaporated. To create artificial diets containing secondary metabolites of the two cyanobacteria, crude extracts from each species of cyanobacteria were dissolved in a small volume of dichloromethane and ethyl acetate (50:50) and added to the *Spirulina* - *Ulva sp.* mixture. The solvents were then evaporated with a rotary evaporator. The proportion of crude extract for *A. torulosa* was 0.9% and 1.1% for *L. majuscula*, which corresponds to the concentrations found naturally in cyanobacteria. The three diet mixtures were poured into a mold (5.5 x 4 cm) that contained a plastic mosquito screen. After the agar had set, equal diet strips of 1 x 1.1 cm (7 x 8 squares = 56 squares in total) were cut and stored in the refrigerator.

Three trials were carried out: 1) Control versus *L. majuscula* crude extract, 2) Control versus *A. torulosa* crude extract and 3) *L. majuscula* versus *A. torulosa* crude extracts. Prior to each alimentary preference trial, the herbivores were starved for 24 h, then 1 individual *S. striatus* and 10 individuals *B. orientalis* were placed in a 6 L aquaria with two food strips equidistant from the herbivore. The experiment ran until >50% of one agar strip had been consumed (i.e. >28 squares), whereupon the number of squares consumed was measured, the time noted, individual length measured and the herbivores returned alive to the lagoon. Individual herbivores were only used in one preference trial, therefore a total of 31 Ss-Lm, 30 Ss-At, 33 Ss-naive and 150 Bo-Lm were used. The number of squares eaten by each herbivore was analyzed using a paired *t*-test.

5.4.5. Preparation of cyanobacterial extracts

5.4.5.1. Feeding assays

Lyophilized biomass of *A. cf torulosa* (159 g) and *L. majuscula* (155 g) were extracted at room temperature 3 times with 600 mL of a mixture of CH₃OH-CH₂Cl₂ (50:50) and sonicated during 10 minutes to yield crude extracts (respectively 7.91 g and 2.98 g) after evaporation.

5.4.5.2. Colonization experiments

Biphasic extractions were carried out on lyophilized biomass of *A. cf torulosa* (134 g) and *L. majuscula* (150 g). To achieve this, the biological material was first extracted at room temperature using 500 mL of a mixture of H₂O-CH₃OH (80:20) and sonicated during 10 min. Then, 900 mL of a CH₃OH-CH₂Cl₂ (55:45) mixture was added followed by ultrasound over 10 min. After filtration, the hydroalcoholic and organic phases were separated, the solvents were then removed by evaporation under reduced pressure and led to the hydroalcoholic

extracts (*A. cf torulosa* 14.22 g and *L. majuscula* 6.62 g) and organic extracts (0.32 g and 1.10 g).

5.4.6. Sea hare dissection

The location of cyanobacterial secondary metabolites in *S. striatus* was investigated. To achieve this, four sea hares were starved for 24 h and then euthanized in iced salt water. Organisms were dissected into head and foot, mantle, hepatopancreas (digestive gland), intestine, buccal bulb, female gland and gizzard. However, more investigation of intestine, buccal bulb, female gland and gizzard are needed to confirm their identification.

5.4.7. Organisms extraction for chromatographic analyses

All the specimens, mollusc eggs and *S. striatus* body parts were lyophilized, extracted at room temperature 3 times with a mixture of CH₃OH-CH₂Cl₂ (1:1) and sonicated during 10 minutes. The solvents were removed under reduced pressure and led to crude extracts. The crude extracts were then desalted on SPE cartridges (Phenomenex Strata C18-SPE, 200 mg, 3 mL, 55 µm, 70 Å) with 3 mL H₂O to remove salts, followed by an elution with 3 mL of CH₃OH-CH₂Cl₂ (50:50) to recover the desalted extracts. Ink mixtures were desalted using the same process. SPE cartridges used in the T-maze experiments were washed with 10 mL H₂O then eluted with 10 mL of CH₃OH-CH₂Cl₂ (50:50). All solutions were filtered on PTFE filters (0.22 µm) before injection. SPE cartridges used in T-maze experiments were eluted with 10 mL H₂O and 10mL of MeOH-CH₂Cl₂ (50:50). The extracts were prepared in MeOH at concentrations of 1 mg/ml for HPLC-ELSD analysis and 0.1 mg/mL for LC-MS analysis.

5.4.8. LC-MS and HPLC-ELSD analysis

LC-MS analyses were carried out using a Thermo Fisher Scientific LC-MS device, Accela HPLC coupled to a LCQ Fleet equipped with an electrospray ionization source and a 3D ion-trap analyzer. HPLC-ELSD analyses were performed with a Waters Alliance HPLC system (W 2695) coupled to an ELS detector (W 2424). The analysis was performed on a reversed-phase column (Thermo Hypersil Gold C-18, 150 x 2.1 mm, 3 µm) employing a gradient of 10% CH₃CN 0.1% formic acid to 100% CH₃CN 0.1% formic acid over 40 min followed by 25 min at 100% CH₃CN 0.1% formic acid with a flow rate of 0.3 mL/min.

5.4.9. Determination of the bioaccumulation factor in *S. striatus* organs

Concentrations of each compound in sea hare organs and in the cyanobacterium were calculated with reporting peak area (obtained by HPLC-ELSD) of each compound to dry mass of tissue (mg) using extraction yield. Relative concentrations (bioaccumulation factor) were calculated with dividing the concentration in sea hare organs by the concentration in cyanobacterium.

5.4.10. Extraction and purification of *S. striatus* compounds

4.24 g of dried *S. striatus* were extracted with a mixture of CH₃OH-CH₂Cl₂ (1:1) and sonicated during 10 minutes to yield a crude extract (1.03 g) after evaporation under reduced pressure. Then the crude extract was subjected to flash RP18 silica gel column eluted with H₂O (A), H₂O-CH₃CN (20:80) (B), CH₃OH-CH₂Cl₂ (80:20) (C) to afford 3 fractions (A, B, and C). Fraction B was fractionated, in turn, with flash chromatography and RP18 silica gel column with a gradient of H₂O-CH₃CN to give 7 fractions. Fractions 2 and 4 were subjected to HPLC purification (Phenomenex Gemini C6-phenyl, 110Å, 250 x 10 mm, 5 µm) using different isocratic conditions of water and acetonitrile acidified with 0.1% formic acid. Fraction 2 was eluted with 32% CH₃CN at a flow rate of 4 mL/min and gave laxaphycin 1211 (4.5 mg, rt = 21.5 min) and laxaphycin 1195 (6.5 mg, rt = 27.5 min), while fraction 4 gave laxaphycin 1228 (5 mg, rt = 19 min) and laxaphycin 1212 (2.5 mg, rt = 22.2 min) with 28% CH₃CN at a flow rate of 4 mL/min.

5.4.11. NMR spectroscopy

1D-NMR and 2D-NMR experiments were acquired on a Jeol ECZ 500 spectrometer, all compounds solubilized in DMSO-*d*₆ (500 µL) at 303 K. All chemical shifts were calibrated on the residual solvent peak [DMSO-*d*₆, 2.50 ppm (¹H) and 39.5 ppm (¹³C)]. The chemical shifts, reported in delta (δ) units, and in parts per million (ppm) are referenced relatively to TMS.

5.5. Conclusion

In conclusion, we have identified a relatively simple ecosystem consisting of two cyanobacteria, three herbivorous molluscs, a carnivorous nudibranch and a carnivorous crab. Our observations revealed that the sea hare *S. striatus* is a generalist herbivore though it was considered as a specialist consumer of *L. majuscula* in previous studies. T-maze and colonization experiments have shown that *S. striatus* and *B. orientalis* used cyanobacterial chemical cues to locate their prey. Chemically induced behavior such as host tracking, foraging or feeding choice usually suggest for a specialist diet. However, we suggest that the two herbivores show generalist diet behaviors due to the results from the feeding choice experiments in which the herbivores chose a different diet from which they were normally consume.

Chemical investigations revealed the sequestration of *L. majuscula* secondary metabolites by *S. striatus* and *B. orientalis* as well as sequestration of laxaphycin A from *A. cf torulosa* by *S. striatus*. Interestingly, we have shown that laxaphycins B and B3 are biotransformed into laxaphycins B1212, B1228, B1195 and B1211, four new laxaphycin-B type lipopeptides. The sequestration of *L. majuscula* compounds mainly occurred in the hepatopancreas of *S. striatus*. It is not clear why sea hares sequestered and biotransformed cyanobacterial secondary metabolites though it is likely a tolerance mechanism to enjoy chemically defended shelter from predation. The location of sequestered and biotransformed compounds in *S. striatus* fed upon *A. cf torulosa* need to be investigated. The biotransformed laxaphycins could be the results of detoxification or may be means of intraspecific communication, even if this hypothesis is less likely. Further experiments are needed to identify the compounds responsible for chemically cueing the herbivores in foraging. Moreover, an exciting challenge is the identification of putative chemical signals attracting the nudibranch *G. ceylonica* towards *L. majuscula*.

Associated content

-Supporting Information

Supplementary data (^1H NMR, ^{13}C , TOCSY, HSQC, HMBC, and ROESY spectra of laxaphycins B1195, B1211, B1212 and B1228) associated with this chapter are available at the end of this thesis (S.5. 1-S5. 20).

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Chapter 6. General conclusion

Marine chemical ecology is a relatively young discipline resulting from the collaboration between ecologists and chemists. Chemists were originally searching for new bioactive natural compounds in the marine realm, but with only 8 compounds approved by FDA or EMA among the 28 000 isolated in approximately 50 years, the discovery of marine drugs could be compared to looking for a needle in a haystack. Thus, combining the discovery of marine compounds for drug application, with the investigation into ecological function, is a smart research strategy. However, as with all new disciplines, marine chemical ecology faces challenges in designing ecological experiments to understand the role of chemical compounds in complex ecological mechanisms, in adapting experimental designs to different species or in finding enough biological material for chemical extraction and ecological assays.

The complexity of marine ecological interactions arises from the broad range of active secondary metabolites whose information varies depending on the receiver. The same compounds can act as chemical deterrents toward generalist consumers, but as chemical cues to specialist species that can tolerate them. Marine gastropods are a group of both specialist and generalist species that have evolved by interacting with chemically defended prey. They have developed strategies to feed upon toxic prey, enjoy the shelter provided by them and steal their chemical defenses. Mechanisms of chemical tolerance and sequestration are carried out by a well organized enzymatic machinery, although only a few studies have highlighted the presence of such a process. Moreover, the remote detection of chemical cues from prey induces dispersing molluscan larvae to settle, enabling their colonization of distant areas, and enables juvenile or adult molluscs to locate their preferred food.

Cyanobacteria are known for forming extensive blooms that can disrupt coral biodiversity and are involved in phase shifts from coral to macroalgal dominance. They constitute a prolific source of secondary metabolites that can be toxic, deterrent, palatable or attractant toward different species. We have identified an ecosystem, our model studies, based on two benthic filamentous cyanobacteria *Lyngbya majuscula* and *Anabaena cf torulosa*, three herbivores, the anaspidean molluscs *Stylocheilus striatus* and *Stylocheilus longicauda*, and the cephalaspidea *Bulla orientalis*, and two carnivores, the nudibranch *Gymnodoris ceylonica* and the crab *Thalamita coerulipes*. The herbivores *S. striatus* and *B. orientalis* are found feeding upon *L. majuscula*, *G. ceylonica* is also present on the cyanobacteria and feed upon *S. striatus* while *T. coerulipes* is an opportunist predator feeding upon the three molluscs. The overall objective of this thesis was to see if the structuration of this model ecosystem was chemically mediated.

In a first step we characterized the secondary metabolites of the primary producers. We isolated and elucidated the structure of 5 new laxaphycins A and six laxaphycins B, some of them being acyclic. Acyclic laxaphycins were never described before this work. Surprisingly, and in contrast to acyclic laxaphycins A, in acyclic laxaphycins B the β -amino fatty acid residue was not on the N-terminal position. This could highlight different uses of the fatty acyl-AMP ligases/polyketide synthases/non-ribosomal peptide synthases (FAAL/PKS/NRPS) machinery and reveal two putative biosynthetic pathways leading to laxaphycin A or B-type peptides.

Laxaphycin A in *Anabaena cf torulosa* is described to be deterrent toward many species, but in our model did not prevent predation by the sea hares *Stylocheilus striatus* and *S. longicauda*. The isolation and characterization of such compounds enables their fate to be investigated along the trophic chain, whether they are transmitted unaltered or transformed, and also enables their biological activity for ecological and/or pharmacological purposes to be directly tested through various experiments.

Although *S. striatus* was considered as a specialist herbivore, we observed and demonstrated that it consumes different cyanobacteria and behaves in a similar manner as generalist sea hares in the *Aplysia* genus. Similarly, *Bulla orientalis* found within *Lyngbya majuscula* did not show specialist behaviors either, as it consumed artificial food with *Anabaena cf torulosa*'s secondary metabolites in feeding choice experiments. As *S. striatus* is able to sequester and biotransform a diversity of secondary metabolite families from various food sources, it demonstrates that the mollusc is adapted to consume a variety of different foods. Furthermore, *S. striatus* and *S. longicauda* show different strategies depending on the metabolite source and strength of toxicity: they do not biotransform laxaphycin A as it is not deterrent to the sea hare, but they do biotransform laxaphycin B and B3, which are cytotoxic on human cell lines, putatively neutralizing these chemical weapons. The purpose of the sequestration of diet-derived compounds by sea hares in an inner organ is still unknown, though it does not seem to be a defensive mechanism but rather a tolerance mechanism.

Except *S. striatus*, no specialist consumer appears, if we consider published data, to feed upon cyanobacteria and it may be because blooms are frequently assemblages of several species and remain ephemeral. However, generalist species, such as *S. striatus*, may become adapted to a specific cyanobacterial food, educate their senses of perception to this species and become able to remotely detect the primary producer's chemical cues. However, despite consumers having a preferred species to feed upon, they may also have developed alternative food sources in times of scarcity of their preferred food. If we consider *Anabaena cf torulosa* as a substitute diet for *L. majuscula*, it would actually be an adaptive advantage for *S. striatus* since its predators, *G. ceylonica* and *Thalamita coerulipes*, are absent from this primary producer.

We further demonstrated that foraging is chemically mediated since the sea hare *S. striatus* and the cephalaspidean *B. orientalis* are able to track the chemical cues released by the cyanobacteria on which they fed. In addition, foraging is specifically stimulated by the lipopeptide rich organic portion of the cyanobacterial metabolome. Feeding choices highlight the generalist behavior of these two herbivores.

The fact that carnivorous predators form multitrophic interactions by preying on herbivores in close contact with certain primary producers, but avoid the same herbivores on other primary producers, suggests that chemicals govern more complex interactions. Indeed, the chemical ritualization of a chemical cue evolving into a chemical signal might explain tri-trophic interactions. The nudibranch *G. ceylonica* may become accustomed to the odour of *L. majuscula* by preying on *S. striatus* which is abundant within the cyanobacterium. Thus, the chemicals cueing the sea hare to locate its prey could be the same chemicals that evolved into chemical signals for the nudibranch. It would be an exciting discovery if the primary producers, when they are being consumed by sea hares, actively signal nudibranchs to prey on their predators.

Our results in this work bring new questions:

- Are the biotransformed laxaphycins less or more toxic than the original ones? Are they used as chemical signals in intraspecific communication?

- What about the role of lipopeptides from *Lyngbya majuscula* and *Anabaena cf. torulosa* in the competition between both cyanobacteria during blooms? Are they allelopathic compounds?

- The question remains if the lipopeptides are the chemical cues attracting the primary predators of the cyanobacteria (*Stylocheilus striatus*, *S. longicauda*, *Bulla orientalis*) and if the same putative chemical cues attract or repel the secondary predator *G. ceylonica* feeding on *S. striatus*.

We will try to answer these questions in the coming months.

Marine chemical ecology is still in its infancy and chemical mediated interactions are sometimes underestimated. Molecules of keystone significance could govern ecological communities over large spatial scales and should therefore be taken into account in ecosystem modelling and could be of economical interest in fisheries management. Moreover, global warming and ocean acidification could impact these interactions, induce behavioral shifts and thus it is essential to understand such interactions and follow their evolution.

From a personal point of view, this PhD thesis enabled me to conduct multidisciplinary research on the borders between chemistry, ecology and microbiology and showed me that these frontiers are terms that do not exist in nature.

Supporting Information

Supporting information - Chapter 3

Figure S3. 1. ^1H and ^{13}C NMR spectra of acyclolaxaphycin B (**3**) in $\text{DMSO-}d_6$ (500 MHz, 303 K).

Figure S3. 2. TOCSY spectrum of acyclolaxaphycin B (**3**) in $\text{DMSO-}d_6$ (500 MHz, 303 K).

Figure S3. 3. ROESY spectrum of acyclolaxaphycin B (**3**) in $\text{DMSO-}d_6$ (500 MHz, 303 K).

Figure S3. 4. HSQC spectrum of acyclolaxaphycin B (**3**) in $\text{DMSO-}d_6$ (500 MHz, 303 K).

Figure S3. 5. HSQC-TOCSY spectrum of acyclolaxaphycin B (**3**) in $\text{DMSO-}d_6$ (500 MHz, 303 K).

Figure S3. 6. HMBC spectrum of acyclolaxaphycin B (**3**) in $\text{DMSO-}d_6$ (500 MHz, 303 K).

Figure S3. 7. ^1H and ^{13}C NMR spectra of acyclolaxaphycin B3 (**4**) in $\text{DMSO-}d_6$ (500 MHz, 303 K).

Figure S3. 8. TOCSY spectrum of acyclolaxaphycin B3 (**4**) in $\text{DMSO-}d_6$ (500 MHz, 303 K).

Figure S3. 9. ROESY spectrum of acyclolaxaphycin B3 (**4**) in $\text{DMSO-}d_6$ (500 MHz, 303 K).

Figure S3. 10. HSQC spectrum of acyclolaxaphycin B3 (**4**) in $\text{DMSO-}d_6$ (500 MHz, 303 K).

Figure S3. 11. HSQC-TOCSY spectrum of acyclolaxaphycin B3 (**4**) in $\text{DMSO-}d_6$ (500 MHz, 303 K).

Figure S3. 12. HMBC spectrum of acyclolaxaphycin B3 (**4**) in $\text{DMSO-}d_6$ (500 MHz, 303 K).

Figure S3. 1. ^1H and ^{13}C NMR spectra of acyclolaxaphycin B (**3**) in $\text{DMSO-}d_6$ (500 MHz, 303 K).

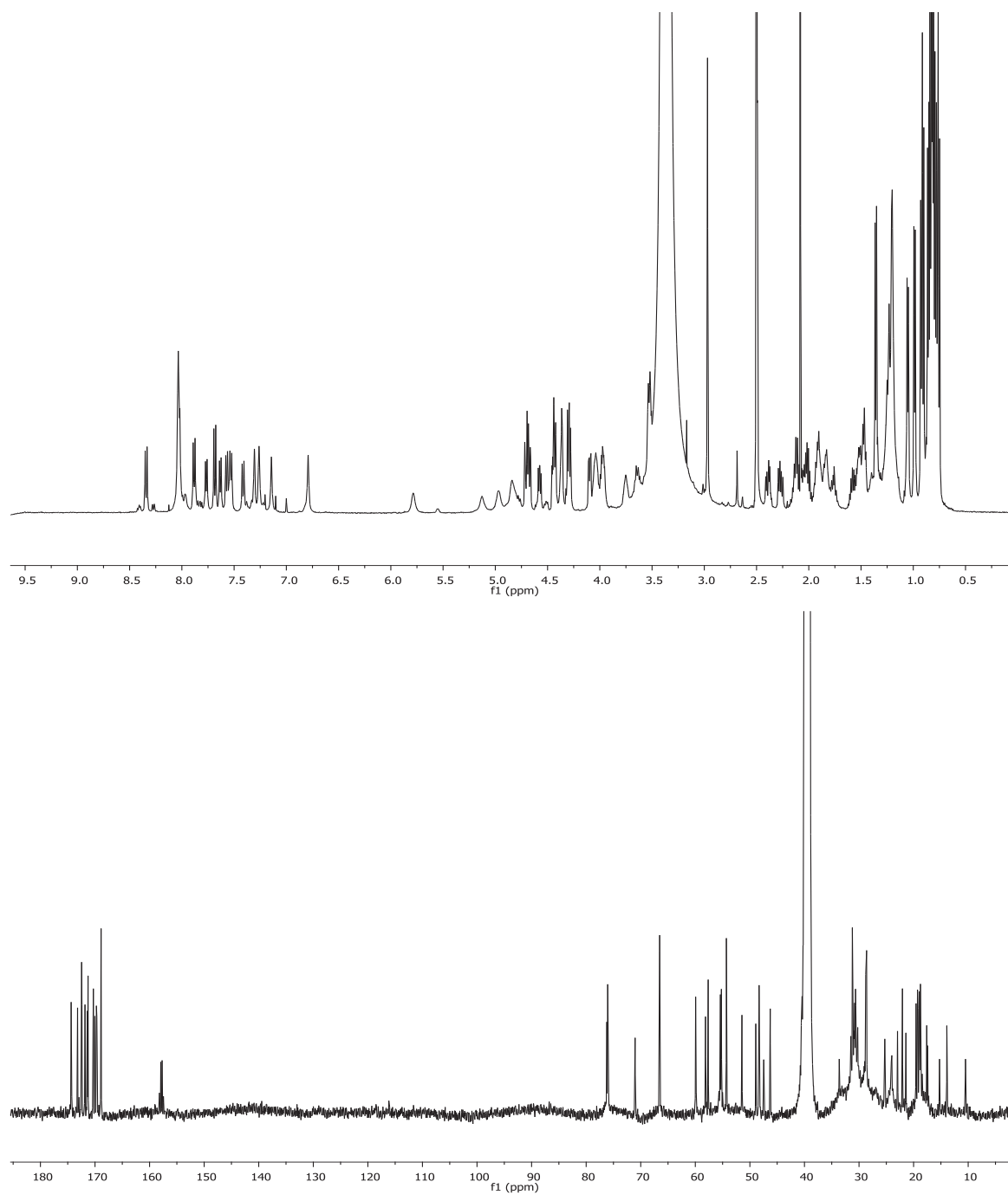


Figure S3. 2. TOCSY spectrum of acyclolaxaphycin B (**3**) in DMSO- d_6 (500 MHz, 303 K).

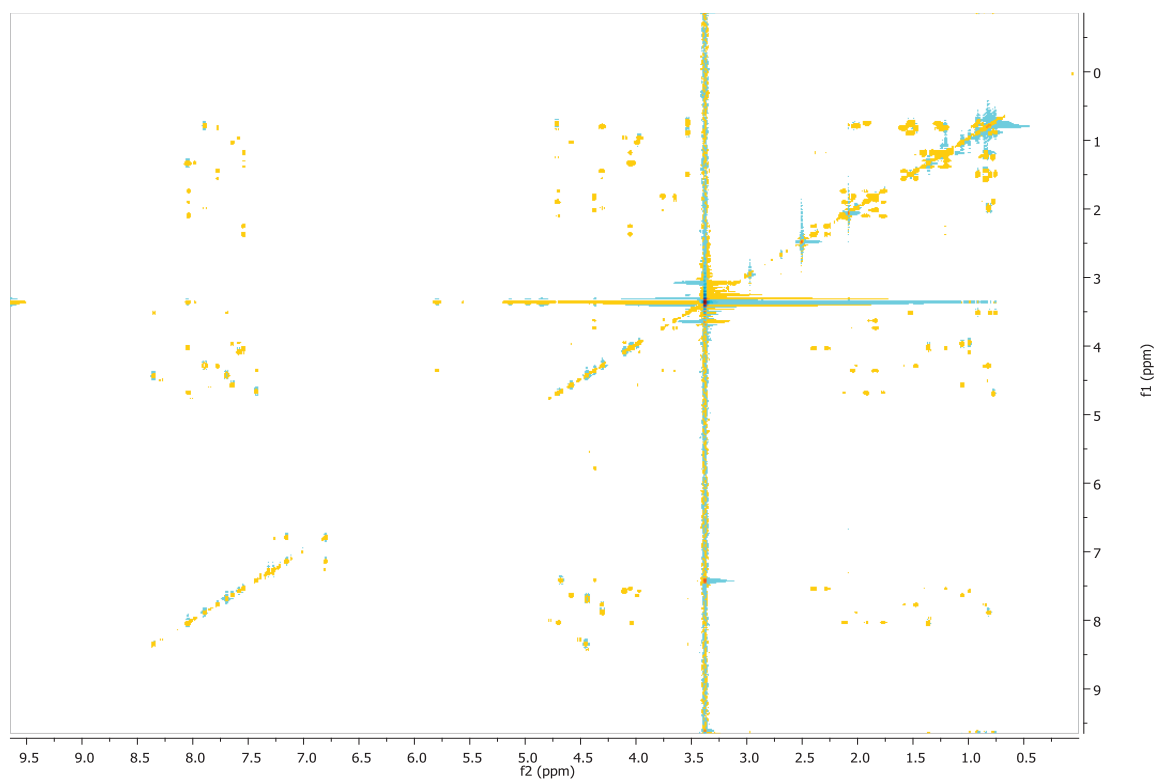


Figure S3. 3. ROESY spectrum of acyclolaxaphycin B (**3**) in DMSO- d_6 (500 MHz, 303 K).

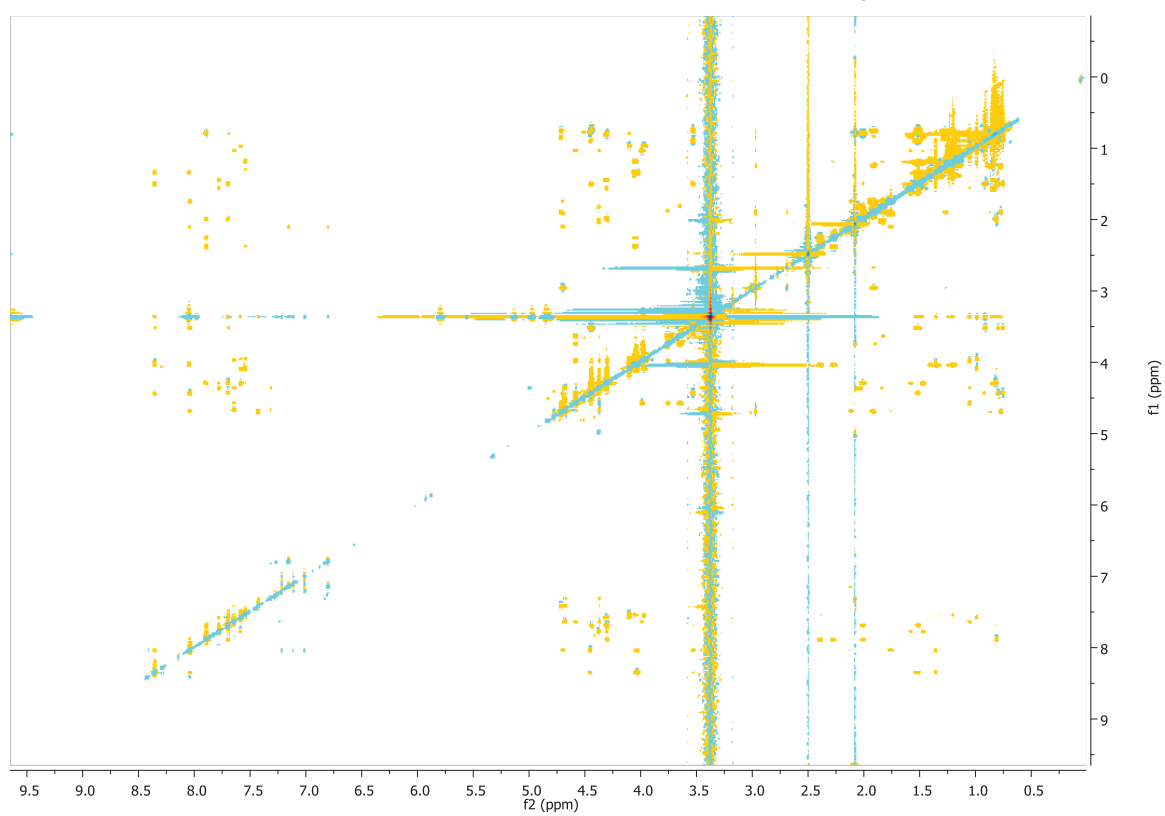


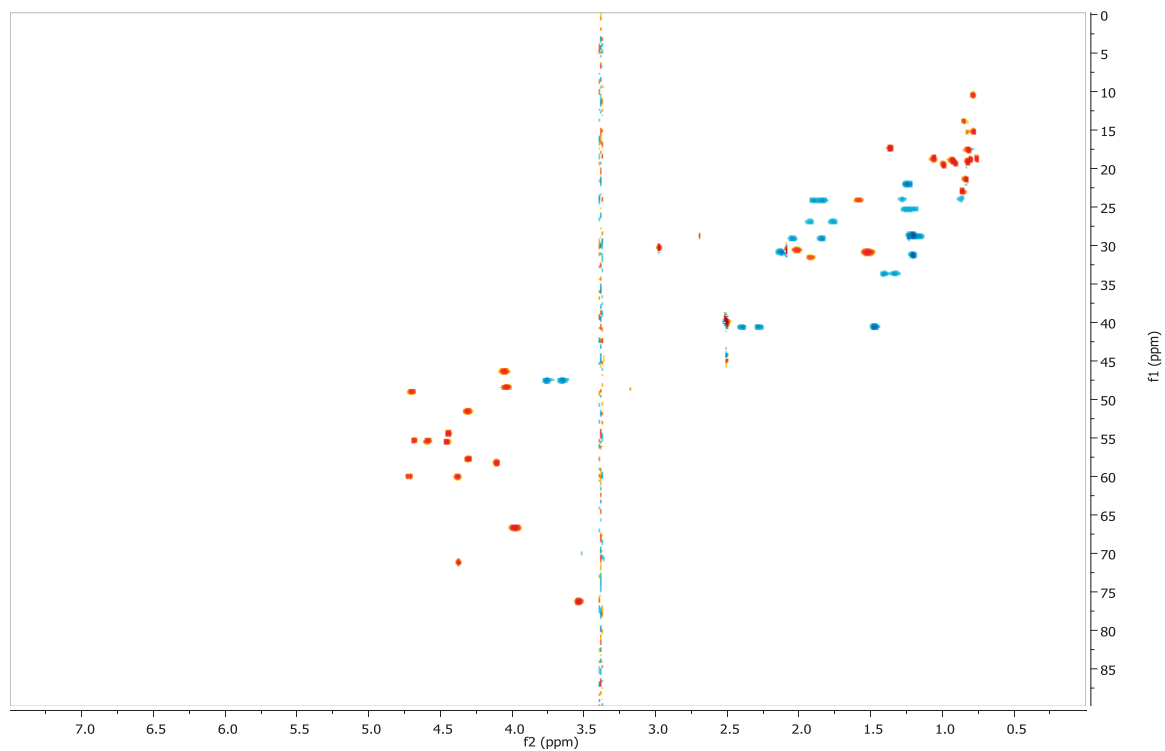
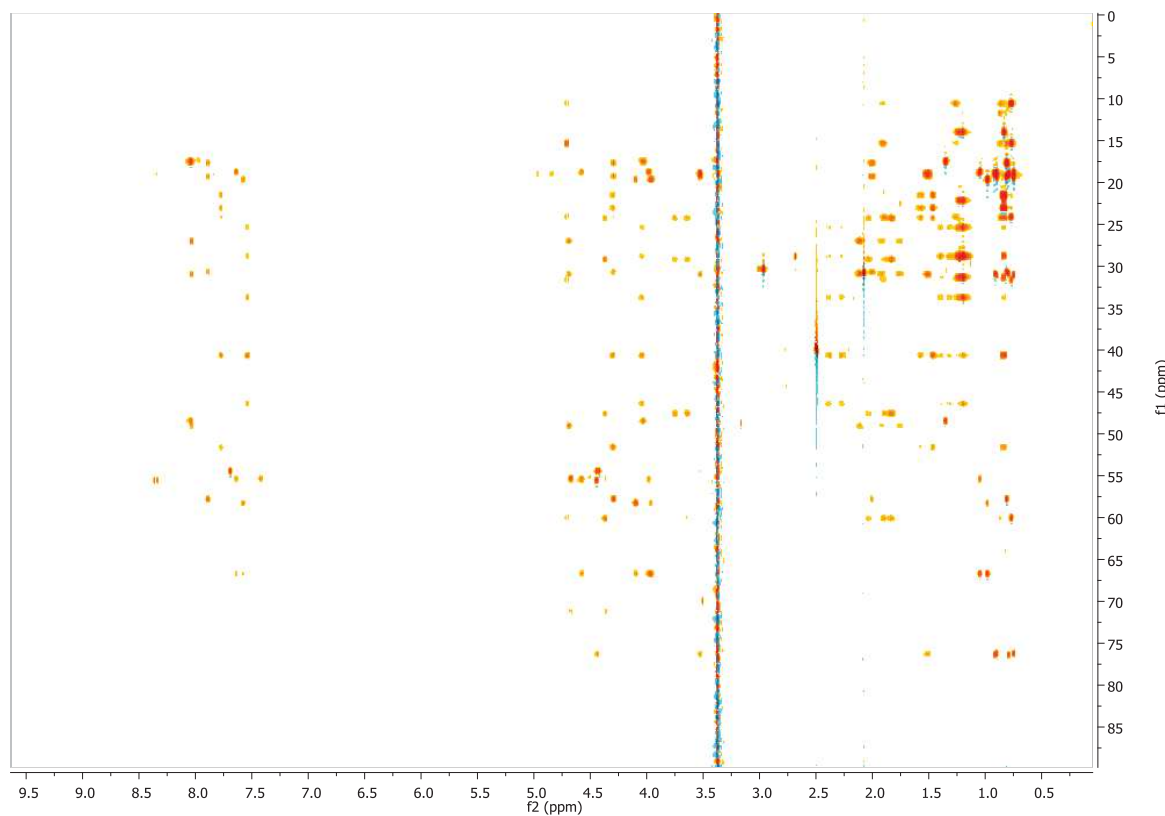
Figure S3. 4. HSQC spectrum of acyclolaxaphycin B (**3**) in DMSO- d_6 (500 MHz, 303 K).**Figure S3. 5.** HSQC-TOCSY spectrum of acyclolaxaphycin B (**3**) in DMSO- d_6 (500 MHz, 303 K).

Figure S3. 6. HMBC spectrum of acyclolaxaphycin B (**3**) in DMSO- d_6 (500 MHz, 303 K).

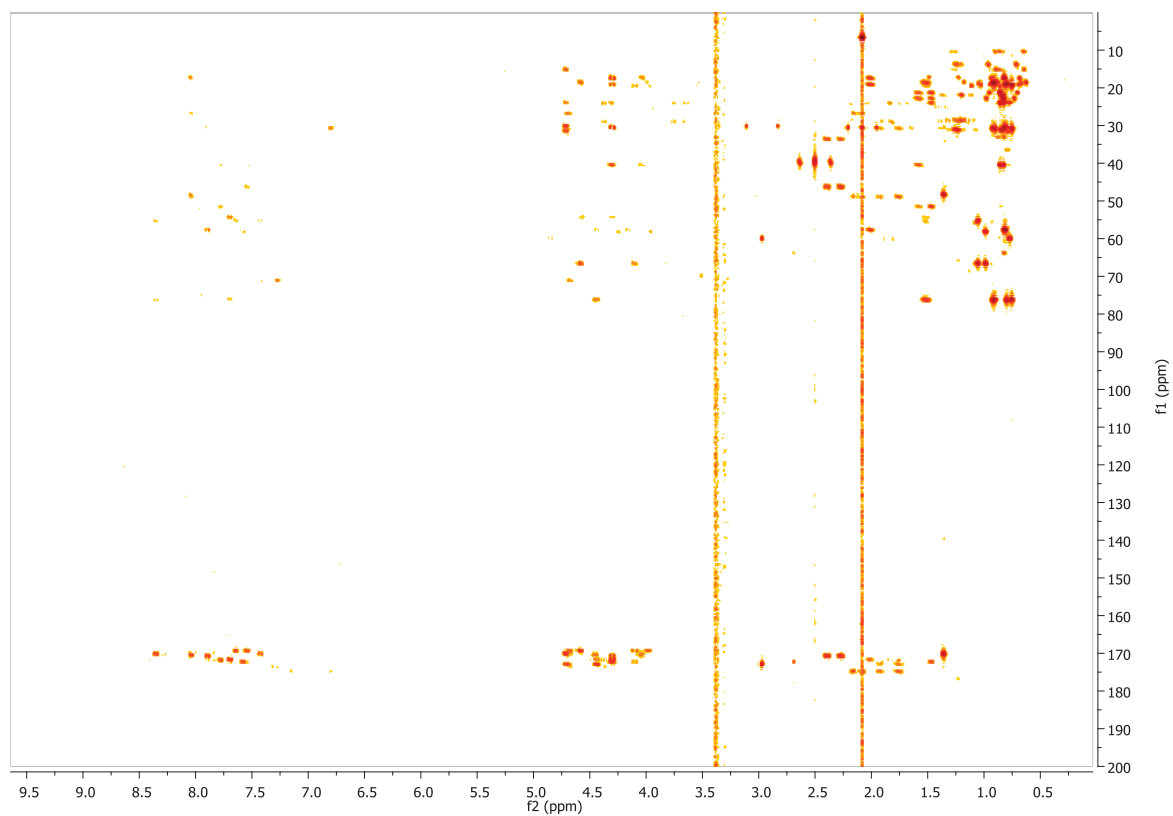


Figure S3. 7. ^1H and ^{13}C NMR spectra of acyclolaxaphycin B3 (**4**) in DMSO- d_6 (500 MHz, 303 K).

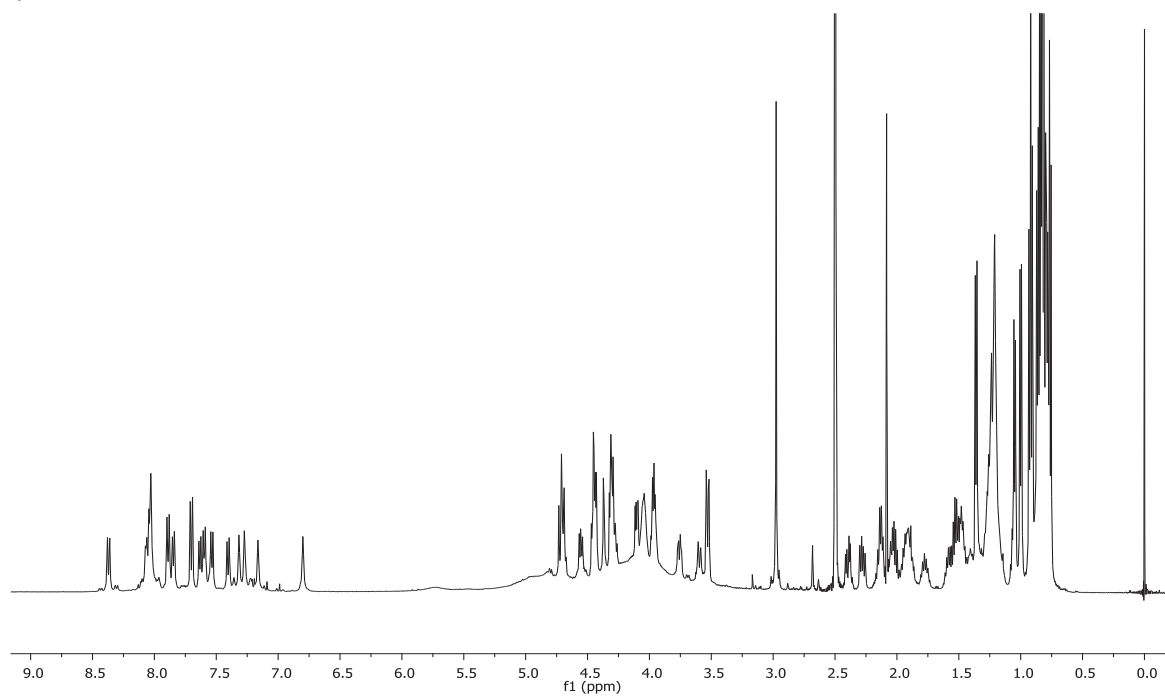


Figure S3. 7. Cont.

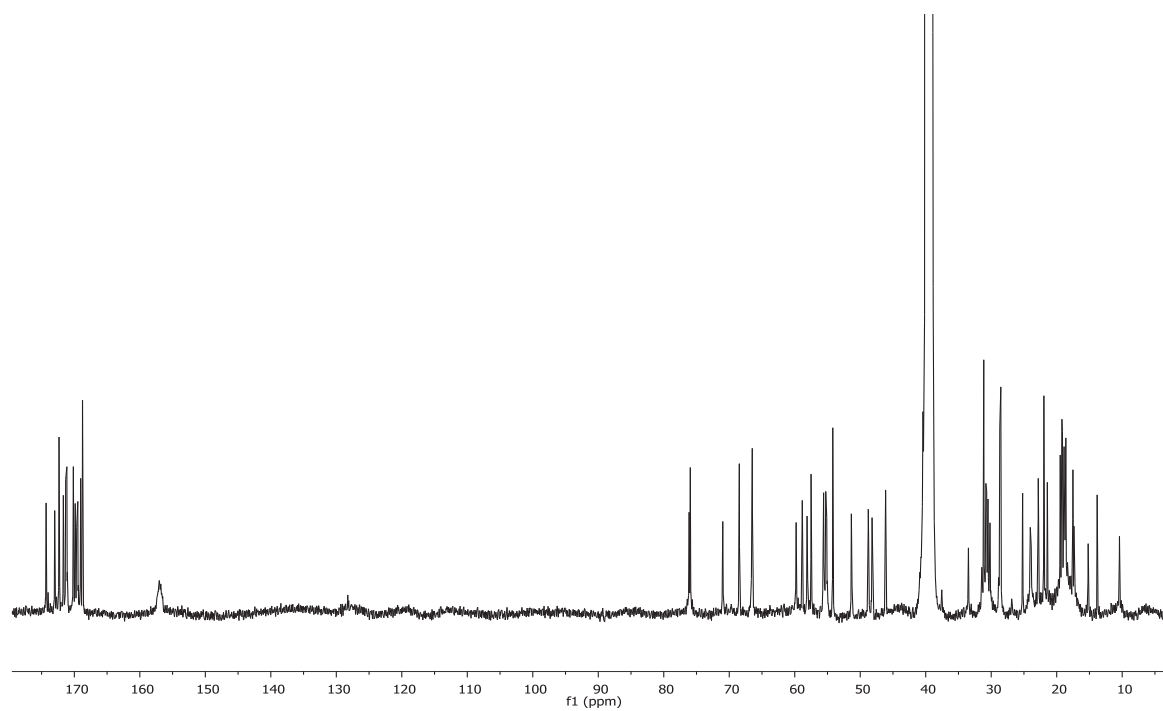
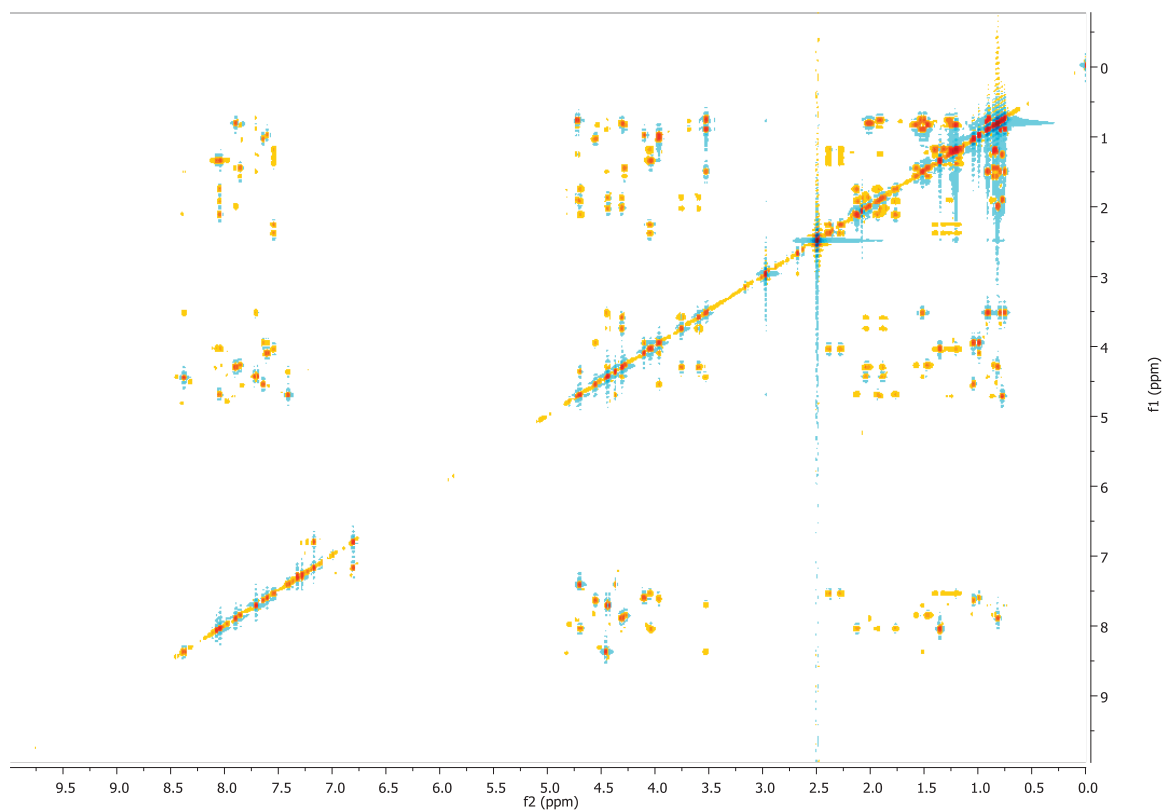
Figure S3. 8. TOCSY spectrum of acyclolaxaphycin B3 (4) in DMSO-*d*₆ (500 MHz, 303 K).

Figure S3. 9. ROESY spectrum of acyclolaxaphycin B3 (**4**) in DMSO- d_6 (500 MHz, 303 K).

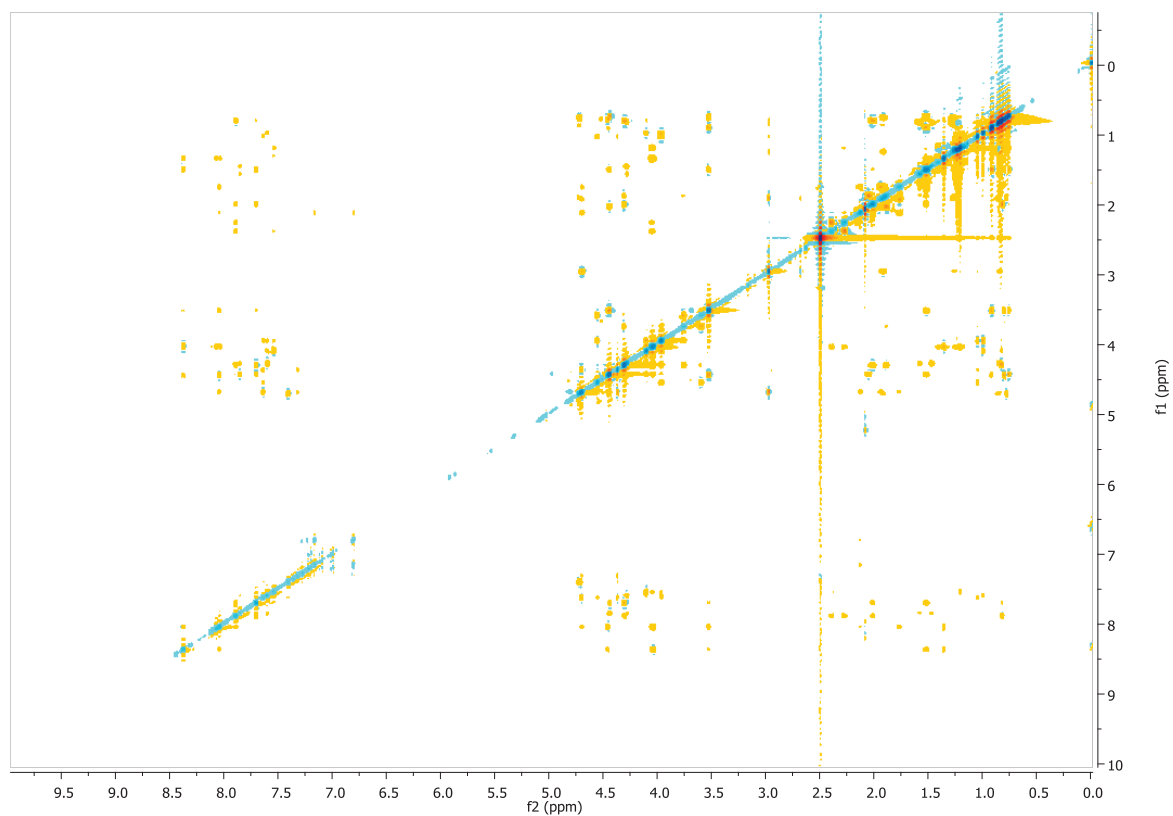


Figure S3. 10. HSQC spectrum of acyclolaxaphycin B3 (**4**) in DMSO- d_6 (500 MHz, 303 K).

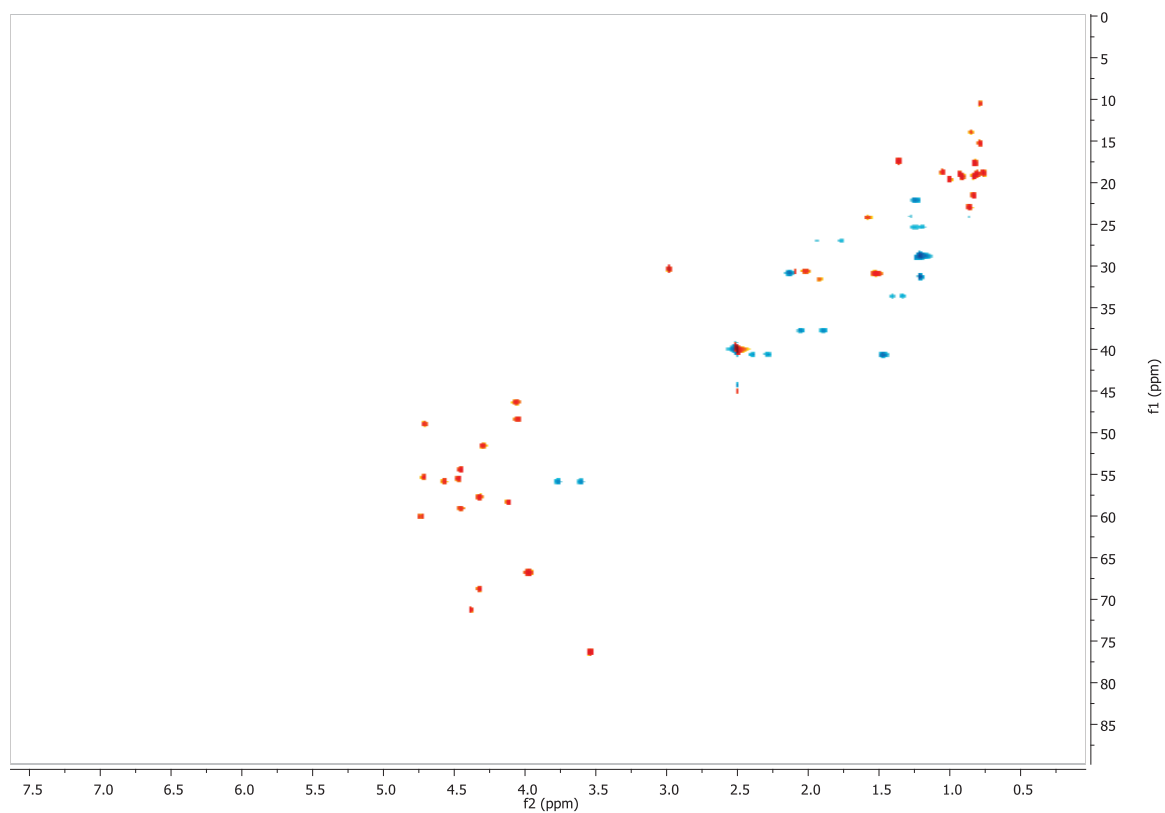


Figure S3. 11. HSQC-TOCSY spectrum of acyclolaxaphycin B3 (**4**) in DMSO- d_6 (500 MHz, 303 K).

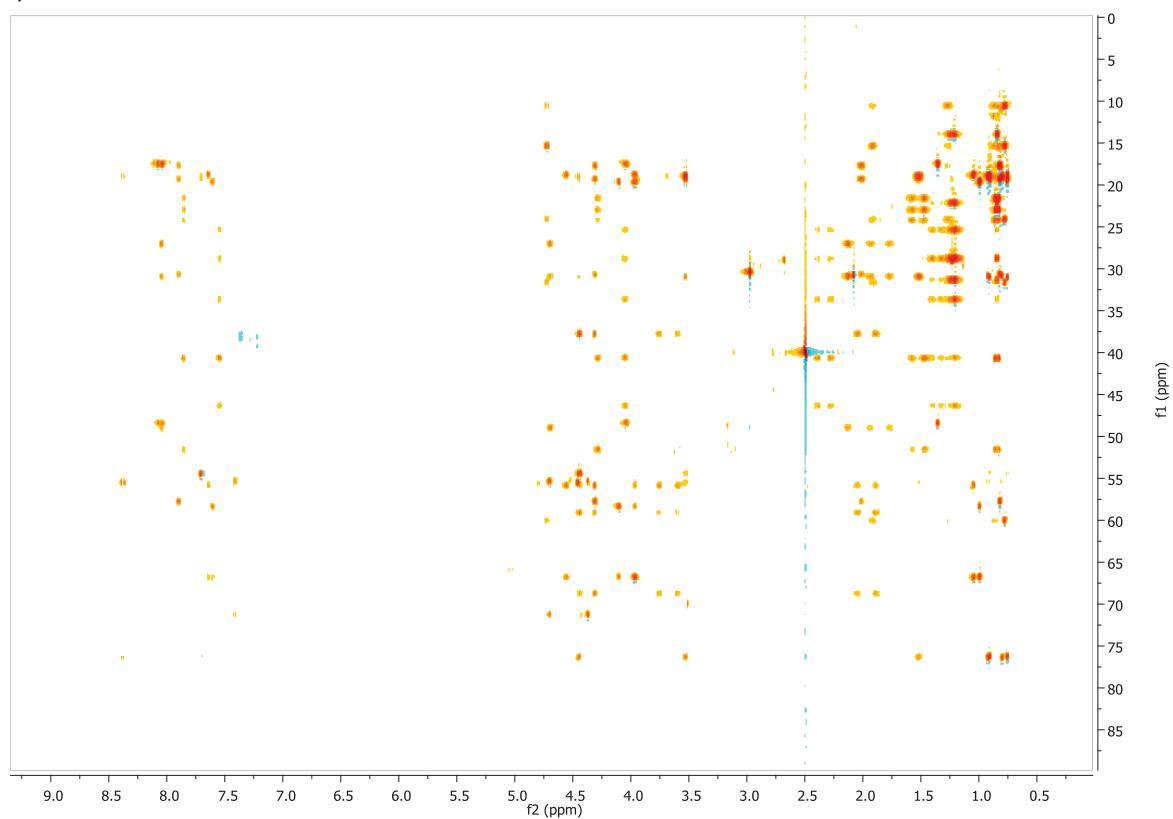
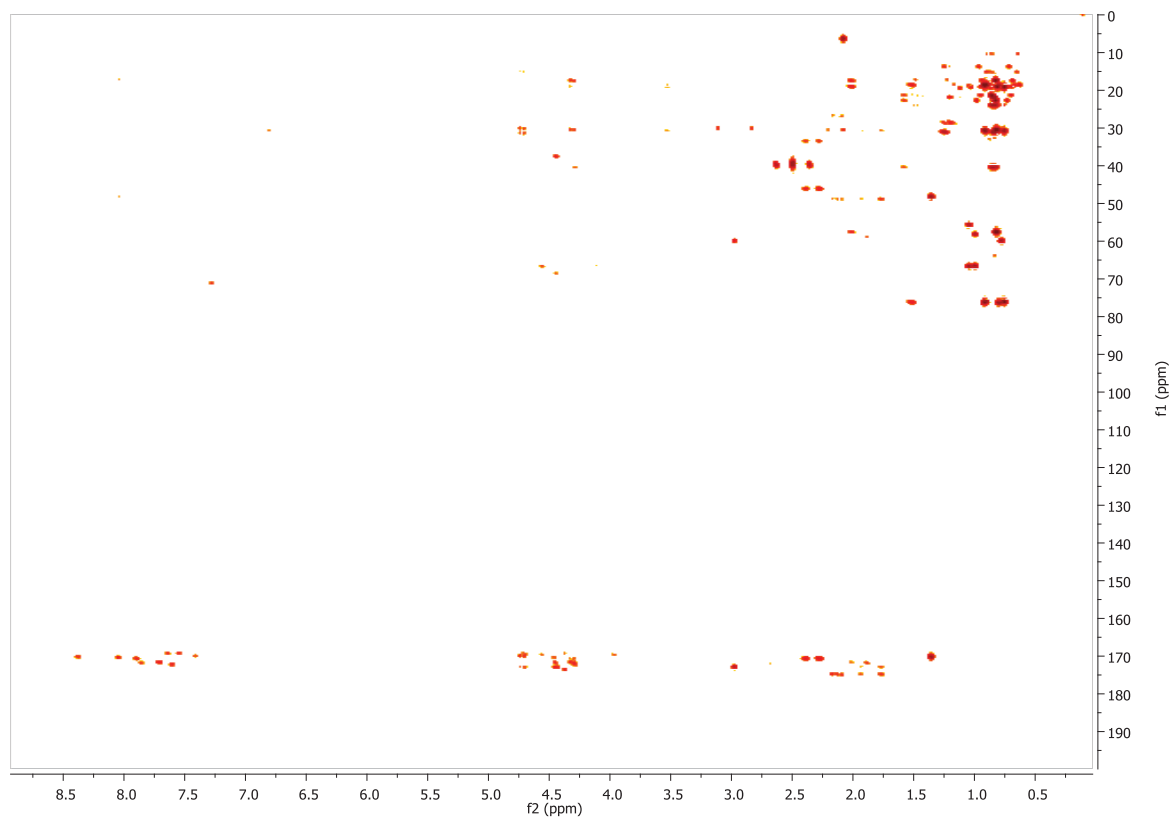


Figure S3. 12. HMBC spectrum of acyclolaxaphycin B3 (**4**) in DMSO- d_6 (500 MHz, 303 K).



Supporting information - Chapter 4

Figure S4. 1. ^1H -NMR spectrum of Acyclolaxaphycin A (**1**) in DMSO (303K)

Figure S4. 2. ^{13}C -NMR spectrum of Acyclolaxaphycin A (**1**) in DMSO (303K)

Figure S4. 3. HSQC spectrum of Acyclolaxaphycin A (**1**) in DMSO (303K)

Figure S4. 4. TOCSY spectrum of Acyclolaxaphycin A (**1**) in DMSO (303K)

Figure S4. 5. HSQC-TOCSY spectrum of Acyclolaxaphycin A (**1**) in DMSO (303K)

Figure S4. 6. HMBC spectrum of Acyclolaxaphycin A (**1**) in DMSO (303K)

Figure S4. 7. ROESY spectrum of Acyclolaxaphycin A(**1**) in DMSO (303K)

Figure S4. 8. Orbitrap MS/MS spectrum of Acyclolaxaphycin A (**1**)

Figure S4. 9. ^1H -NMR spectrum [des-(Gly 11)]acyclolaxaphycin A (**2**) in DMSO (303K)

Figure S4. 10. ^{13}C -NMR spectrum of [des-(Gly 11)]acyclolaxaphycin A (**2**) in DMSO (303K)

Figure S4. 11. HSQC spectrum of [des-(Gly 11)]acyclolaxaphycin A (**2**) in DMSO (303K)

Figure S4. 12. TOCSY spectrum of [des-(Gly 11)]acyclolaxaphycin A (**2**) in DMSO (303K)

Figure S4. 13. HSQC-TOCSY spectrum of [des-(Gly 11)]acyclolaxaphycin A (**2**) in DMSO (303K)

Figure S4. 14. HMBC spectrum of [des-(Gly 11)]acyclolaxaphycin A (**2**) in DMSO (303K)

Figure S4. 15. ROESY spectrum of [des-(Gly 11)]acyclolaxaphycin A (**2**) in DMSO (303K)

Figure S4. 16. Orbitrap MS/MS spectrum of [des-(Gly 11)]acyclolaxaphycin A (**2**)

Figure S4. 17. ^1H -NMR spectrum of [des-(Leu 10 -Gly 11)]acyclolaxaphycin A (**3**) in DMSO (303K)

Figure S4. 18. ^{13}C -NMR spectrum of [des-(Leu 10 -Gly 11)]acyclolaxaphycin A (**3**) in DMSO (303K)

Figure S4. 19. HSQC spectrum of [des-(Leu 10 -Gly 11)]acyclolaxaphycin A (**3**) in DMSO (303K)

Figure S4. 20. TOCSY spectrum of [des-(Leu 10 -Gly 11)]acyclolaxaphycin A (**3**) in DMSO (303K)

Figure S4. 21. HSQC-TOCSY spectrum of [des-(Leu 10 -Gly 11)]acyclolaxaphycin A (**3**) in DMSO (303K)

Figure S4. 22. HMBC spectrum of [des-(Leu 10 -Gly 11)]acyclolaxaphycin A (**3**) in DMSO (303K)

Figure S4. 23. ROESY spectrum of [des-(Leu 10 -Gly 11)]acyclolaxaphycin A (**3**) in DMSO (303K)

Figure S4. 24. Orbitrap MS/MS spectrum of [des-(Leu 10 -Gly 11)]acyclolaxaphycin A (**3**)

Figure S4. 25. Advanced Marfey's analysis of [des-(Gly¹¹)]acyclolaxaphycin A (**2**) and [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A (**3**)

Figure S4. 26. ¹H-NMR spectrum of [L-Val⁸]laxaphycin A (**4**) in DMSO (303K)

Figure S4. 27. ¹³C-NMR spectrum of [L-Val⁸]laxaphycin A (**4**) in DMSO (303K)

Figure S4. 28. HSQC spectrum of [L-Val⁸]laxaphycin A (**4**) in DMSO (303K)

Figure S4. 29. TOCSY spectrum of [L-Val⁸]laxaphycin A (**4**) in DMSO (303K)

Figure S4. 30. HSQC-TOCSY spectrum of [L-Val⁸]laxaphycin A (**4**) in DMSO (303K)

Figure S4. 31. HMBC spectrum of [L-Val⁸]laxaphycin A (**4**) in DMSO (303K)

Figure S4. 32. ROESY spectrum of [L-Val⁸]laxaphycin A (**4**) in DMSO (303K)

Figure S4. 33. Orbitrap MS/MS spectrum of [L-Val⁸]laxaphycin A (**4**)

Figure S4. 34. Advanced Marfey's analysis of [L-Val⁸]laxaphycin A (**4**)

Figure S4. 35. ¹H-NMR spectrum of [D-Val⁹]laxaphycin A (**5**) in DMSO (303K)

Figure S4. 36. ¹³C-NMR spectrum of [D-Val⁹]laxaphycin A (**5**) in DMSO (303K)

Figure S4. 37. HSQC spectrum of [D-Val⁹]laxaphycin A (**5**) in DMSO (303K)

Figure S4. 38. TOCSY spectrum of [D-Val⁹]laxaphycin A (**5**) in DMSO (303K)

Figure S4. 39. HSQC-TOCSY spectrum of [D-Val⁹]laxaphycin A (**5**) in DMSO (303K)

Figure S4. 40. HMBC spectrum of [D-Val⁹]laxaphycin A (**5**) in DMSO (303K)

Figure S4. 41. ROESY spectrum of [D-Val⁹]laxaphycin A (**5**) in DMSO (303K)

Figure S4. 42. Orbitrap MS/MS spectrum of [D-Val⁹]laxaphycin A (**5**)

Figure S4. 43. Advanced Marfey's analysis of [D-Val⁹]laxaphycin A (**5**)

Figure S4. 44. Advanced Marfey's analysis of acyclolaxaphycin B (**6**) and acyclolaxaphycin B3 (**7**)

Figure S4. 1. ^1H -NMR spectrum of Acyclolaxaphycin A(1) in DMSO (303K)

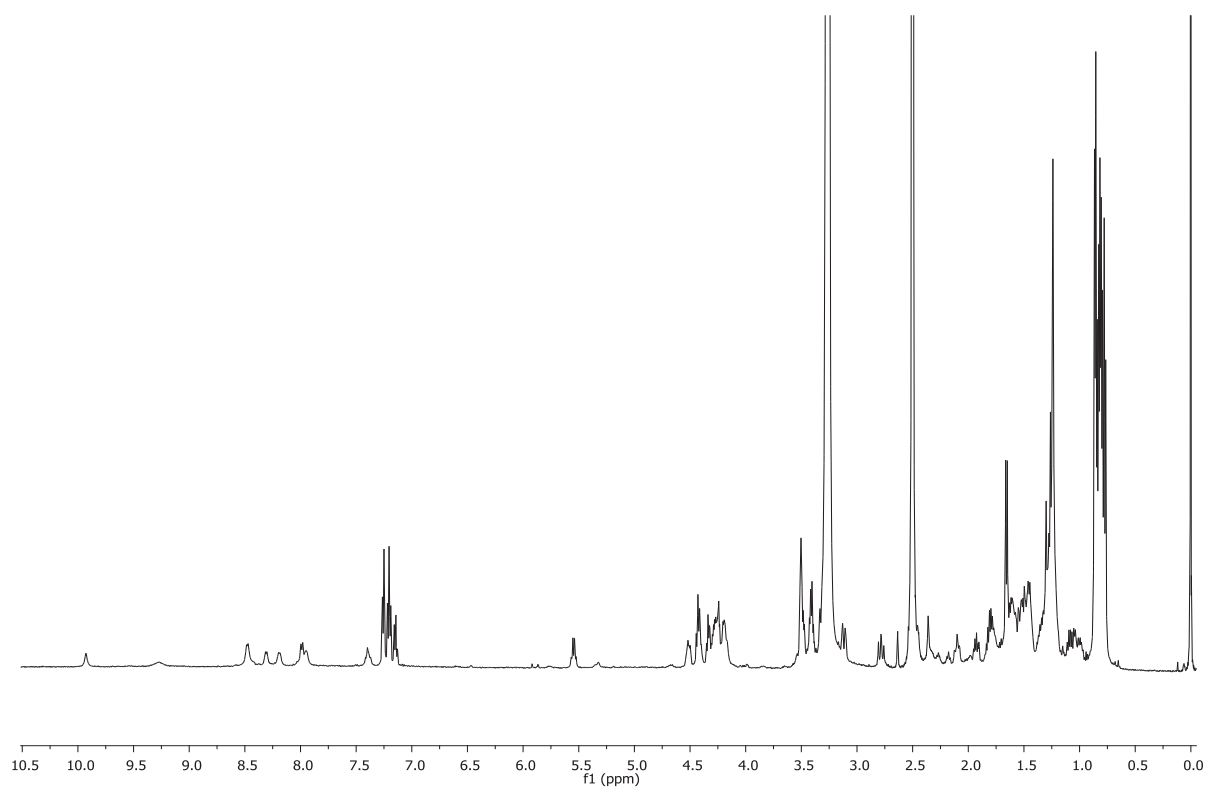


Figure S4. 2. ^{13}C -NMR spectrum of Acyclolaxaphycin A(1) in DMSO (303K)

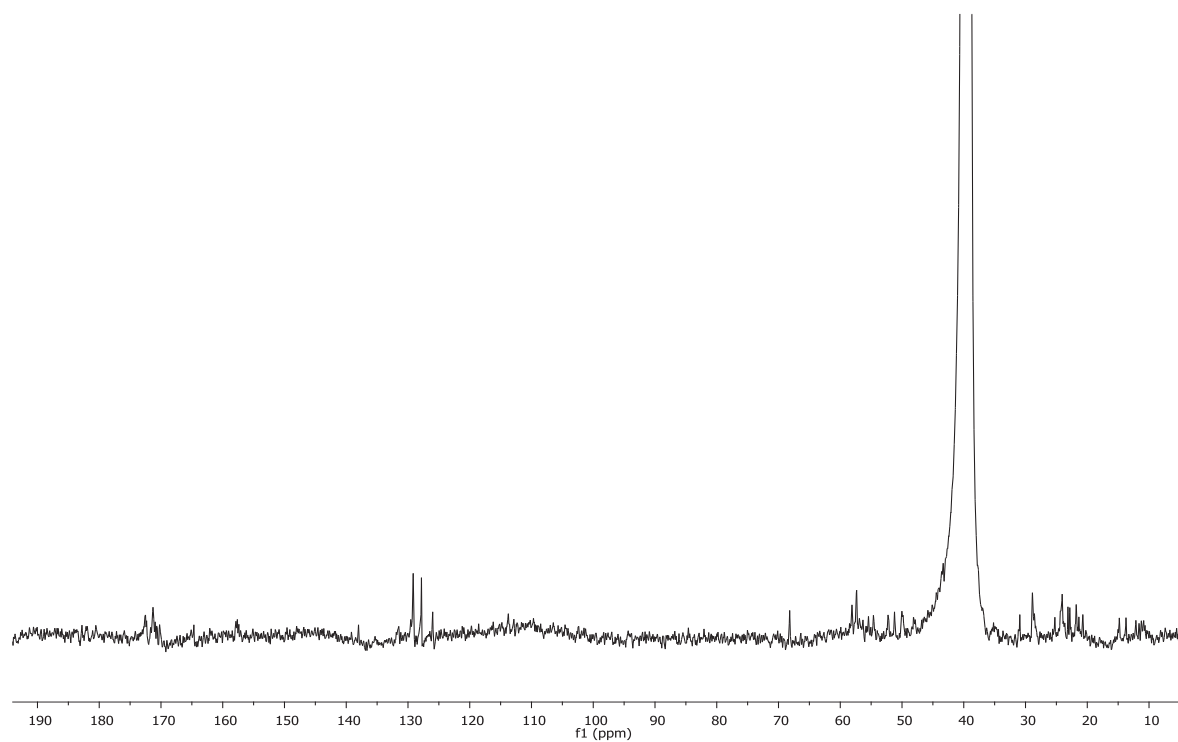


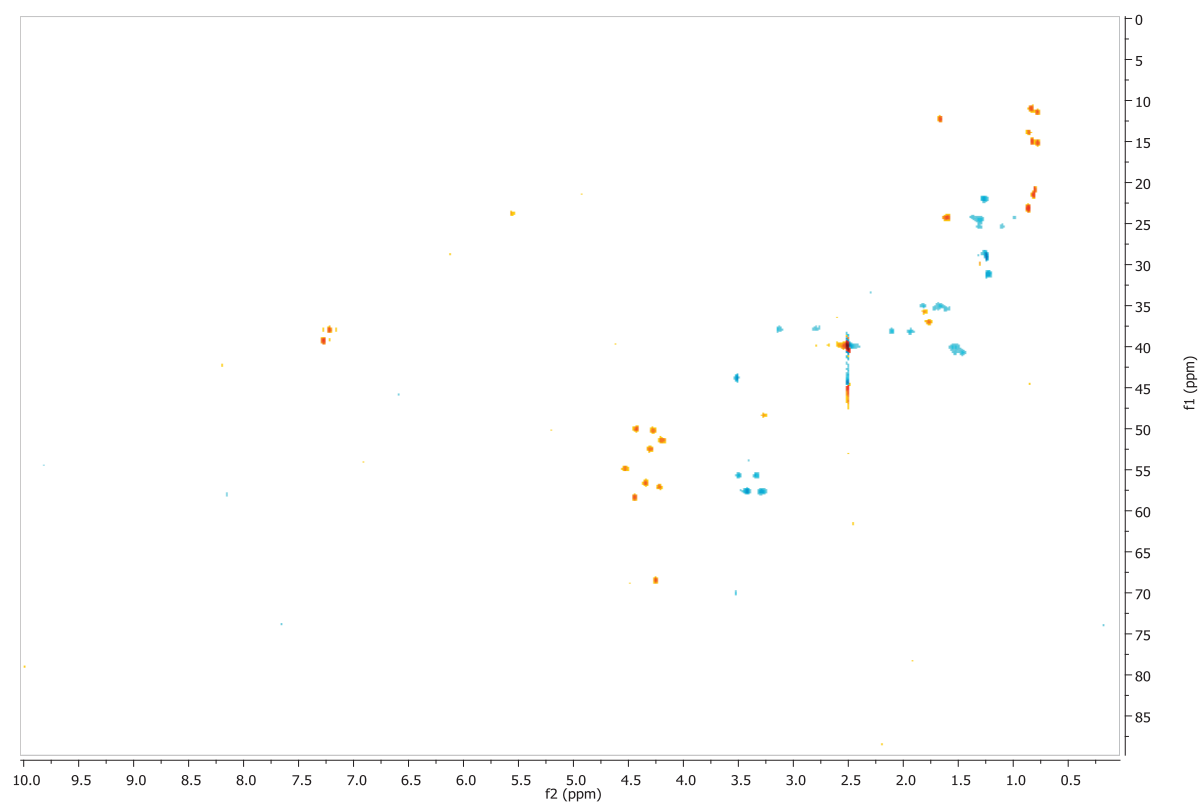
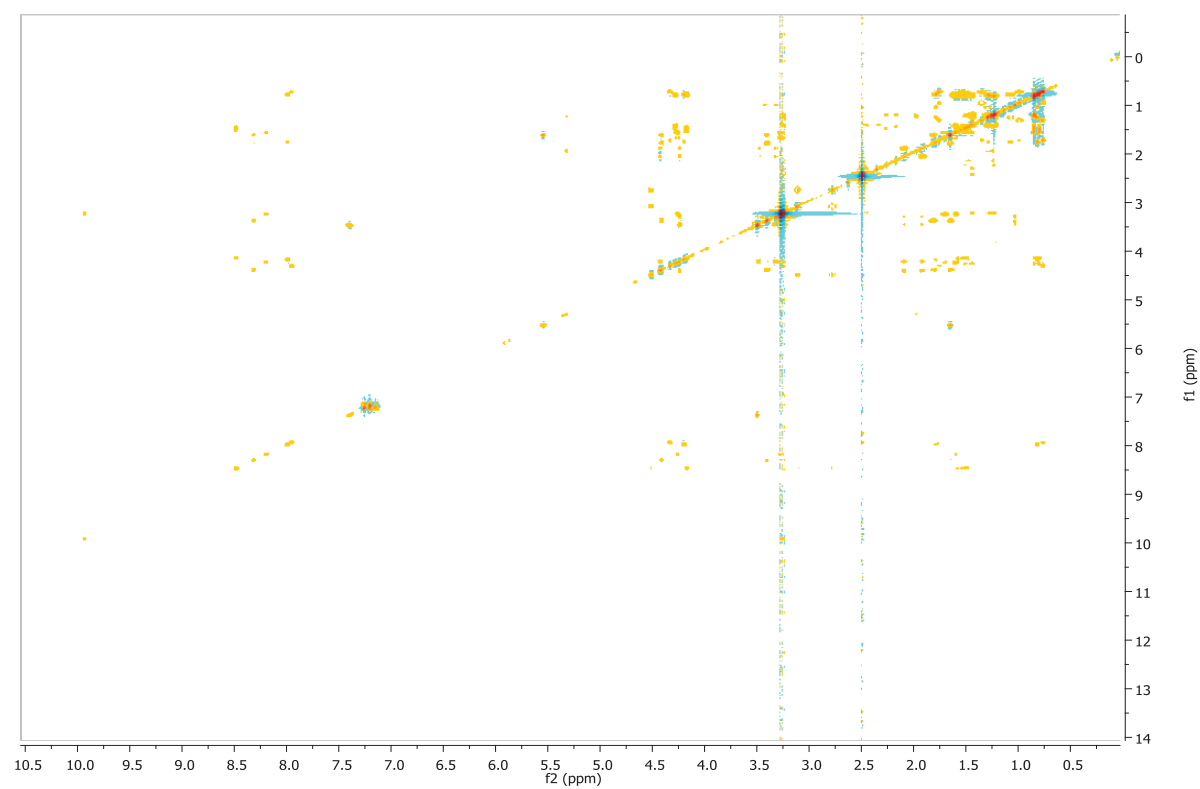
Figure S4. 3. HSQC spectrum of Acyclolaxaphycin A(1) in DMSO (303K)**Figure S4. 4.** TOCSY spectrum of Acyclolaxaphycin A(1) in DMSO (303K)

Figure S4. 5. HSQC-TOCSY spectrum of Acyclolaxaphycin A(1) in DMSO (303K)

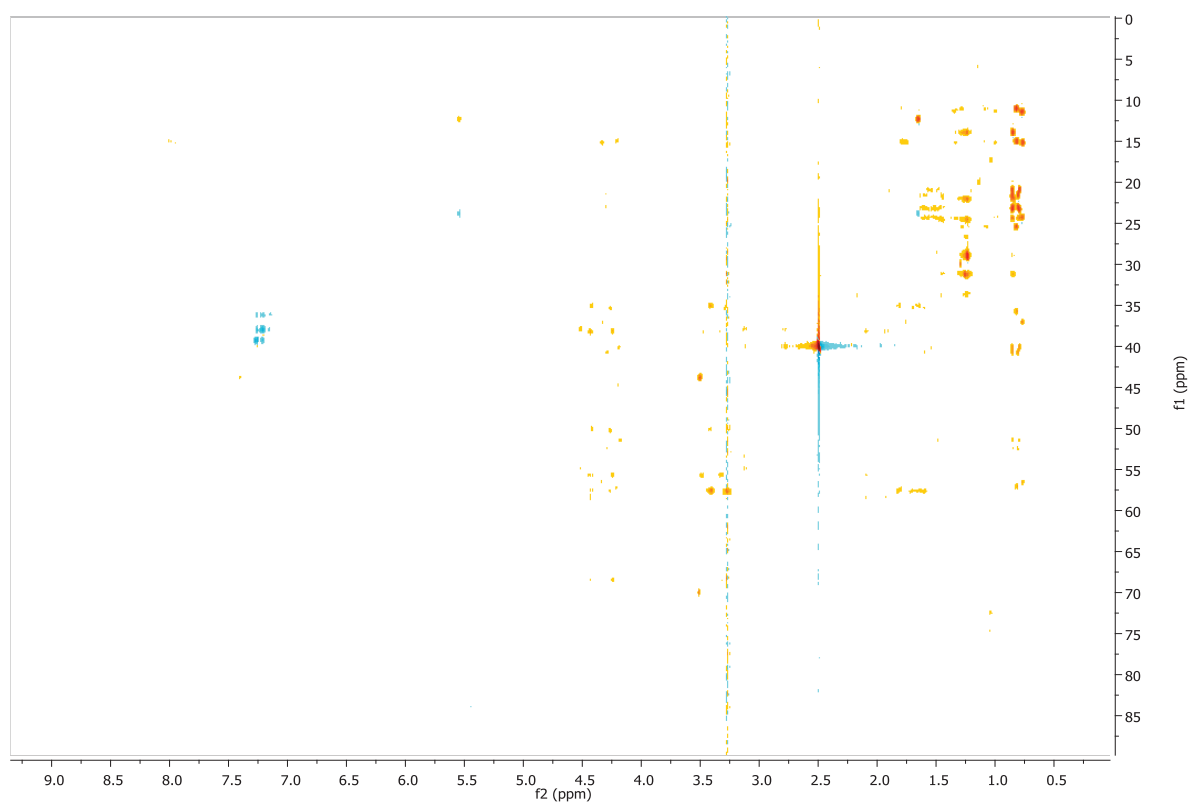


Figure S4. 6. HMBC spectrum of Acyclolaxaphycin A(1) in DMSO (303K)

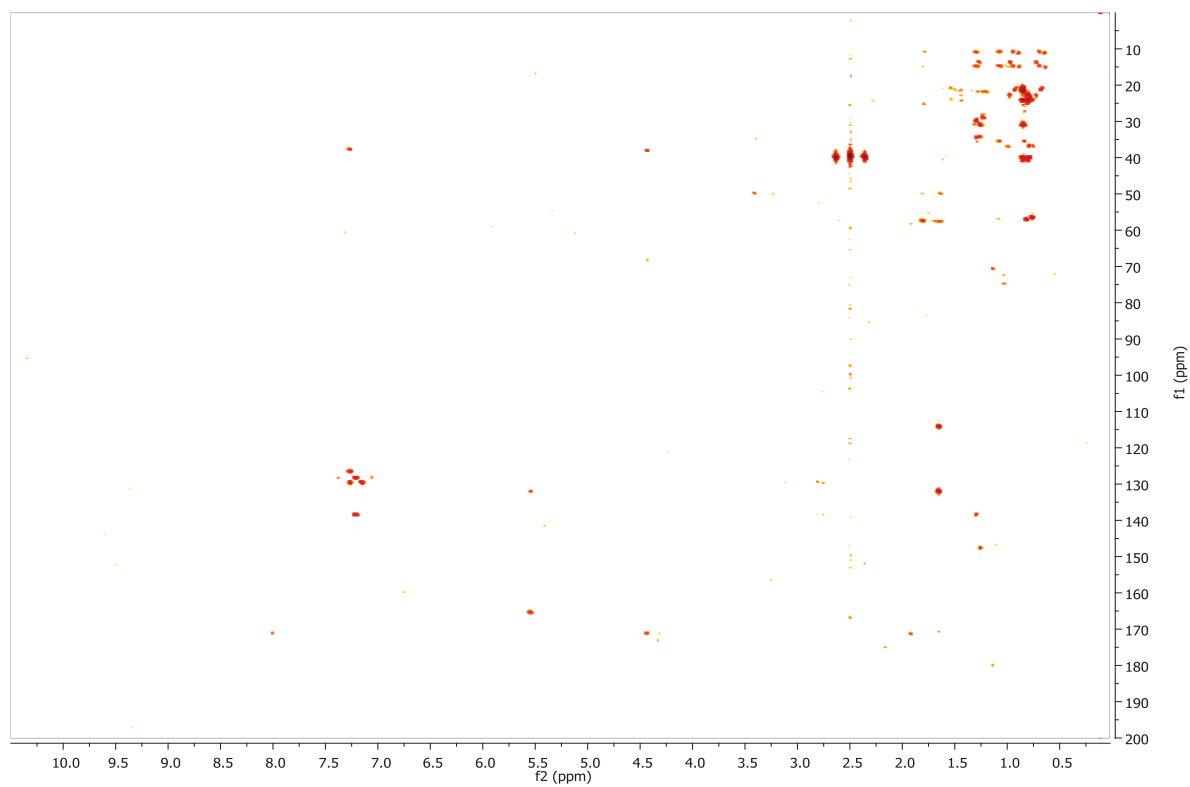


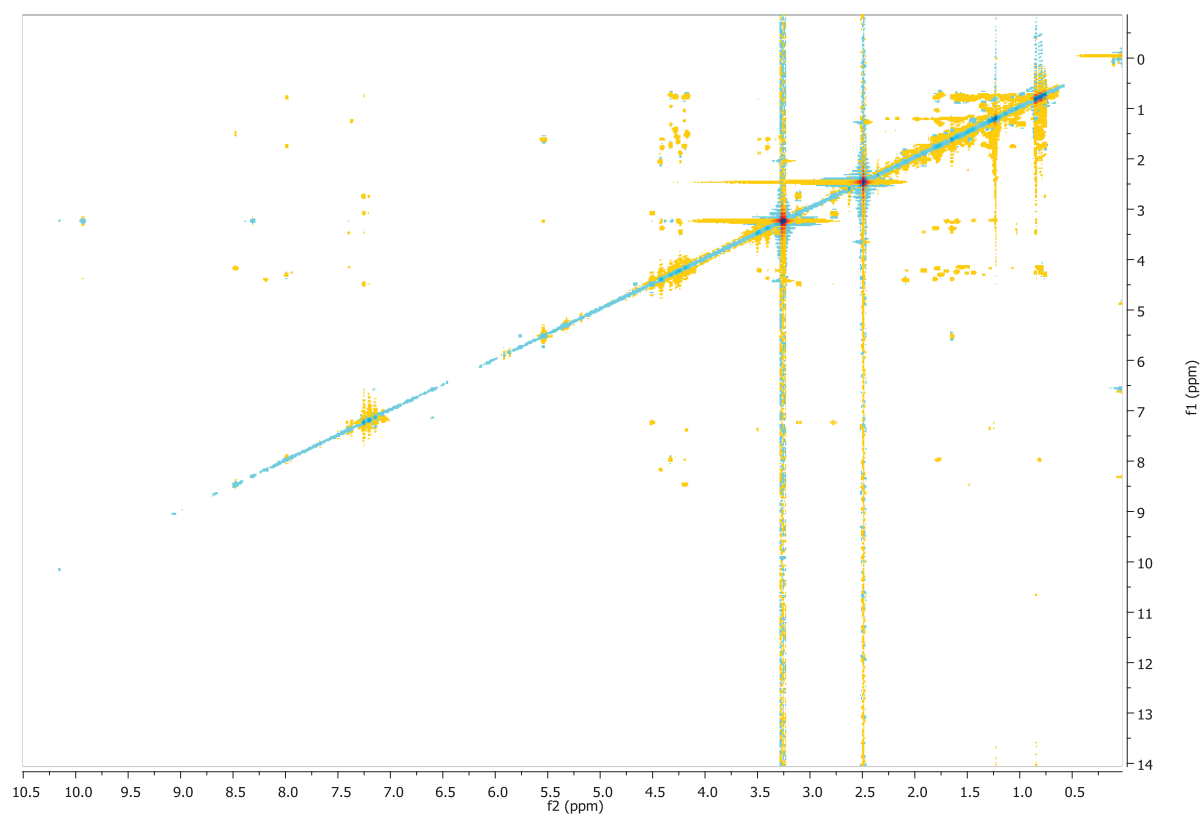
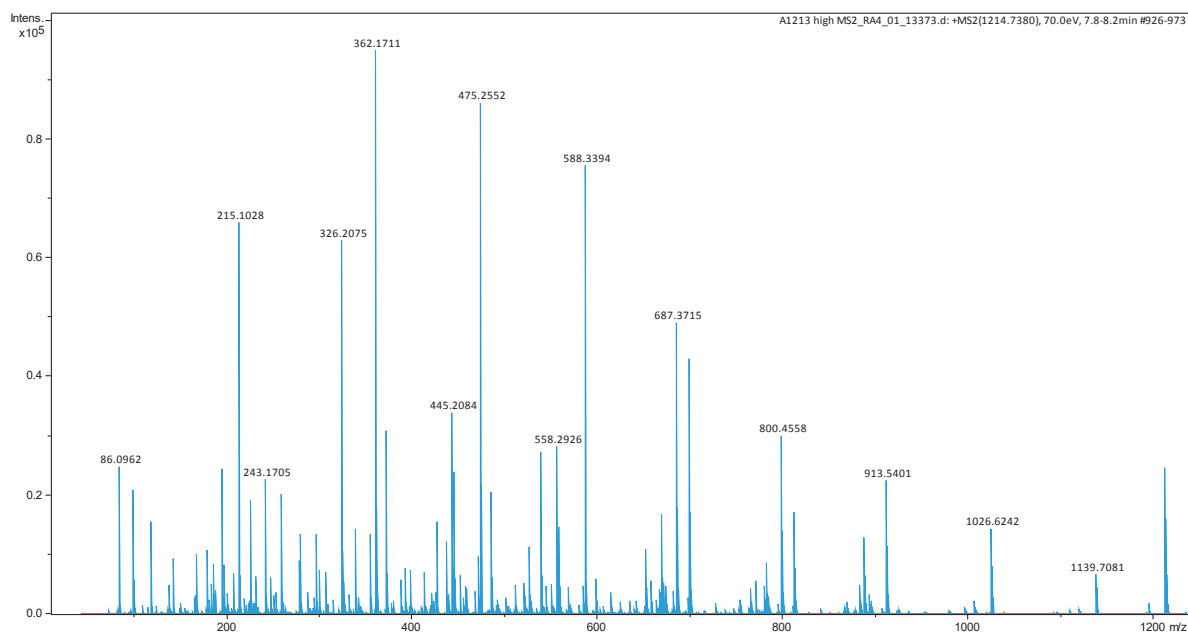
Figure S4. 7. ROESY spectrum of Acyclolaxaphycin A(1) in DMSO (303K)**Figure S4. 8.** Orbitrap MS/MS spectrum of Acyclolaxaphycin A (1)

Figure S4. 9. ^1H -NMR spectrum of [des-(Gly¹¹)]acyclolaxaphycin A (**2**) in DMSO (303K)

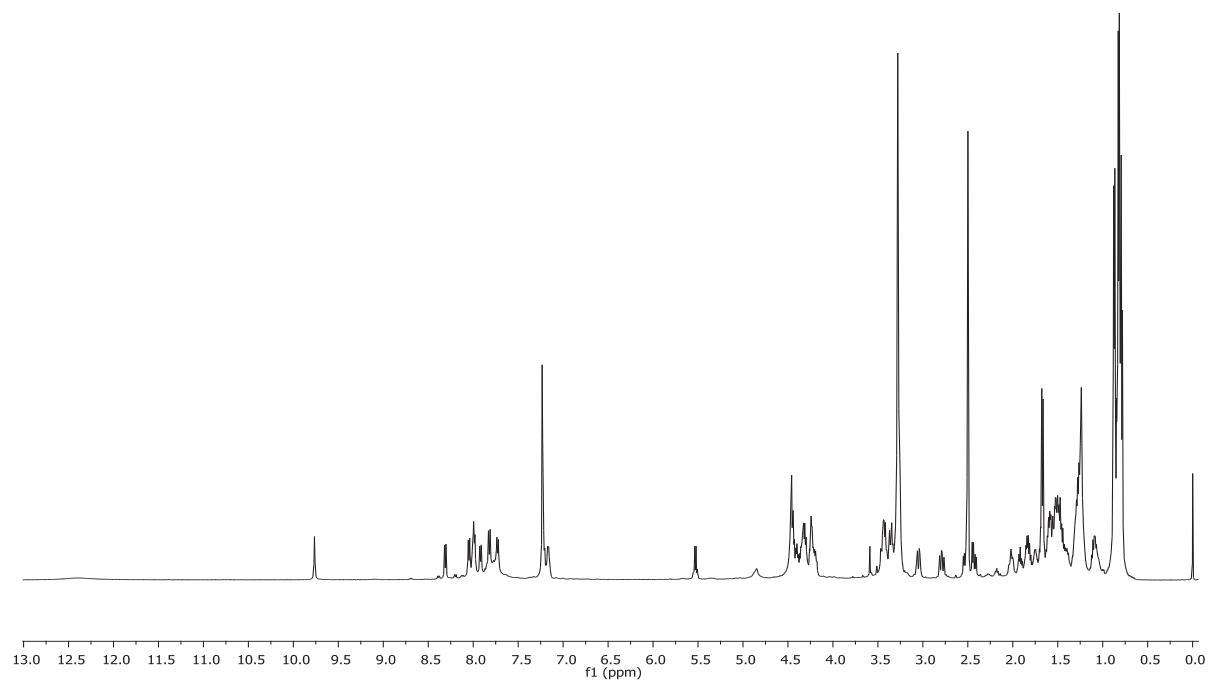


Figure S4. 10. ^{13}C -NMR spectrum of [des-(Gly¹¹)]acyclolaxaphycin A (**2**) in DMSO (303K)

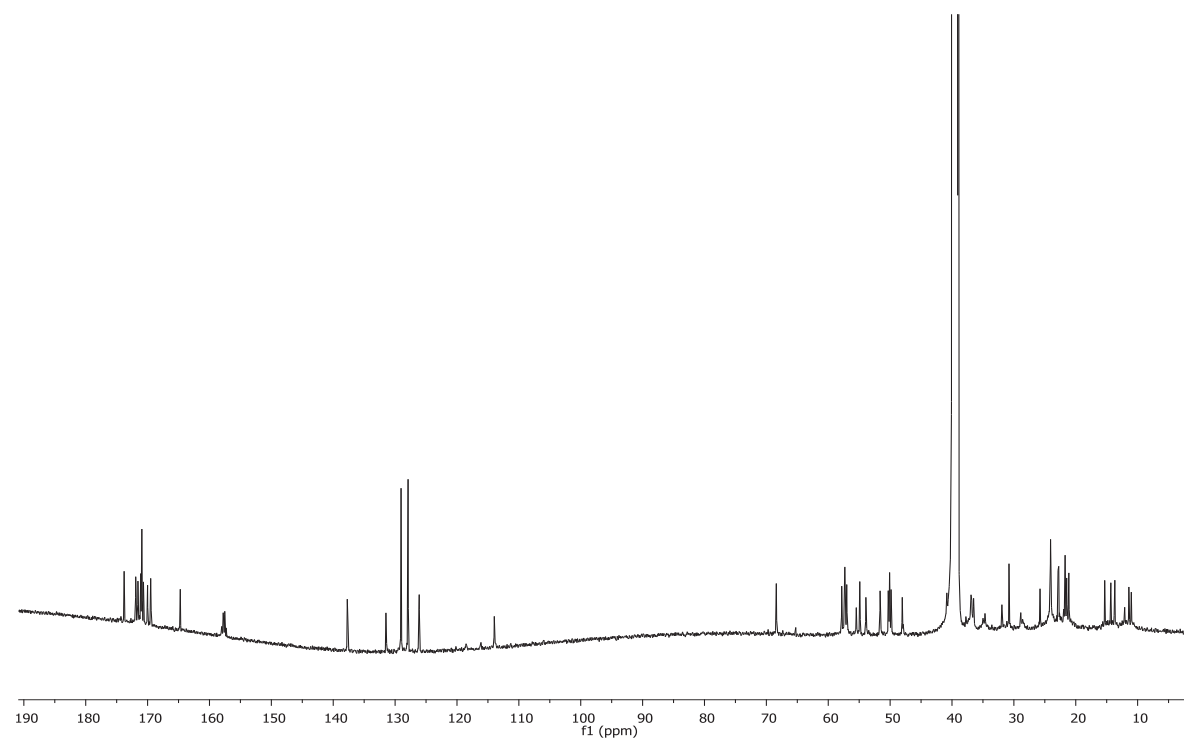


Figure S4. 11. HSQC spectrum of [des-(Gly¹¹)]acyclolaxaphycin A (**2**) in DMSO (303K)

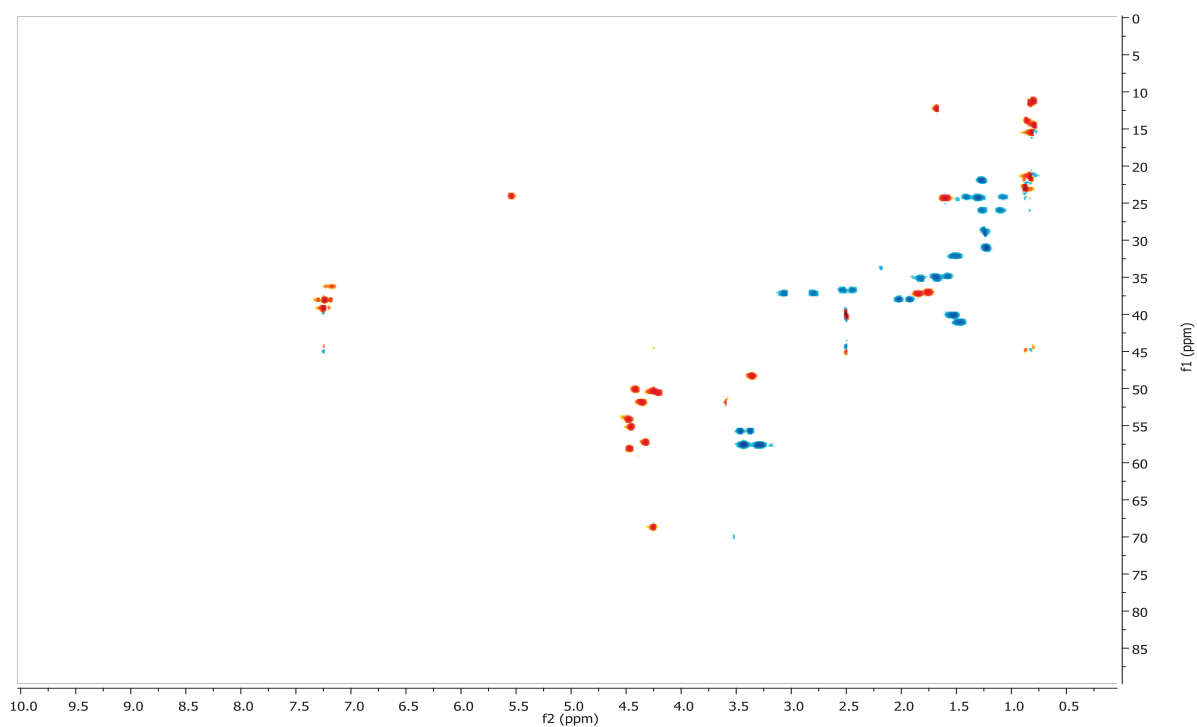


Figure S4. 12. TOCSY spectrum of [des-(Gly¹¹)]acyclolaxaphycin A (**2**) in DMSO (303K)

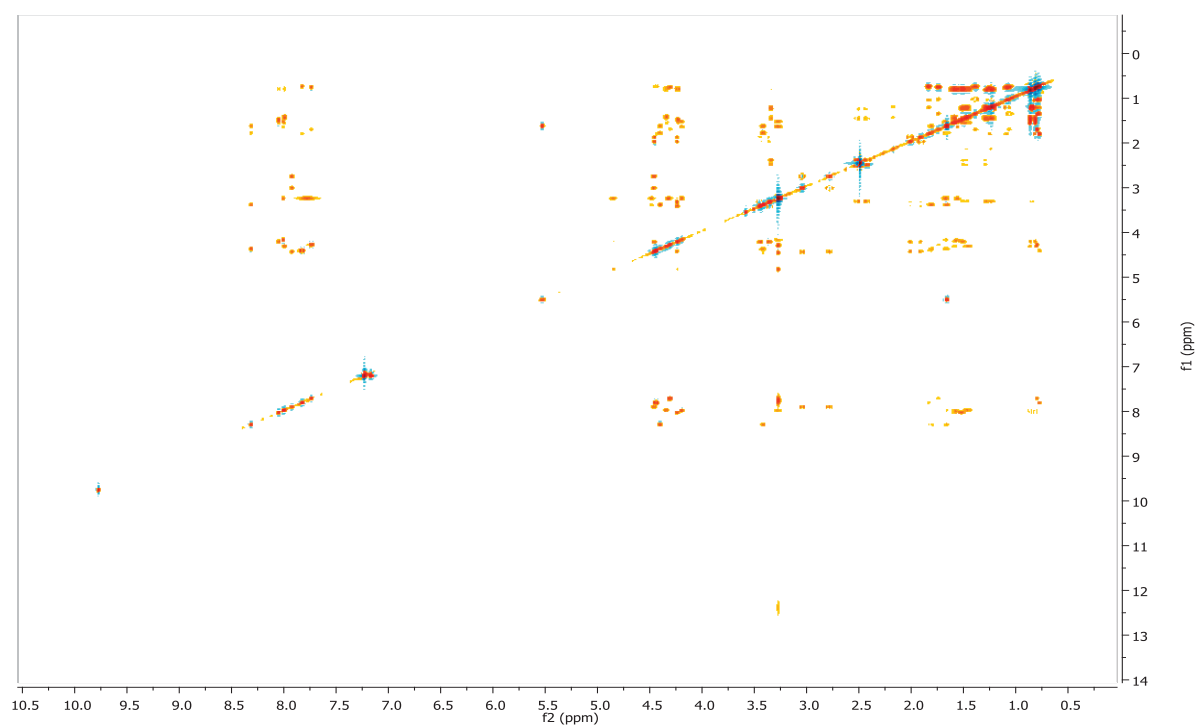


Figure S4. 13. HSQC-TOCSY spectrum of [des-(Gly¹¹)]acyclolaxaphycin A (**2**) in DMSO (303K)

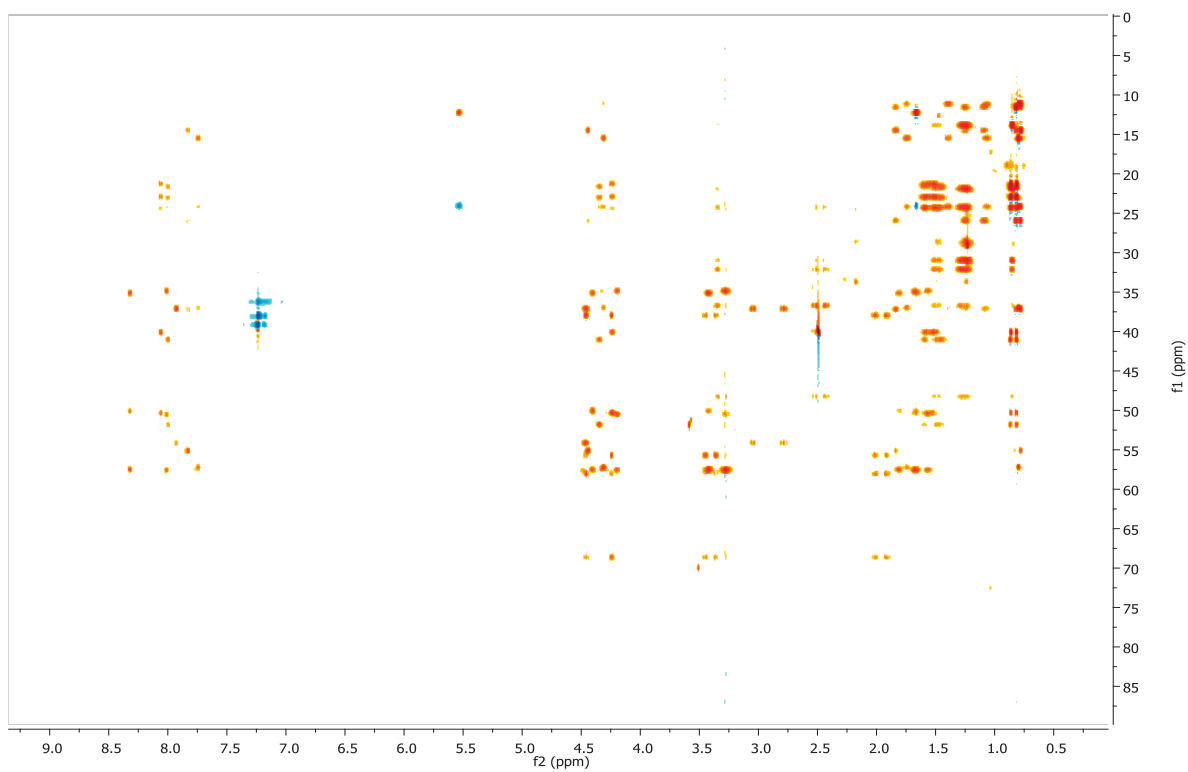


Figure S4. 14. HMBC spectrum of [des-(Gly¹¹)]acyclolaxaphycin A (**2**) in DMSO (303K)

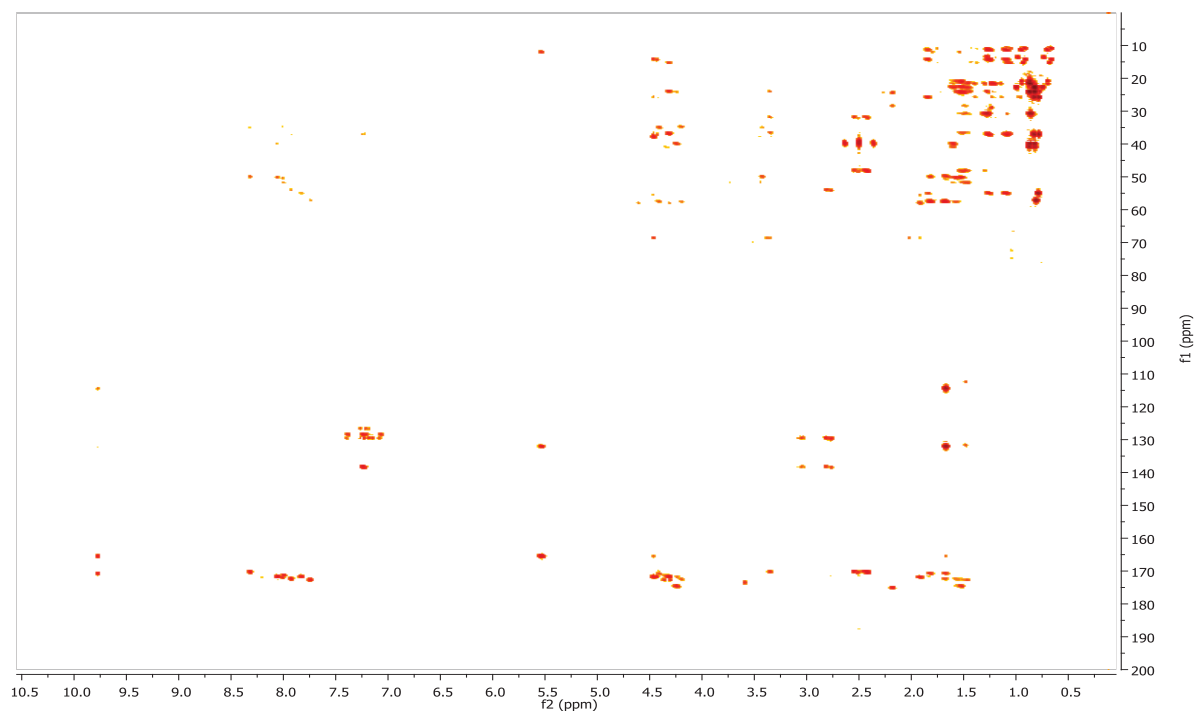


Figure S4. 15. ROESY spectrum of [des-(Gly¹¹)]acyclolaxaphycin A (**2**) in DMSO (303K)

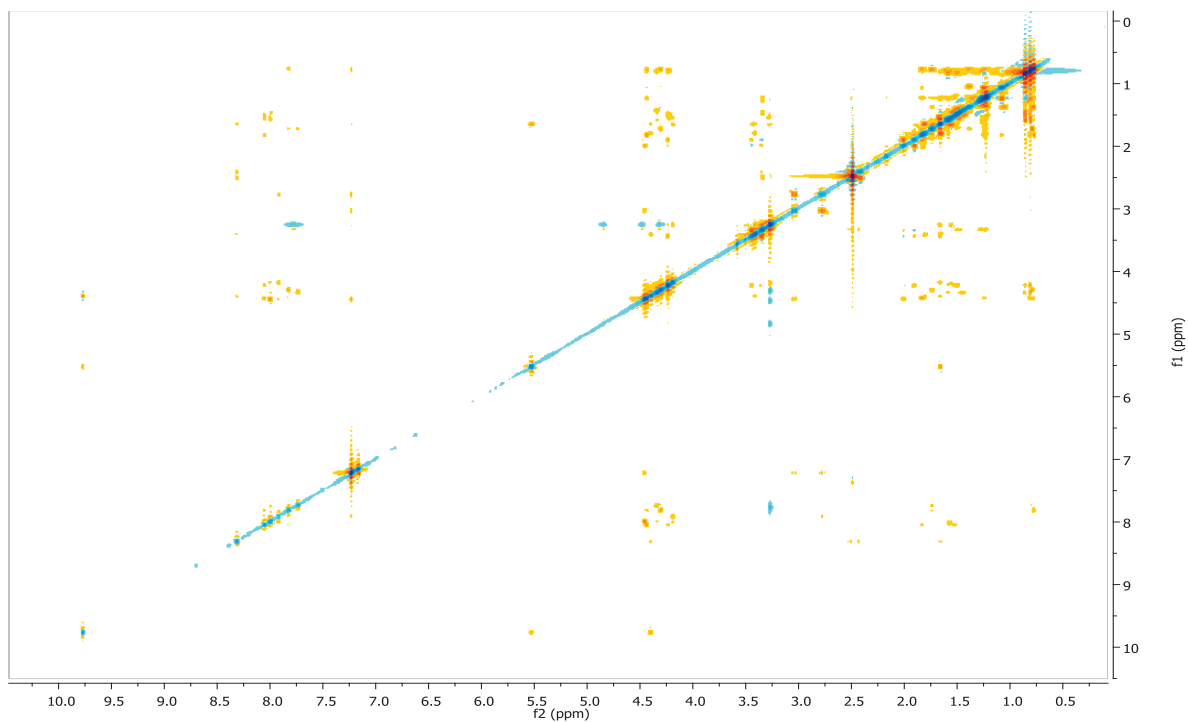


Figure S4. 16. Orbitrap MS/MS spectrum of [des-(Gly¹¹)]acyclolaxaphycin A (2)

20141007OT_140710151527 #8 RT: 0.07 AV: 1 NL: 1.51E7
T: FTMS + p ESI w Full ms2 1157.70@cid35.00 [315.00-116:

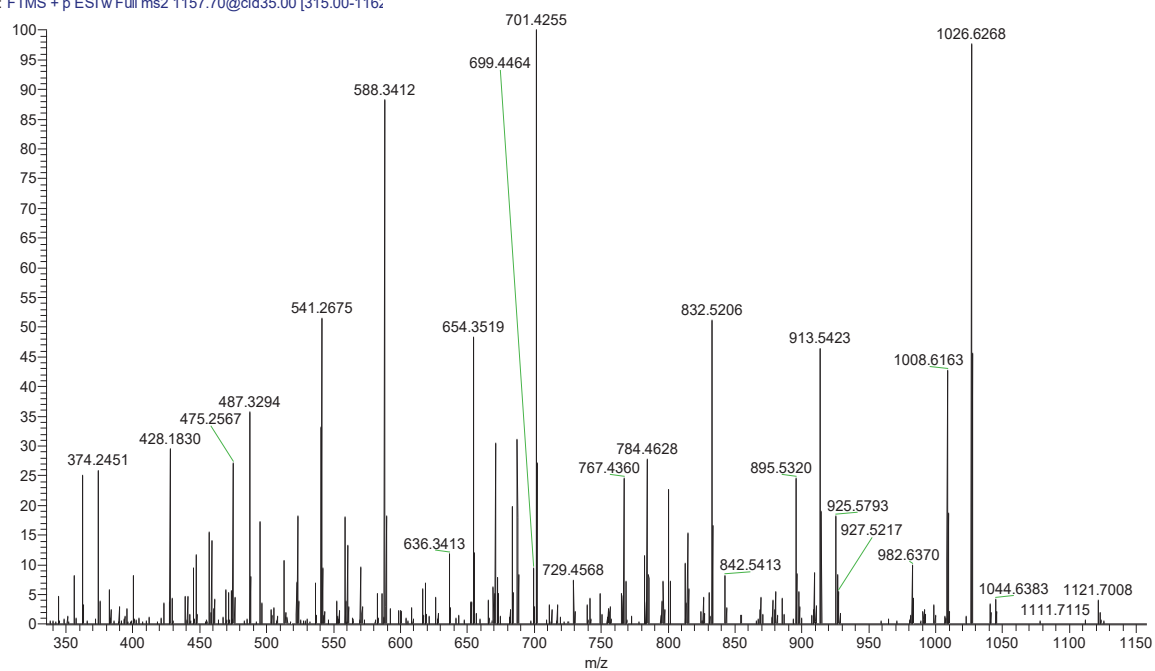


Figure S4. 17. ^1H -NMR spectrum of [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A (**3**) in DMSO (303K)

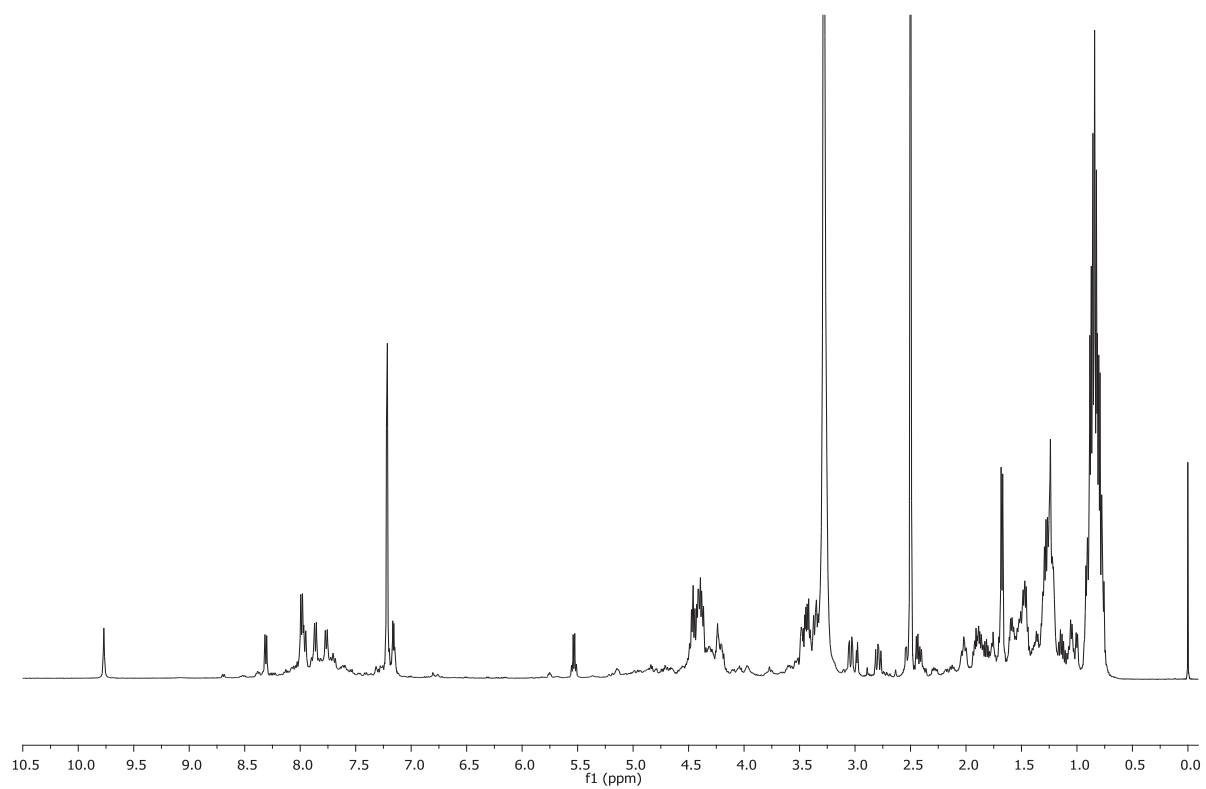


Figure S4. 18. ^{13}C -NMR spectrum of [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A (**3**) in DMSO (303K)

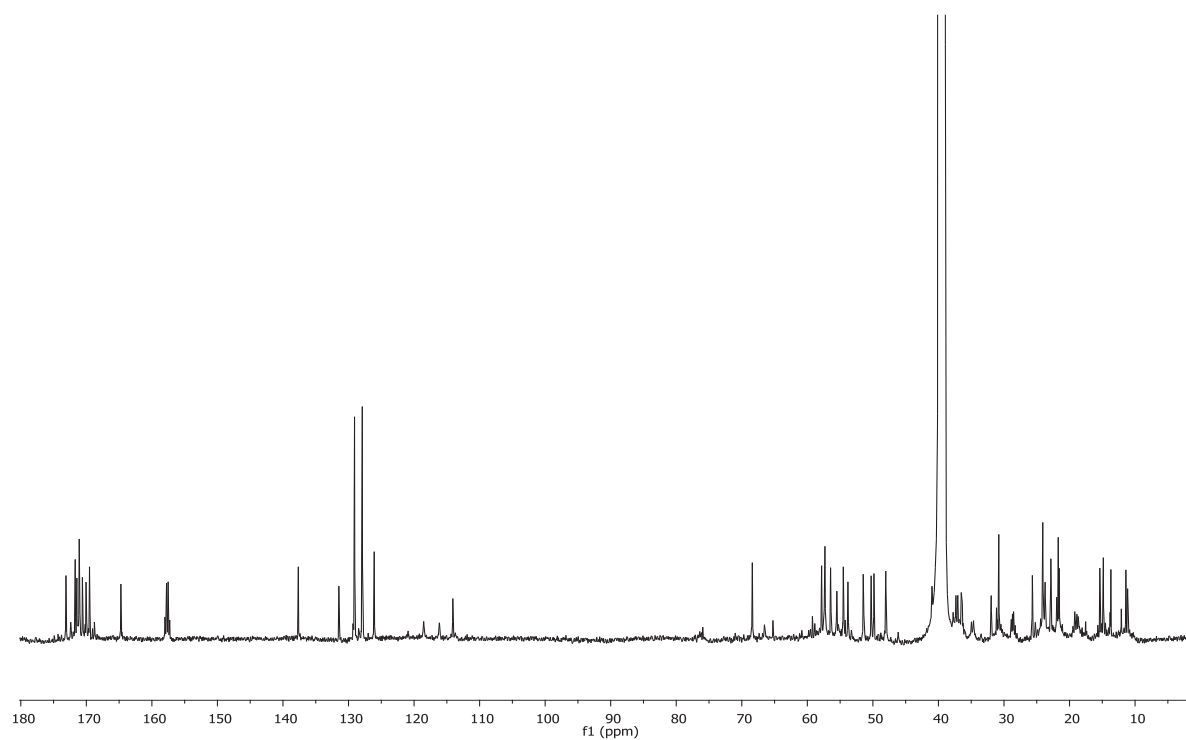


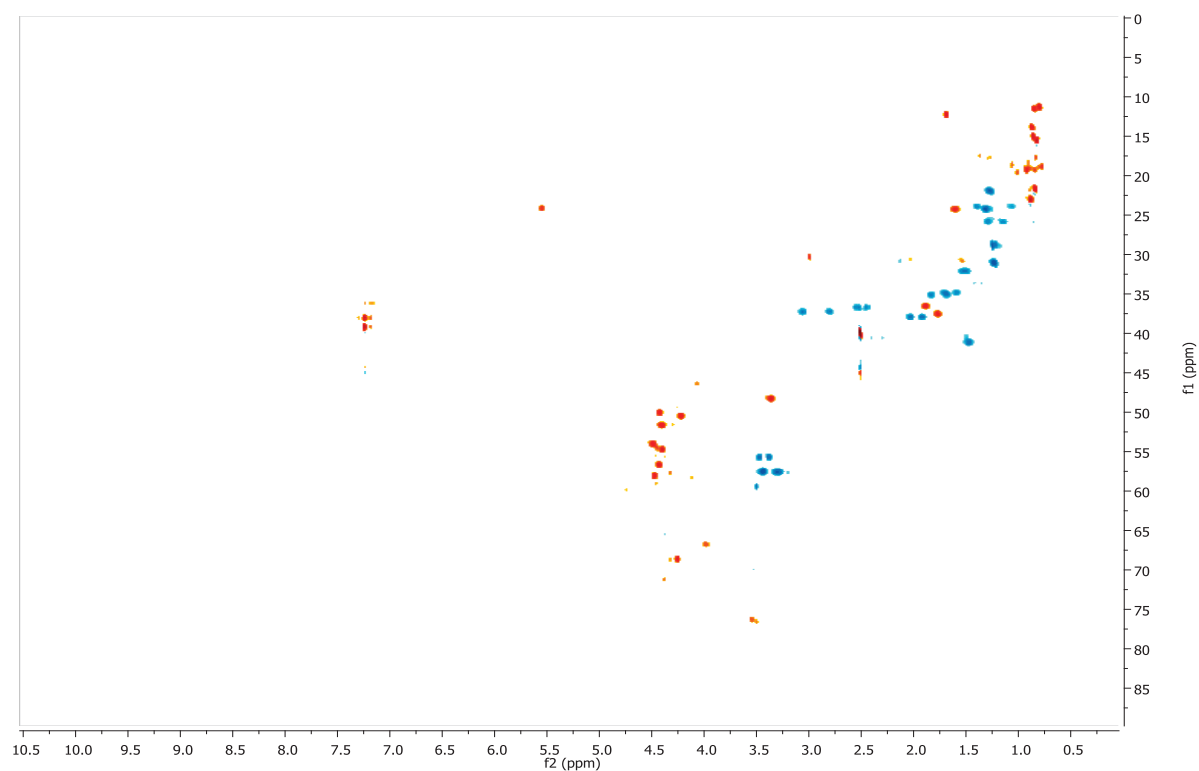
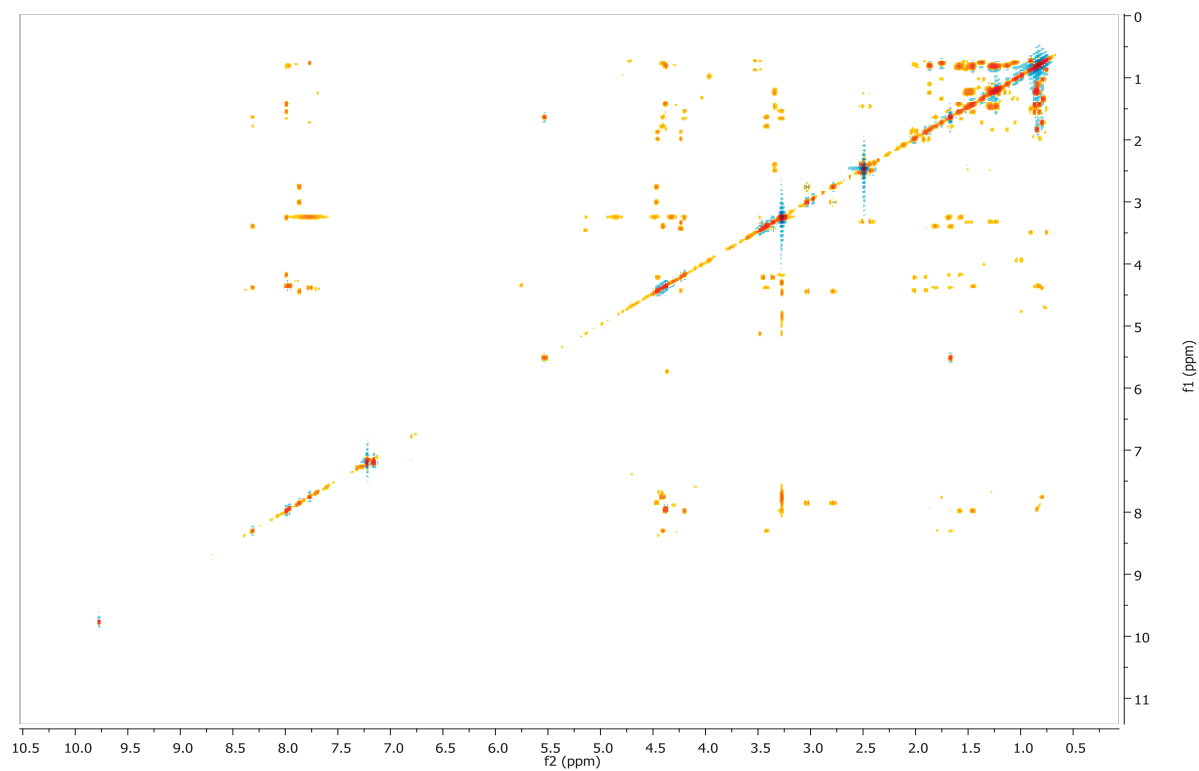
Figure S4. 19. HSQC spectrum of [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A (**3**) in DMSO (303K)**Figure S4. 20.** TOCSY spectrum of [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A (**3**) in DMSO (303K)

Figure S4. 21. HSQC-TOCSY spectrum of [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A (**3**) in DMSO (303K)

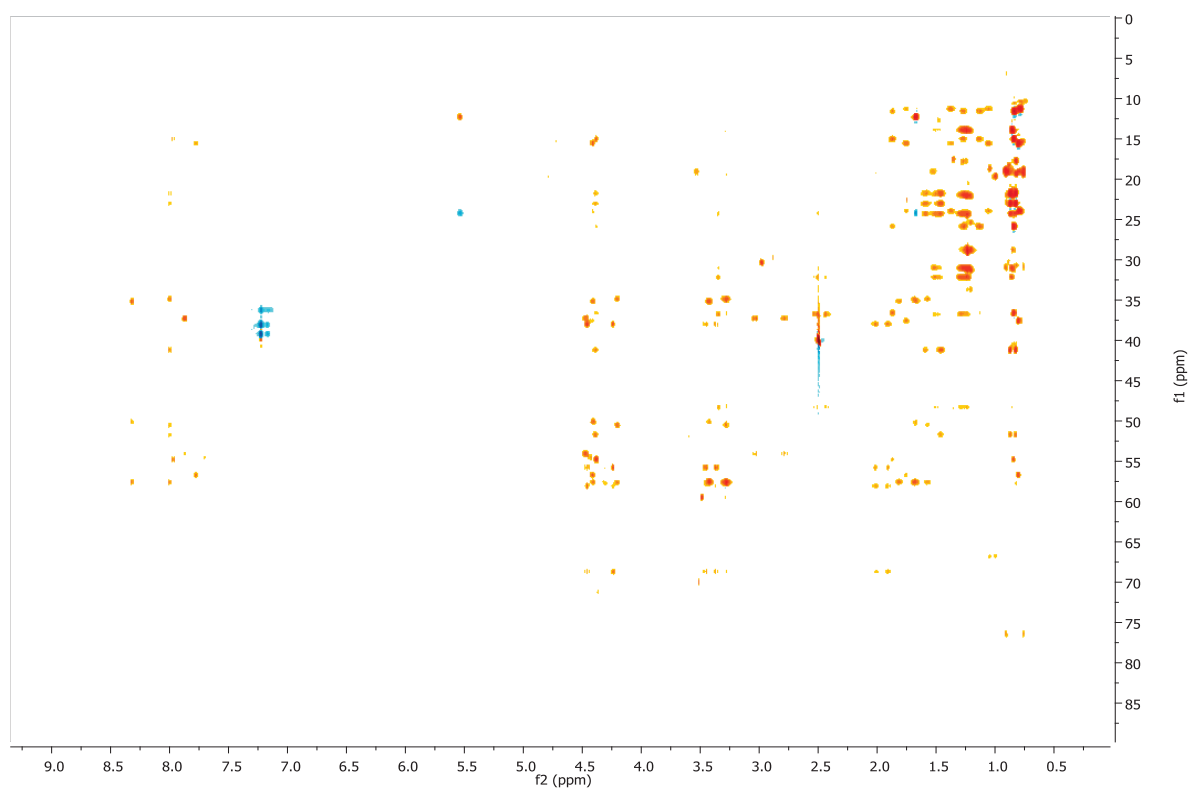


Figure S4. 22. HMBC spectrum of [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A (**3**) in DMSO (303K)

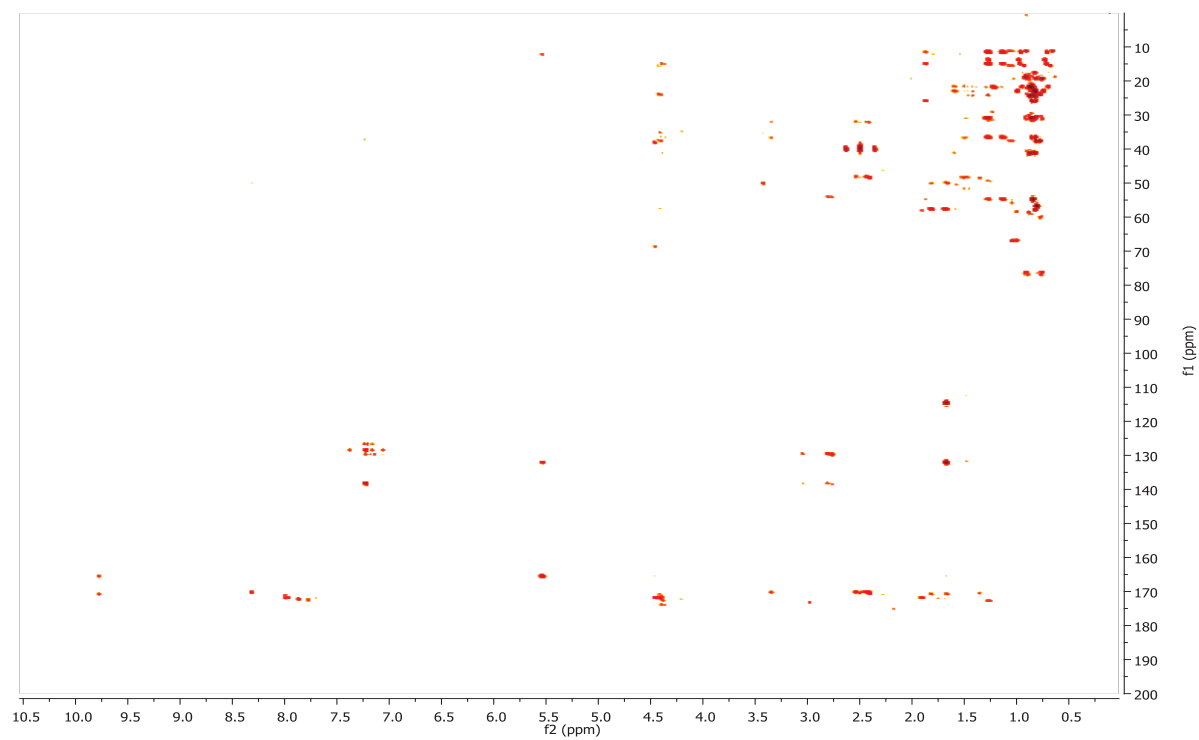
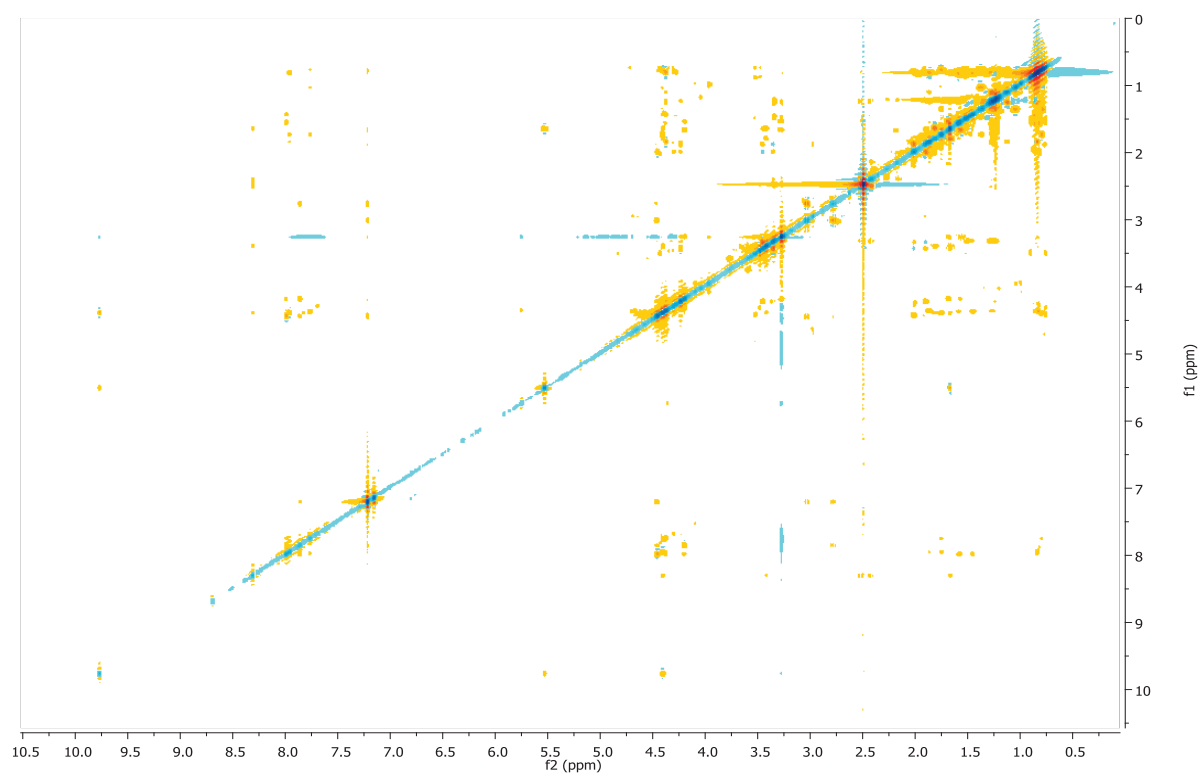


Figure S4. 23. ROESY spectrum of [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A (**3**) in DMSO (303K)**Figure S4. 24.** Orbitrap MS/MS spectrum of [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A (**3**)

20141007OT_140710153245 #9 RT: 0.09 AV: 1 NL: 1.42E7
T: FTMS + p ESIw Full ms2 1044.60@cid30.00 [285.00-1045]

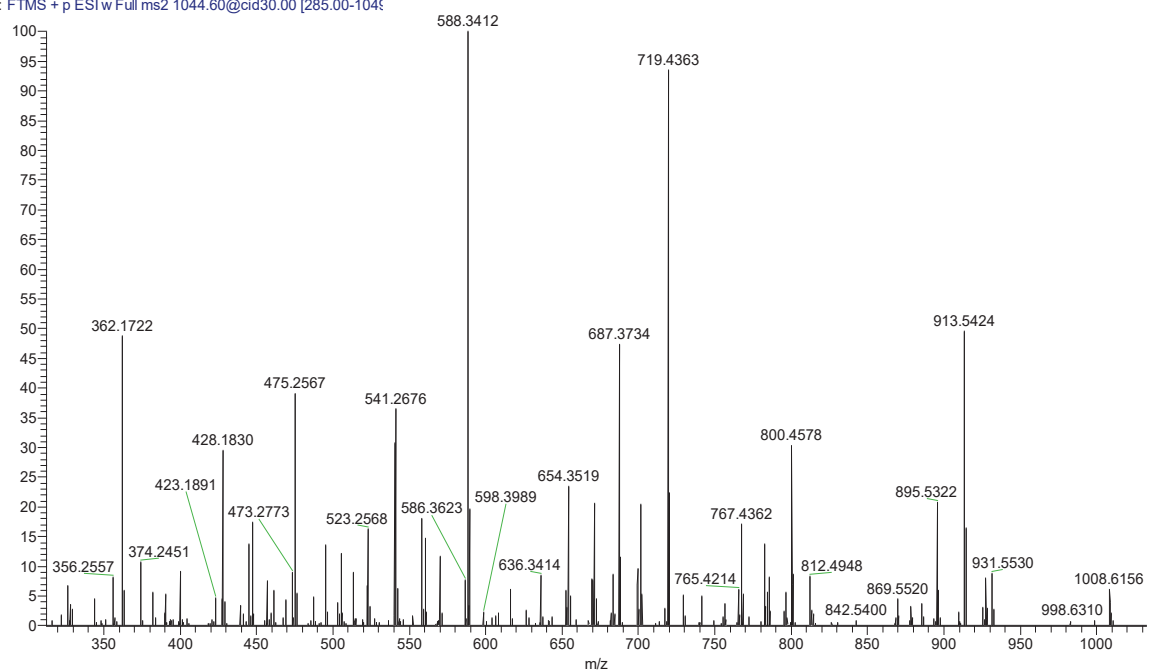


Figure S4. 25. Advanced Marfey's analysis of [des-(Gly¹¹)]acyclolaxaphycin A (**2**) and [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A (**3**)

[des-(Gly¹¹)]acyclolaxaphycin A				[des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A																																																																																														
	RT for each Marfey's derivatives (min)		Absolute configuration		RT for each Marfey's derivatives (min)		Absolute configuration																																																																																											
	Aoc	L-FDLA			54,45 (1)	3R		Aoc	L-FDLA	54,46 (1)	3R																																																																																							
	D-FDLA	42,98 (1)	D-FDLA	42,95 (1)	Hse		L-FDLA	35,33 (2)	2x L (2S)	Hse		L-FDLA	35,36 (2)	2x L (2S)		D-FDLA	37,94 (2)	D-FDLA	37,95 (2)	Hyp	L-FDLA	30,39 (2)	L (2S)	Hyp	L-FDLA	30,38 (2)	L (2S)		D-FDLA	29,40 (2)	D-FDLA	29,39 (2)	Phe	L-FDLA	65,13 (2)	D (2R)	Phe	L-FDLA	65,17 (2)	D (2R)		D-FDLA	53,90 (2)	D-FDLA	53,91 (2)	Leu	L-FDLA	66,30 (2)	D (2R)	Leu	L-FDLA	66,29 (2)	D (2R)		D-FDLA	52,65 (2)	D-FDLA	52,62 (2)	Ile	L-FDLA	51,84 (2)	(2S,3S)	Ile	L-FDLA	51,84 (2)	(2S,3S)		D-FDLA	65,41 (2)	D-FDLA	65,42 (2)	Ile	L-FDLA	65,55 (2)	(2R,3S)	Ile	L-FDLA	65,53 (2)	(2R,3S)		D-FDLA	51,50 (2)	D-FDLA	51,48 (2)	Leu	L-FDLA	52,61 (2)	L (2S)						D-FDLA	66,31 (2)			
Hse	L-FDLA	35,33 (2)	2x L (2S)	Hse	L-FDLA	35,36 (2)	2x L (2S)																																																																																											
	D-FDLA	37,94 (2)		D-FDLA	37,95 (2)	Hyp		L-FDLA	30,39 (2)	L (2S)	Hyp	L-FDLA	30,38 (2)	L (2S)		D-FDLA	29,40 (2)	D-FDLA	29,39 (2)	Phe	L-FDLA	65,13 (2)	D (2R)	Phe	L-FDLA	65,17 (2)	D (2R)		D-FDLA	53,90 (2)	D-FDLA	53,91 (2)	Leu	L-FDLA	66,30 (2)	D (2R)	Leu	L-FDLA	66,29 (2)	D (2R)		D-FDLA	52,65 (2)	D-FDLA	52,62 (2)	Ile	L-FDLA	51,84 (2)	(2S,3S)	Ile	L-FDLA	51,84 (2)	(2S,3S)		D-FDLA	65,41 (2)	D-FDLA	65,42 (2)	Ile	L-FDLA	65,55 (2)	(2R,3S)	Ile	L-FDLA	65,53 (2)	(2R,3S)		D-FDLA	51,50 (2)	D-FDLA	51,48 (2)	Leu	L-FDLA	52,61 (2)	L (2S)						D-FDLA	66,31 (2)																
Hyp	L-FDLA	30,39 (2)	L (2S)	Hyp	L-FDLA	30,38 (2)	L (2S)																																																																																											
	D-FDLA	29,40 (2)		D-FDLA	29,39 (2)	Phe		L-FDLA	65,13 (2)	D (2R)	Phe	L-FDLA	65,17 (2)	D (2R)		D-FDLA	53,90 (2)	D-FDLA	53,91 (2)	Leu	L-FDLA	66,30 (2)	D (2R)	Leu	L-FDLA	66,29 (2)	D (2R)		D-FDLA	52,65 (2)	D-FDLA	52,62 (2)	Ile	L-FDLA	51,84 (2)	(2S,3S)	Ile	L-FDLA	51,84 (2)	(2S,3S)		D-FDLA	65,41 (2)	D-FDLA	65,42 (2)	Ile	L-FDLA	65,55 (2)	(2R,3S)	Ile	L-FDLA	65,53 (2)	(2R,3S)		D-FDLA	51,50 (2)	D-FDLA	51,48 (2)	Leu	L-FDLA	52,61 (2)	L (2S)						D-FDLA	66,31 (2)																													
Phe	L-FDLA	65,13 (2)	D (2R)	Phe	L-FDLA	65,17 (2)	D (2R)																																																																																											
	D-FDLA	53,90 (2)		D-FDLA	53,91 (2)	Leu		L-FDLA	66,30 (2)	D (2R)	Leu	L-FDLA	66,29 (2)	D (2R)		D-FDLA	52,65 (2)	D-FDLA	52,62 (2)	Ile	L-FDLA	51,84 (2)	(2S,3S)	Ile	L-FDLA	51,84 (2)	(2S,3S)		D-FDLA	65,41 (2)	D-FDLA	65,42 (2)	Ile	L-FDLA	65,55 (2)	(2R,3S)	Ile	L-FDLA	65,53 (2)	(2R,3S)		D-FDLA	51,50 (2)	D-FDLA	51,48 (2)	Leu	L-FDLA	52,61 (2)	L (2S)						D-FDLA	66,31 (2)																																										
Leu	L-FDLA	66,30 (2)	D (2R)	Leu	L-FDLA	66,29 (2)	D (2R)																																																																																											
	D-FDLA	52,65 (2)		D-FDLA	52,62 (2)	Ile		L-FDLA	51,84 (2)	(2S,3S)	Ile	L-FDLA	51,84 (2)	(2S,3S)		D-FDLA	65,41 (2)	D-FDLA	65,42 (2)	Ile	L-FDLA	65,55 (2)	(2R,3S)	Ile	L-FDLA	65,53 (2)	(2R,3S)		D-FDLA	51,50 (2)	D-FDLA	51,48 (2)	Leu	L-FDLA	52,61 (2)	L (2S)						D-FDLA	66,31 (2)																																																							
Ile	L-FDLA	51,84 (2)	(2S,3S)	Ile	L-FDLA	51,84 (2)	(2S,3S)																																																																																											
	D-FDLA	65,41 (2)		D-FDLA	65,42 (2)	Ile		L-FDLA	65,55 (2)	(2R,3S)	Ile	L-FDLA	65,53 (2)	(2R,3S)		D-FDLA	51,50 (2)	D-FDLA	51,48 (2)	Leu	L-FDLA	52,61 (2)	L (2S)						D-FDLA	66,31 (2)																																																																				
Ile	L-FDLA	65,55 (2)	(2R,3S)	Ile	L-FDLA	65,53 (2)	(2R,3S)																																																																																											
	D-FDLA	51,50 (2)		D-FDLA	51,48 (2)	Leu		L-FDLA	52,61 (2)	L (2S)						D-FDLA	66,31 (2)																																																																																	
Leu	L-FDLA	52,61 (2)	L (2S)																																																																																															
	D-FDLA	66,31 (2)																																																																																																

RT of Marfey's derivatives for each standard isoleucines (min)							
(2R,3R)-Ile	D-Ile	(2R,3S)-Ile	D-Allo-Ile	(2S,3R)-Ile	L-Allo-Ile	(2S,3S)-Ile	L-Ile
L-FDLA	65,37	L-FDLA	65,42	L-FDLA	51,62	L-FDLA	51,80
D-FDLA	51,79	D-FDLA	51,58	D-FDLA	65,44	D-FDLA	65,40

RT of Marfey's derivatives for each standard leucines (min)			
(2S)-Leu	L-Leu	(2R)-Leu	D-Leu
L-FDLA	52,57	L-FDLA	66,21
D-FDLA	66,27	D-FDLA	52,6

(1) Gradient 1: from 20% CH₃CN-.% 0.01 M formic acid to 60% CH₃CN-40% 0.01 M formic acid at 0.3 mL/min over 70 min

(2) Gradient 2: from 10% CH₃CN-.% 0.01 M formic acid to 50% CH₃CN-50% 0.01 M formic acid at 0.3 mL/min over 70 min, then to 80% CH₃CN-20% over 10 min

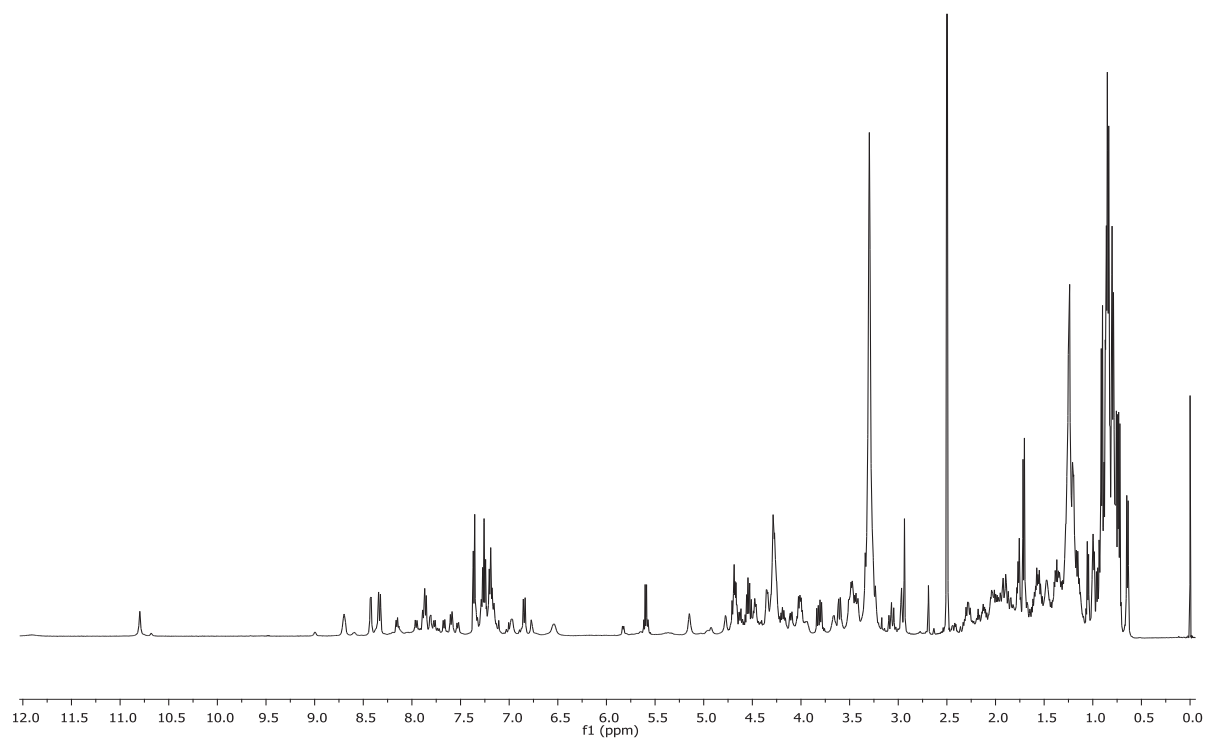
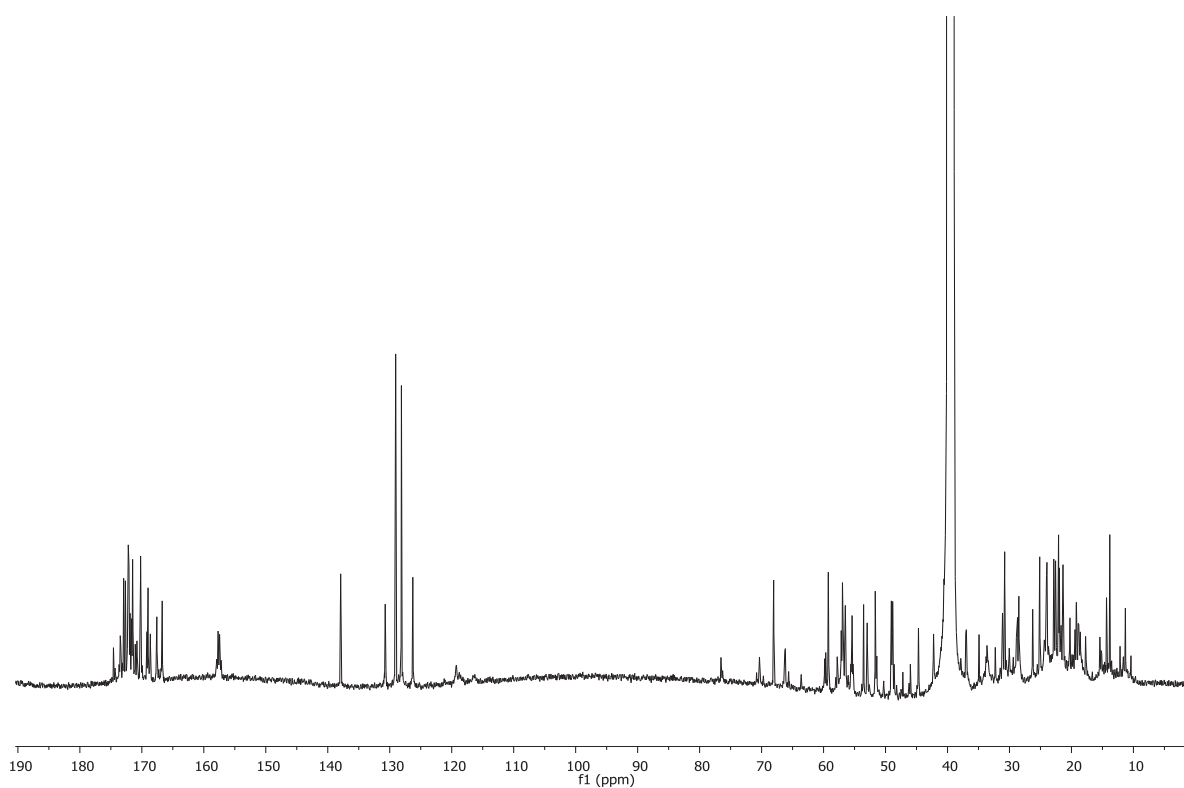
Figure S4. 26. ^1H -NMR spectrum of [L-Val⁸]Iaxaphycin A (**4**) in DMSO (303K)**Figure S4. 27.** ^{13}C -NMR spectrum of [L-Val⁸]Iaxaphycin A (**4**) in DMSO (303K)

Figure S4. 28. HSQC spectrum of [L-Val⁸]Iaxaphycin A (**4**) in DMSO (303K)

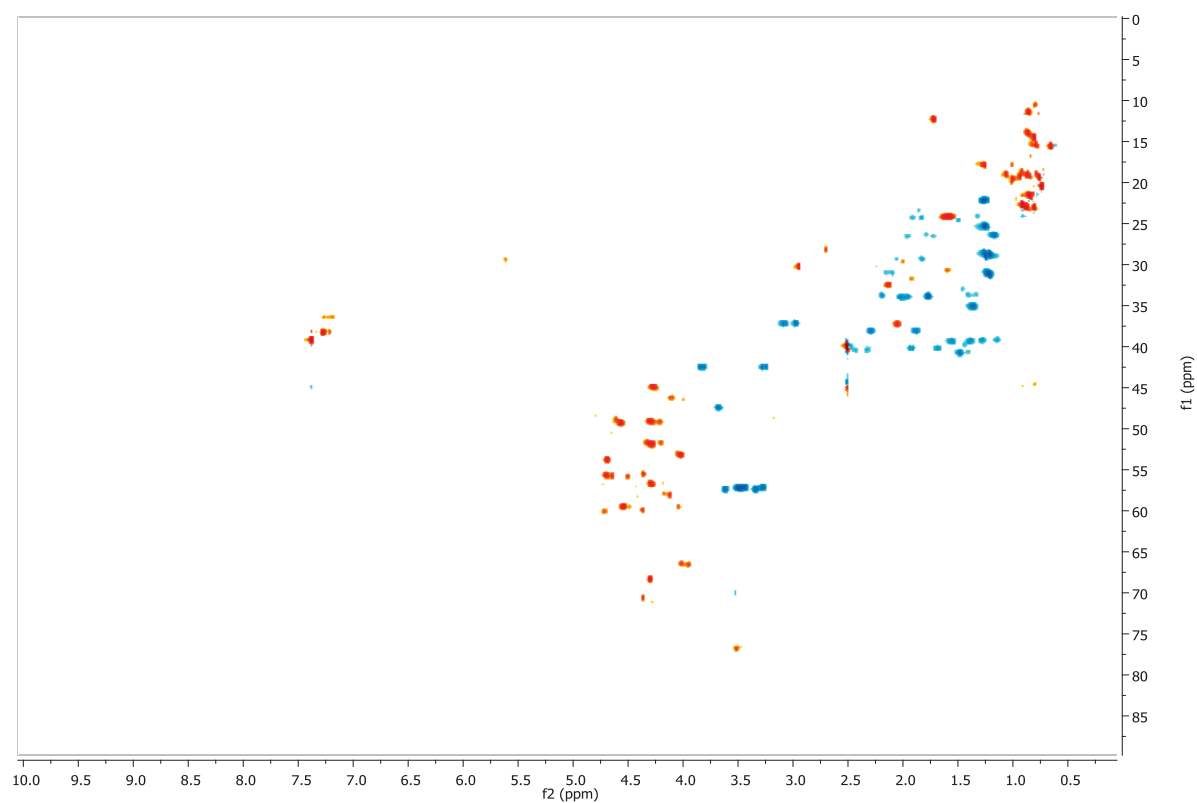


Figure S4. 29. TOCSY spectrum of [L-Val⁸]Iaxaphycin A (**4**) in DMSO (303K)

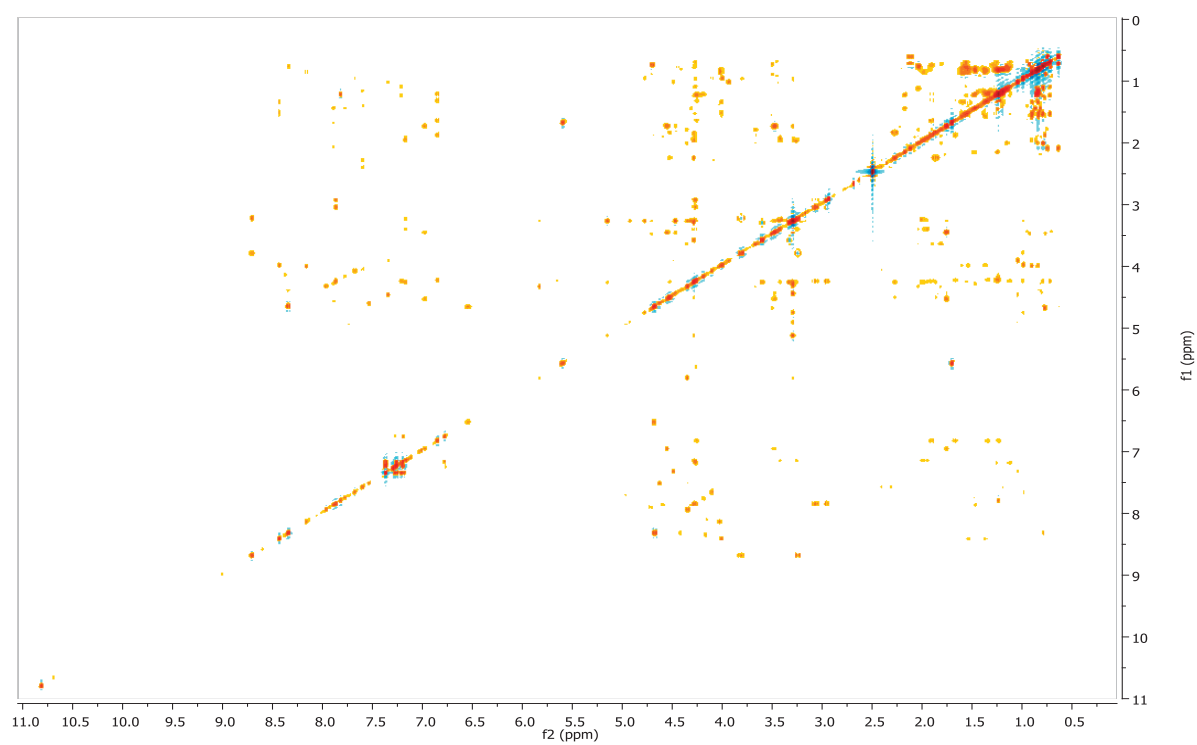


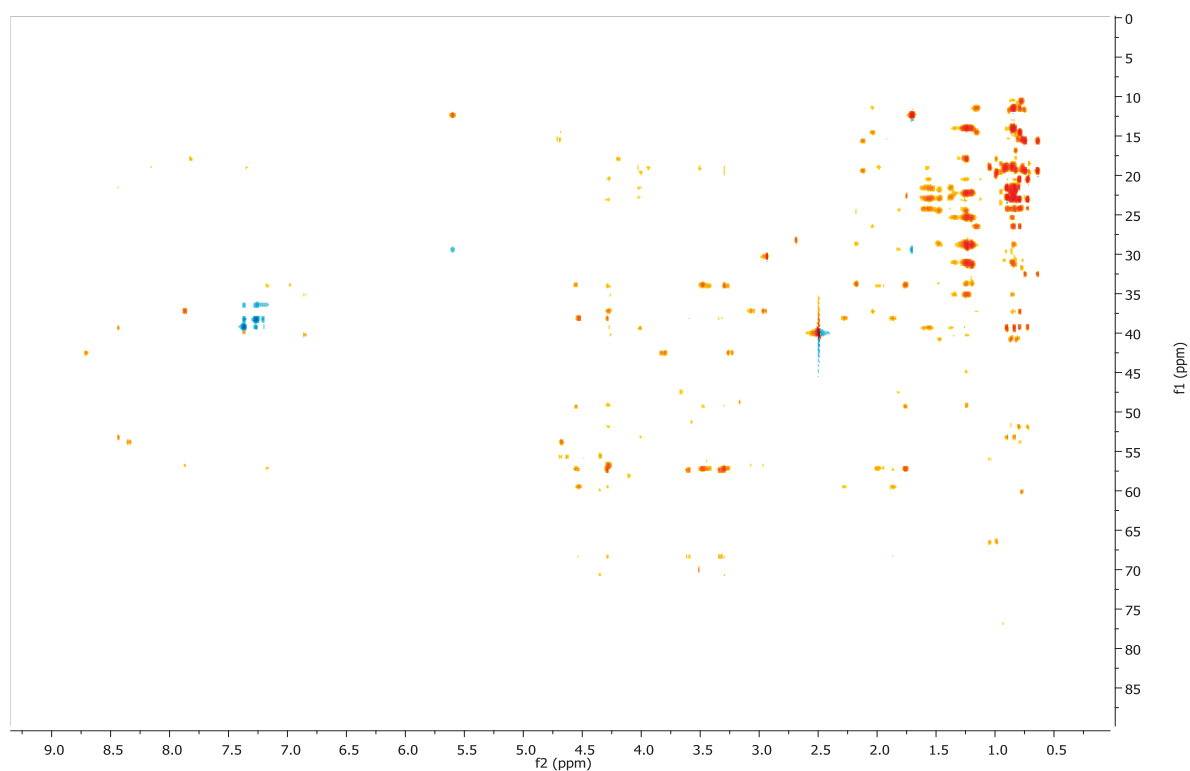
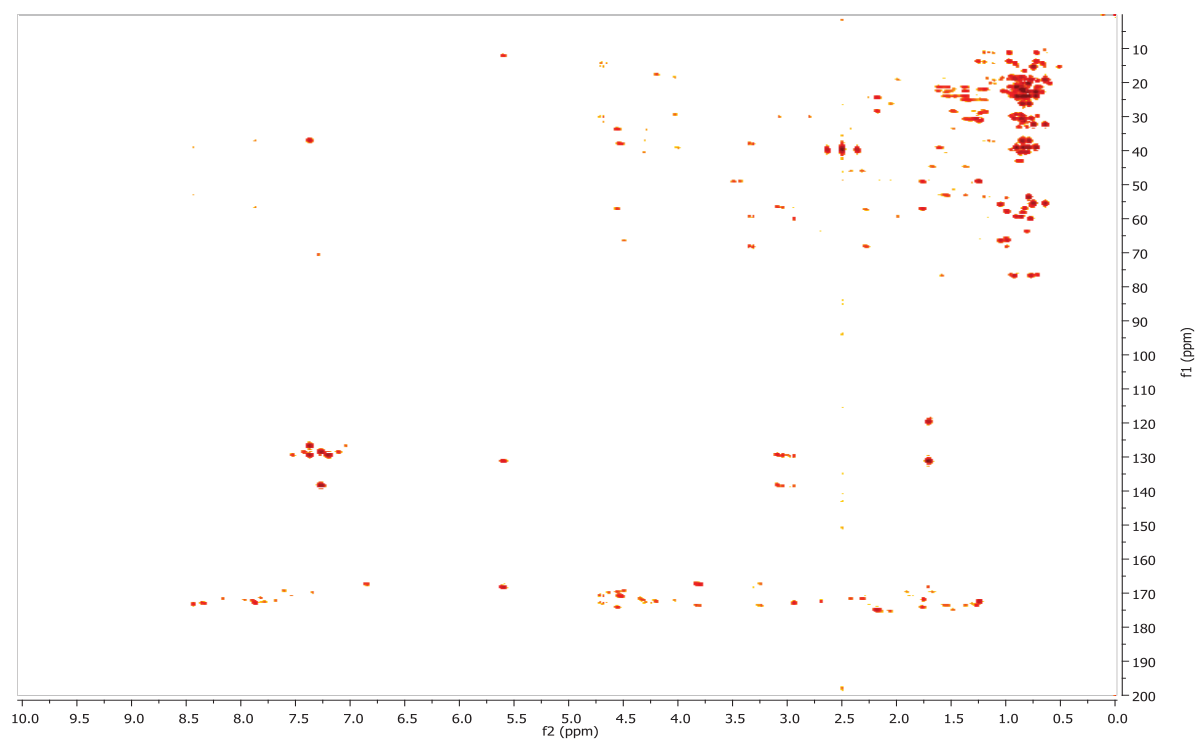
Figure S4. 30. HSQC-TOCSY spectrum of [L-Val⁸]Iaxaphycin A (**4**) in DMSO (303K)**Figure S4. 31.** HMBC spectrum of [L-Val⁸]Iaxaphycin A (**4**) in DMSO (303K)

Figure S4. 32. ROESY spectrum of [L-Val⁸]laxaphycin A (**4**) in DMSO (303K)

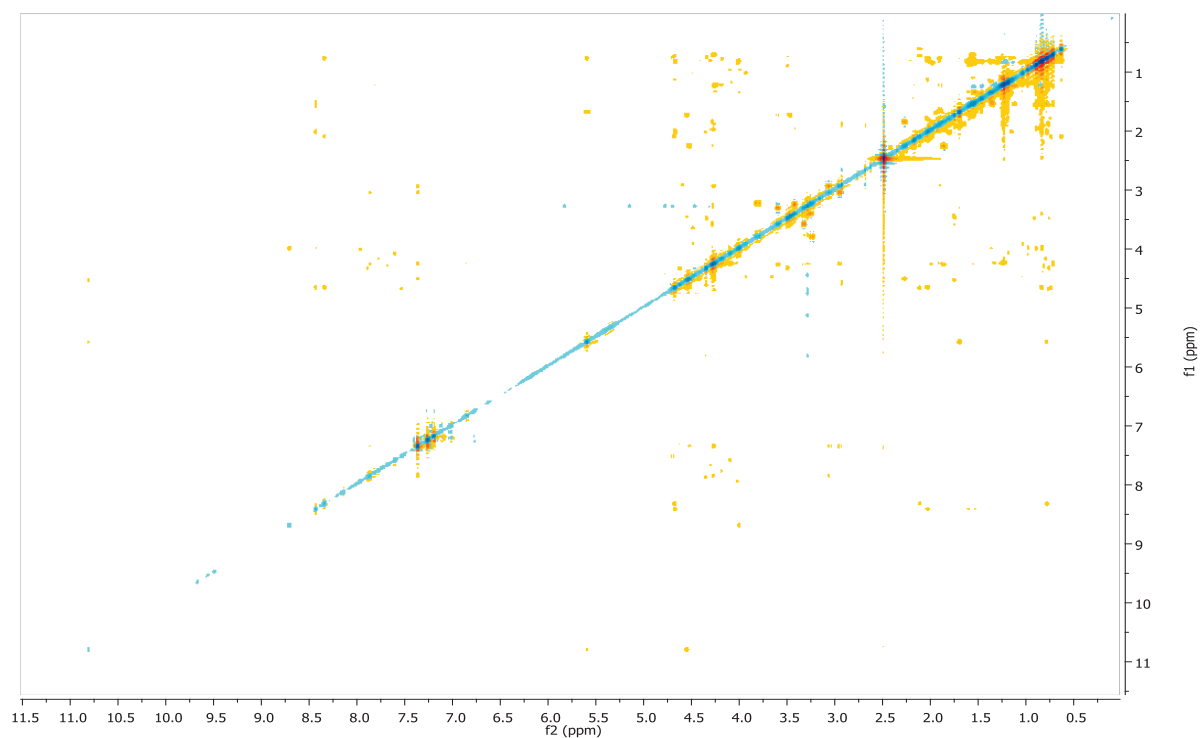


Figure S4. 33. Orbitrap MS/MS spectrum of [L-Val⁸]laxaphycin A (**4**)

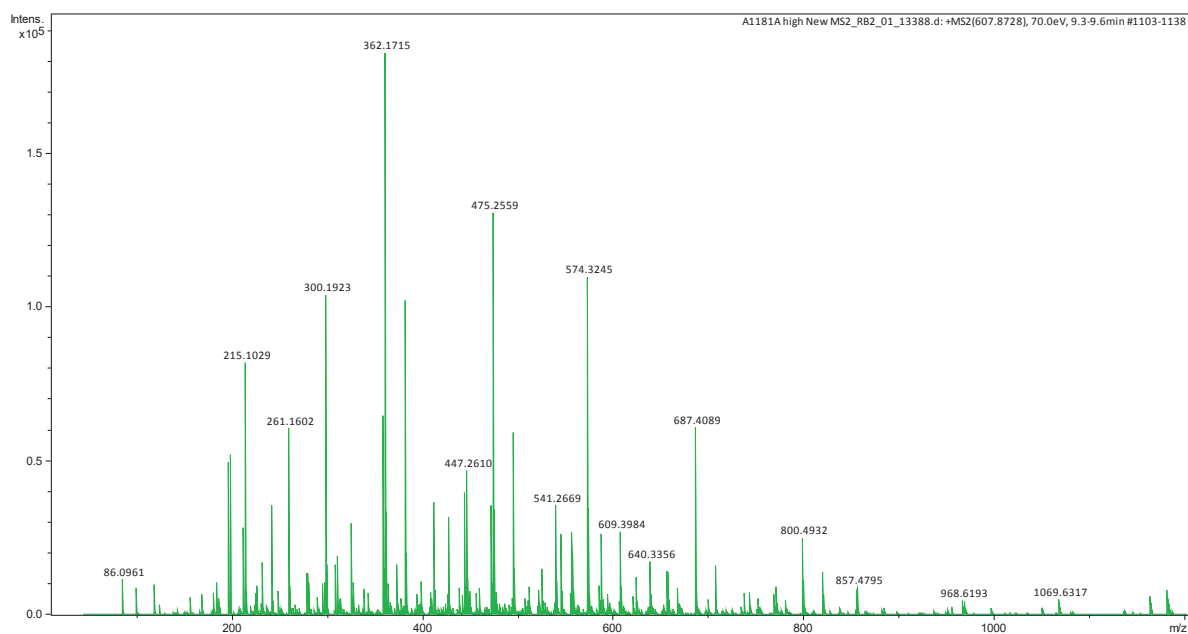


Figure S4. 34. Advanced Marfey's analysis of [L-Val⁸]Iaxaphycin A (**4**)

[L-Val⁸]Iaxaphycin A			
		RT for each Marfey's derivatives (min)	Absolute configuration
Aoc	L-FDLA	54,46 (1)	3 <i>R</i>
	D-FDLA	42,97 (1)	
Hse	L-FDLA	35,33 (2)	2x L (2 <i>S</i>)
	D-FDLA	37,96 (2)	
Hyp	L-FDLA	30,43 (2)	L (2 <i>S</i>)
	D-FDLA	29,46 (2)	
Phe	L-FDLA	65,09 (2)	D (2 <i>R</i>)
	D-FDLA	53,88 (2)	
Leu	L-FDLA	66,28 (2)	D (2 <i>R</i>)
	D-FDLA	52,61 (2)	
Val	L-FDLA	28,34 (1)	L (2 <i>S</i>)
	D-FDLA	40,46 (1)	
Ile	L-FDLA	65,55 (2)	(2 <i>R</i> ,3 <i>S</i>)
	D-FDLA	51,50 (2)	
Leu	L-FDLA	52,59 (2)	L (2 <i>S</i>)
	D-FDLA	66,34 (2)	

(1) Gradient 1: from 20% CH₃CN-...% 0.01 M formic acid to 60% CH₃CN-40% 0.01 M formic acid at 0.3 mL/min over 70 min

(2) Gradient 2: from 10% CH₃CN-...% 0.01 M formic acid to 50% CH₃CN-50% 0.01 M formic acid at 0.3 mL/min over 70 min, then to 80% CH₃CN-20% over 10 min

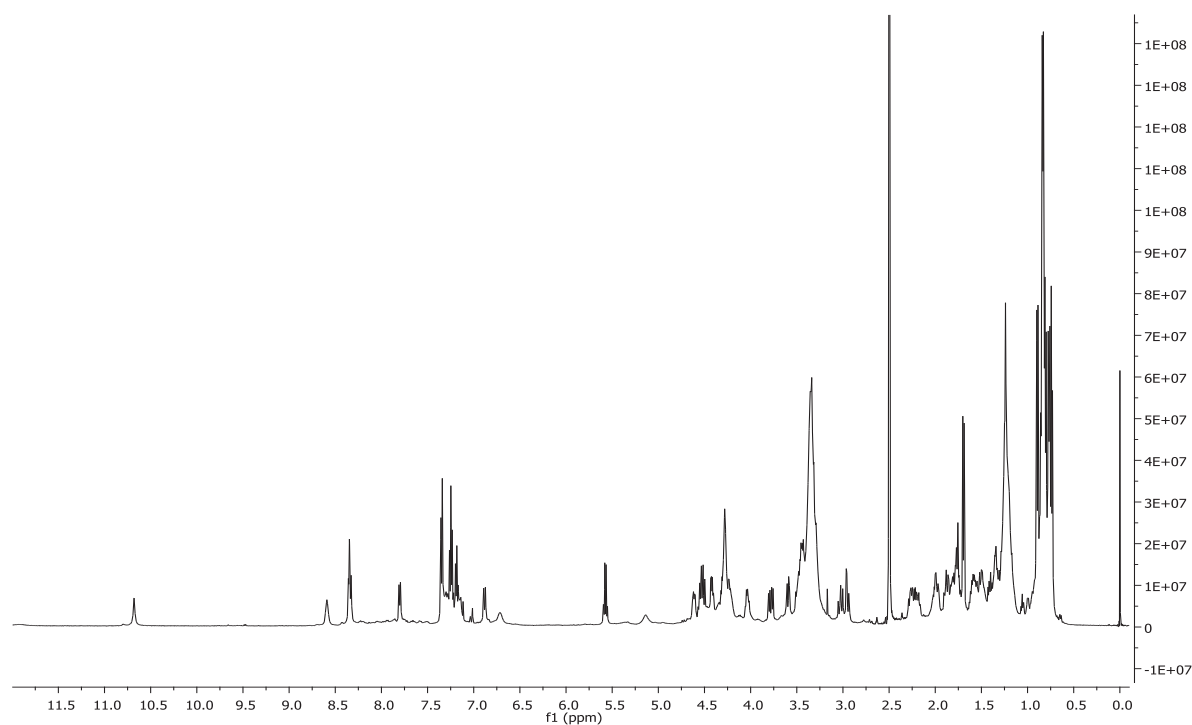
Figure S4. 35. ¹H-NMR spectrum of [D-Val⁹]Iaxaphycin A (**5**) in DMSO (303K)

Figure S4. 36. ^{13}C -NMR spectrum of [D-Val⁹]laxaphycin A (**5**) in DMSO (303K)

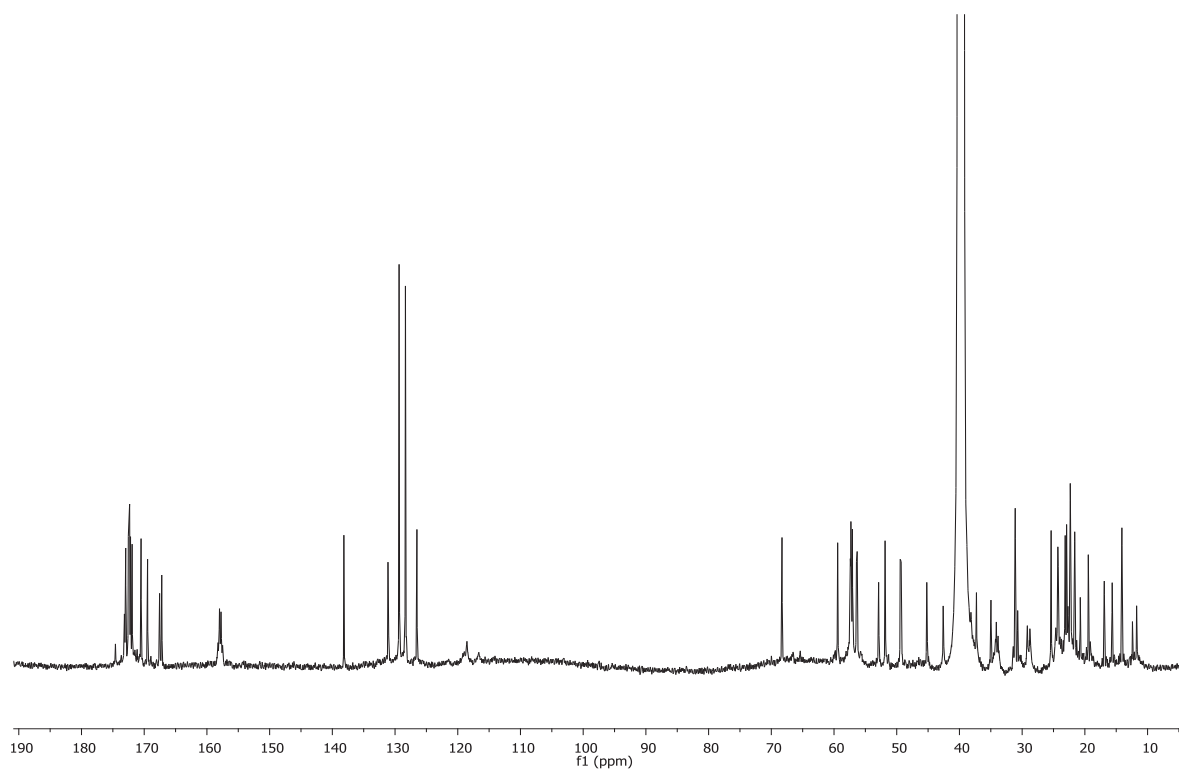


Figure S4. 37. HSQC spectrum of [D-Val⁹]laxaphycin A (**5**) in DMSO (303K)

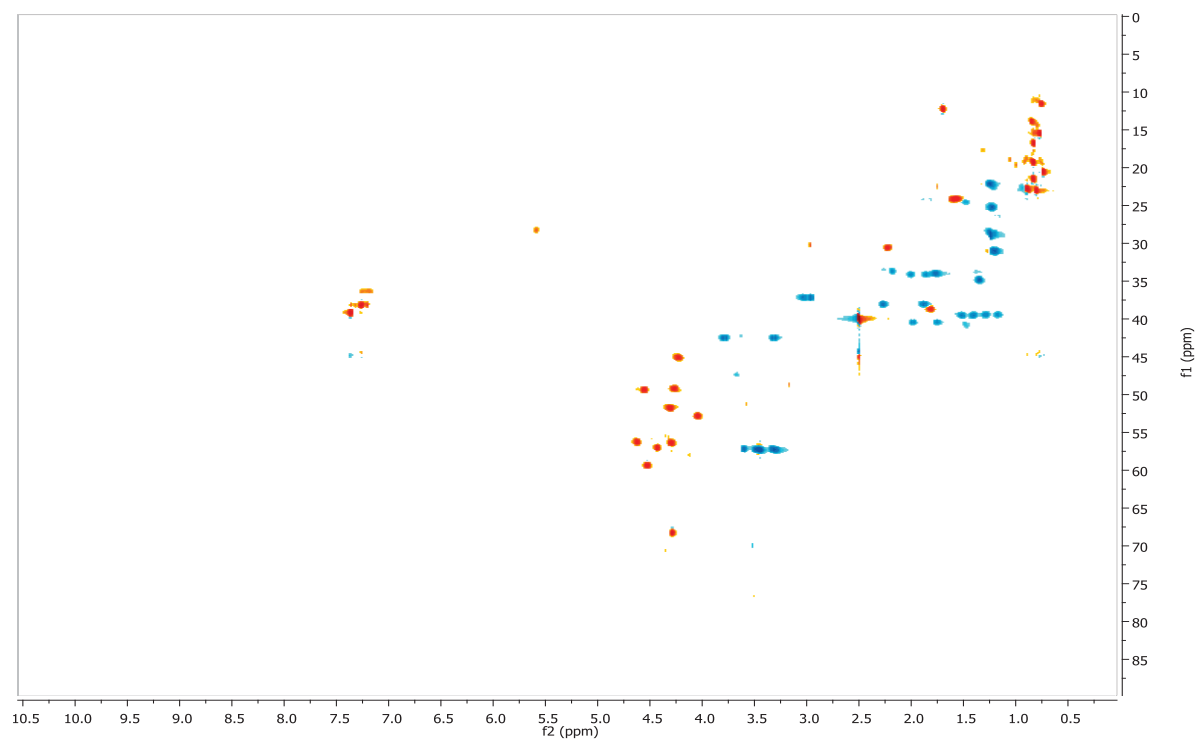


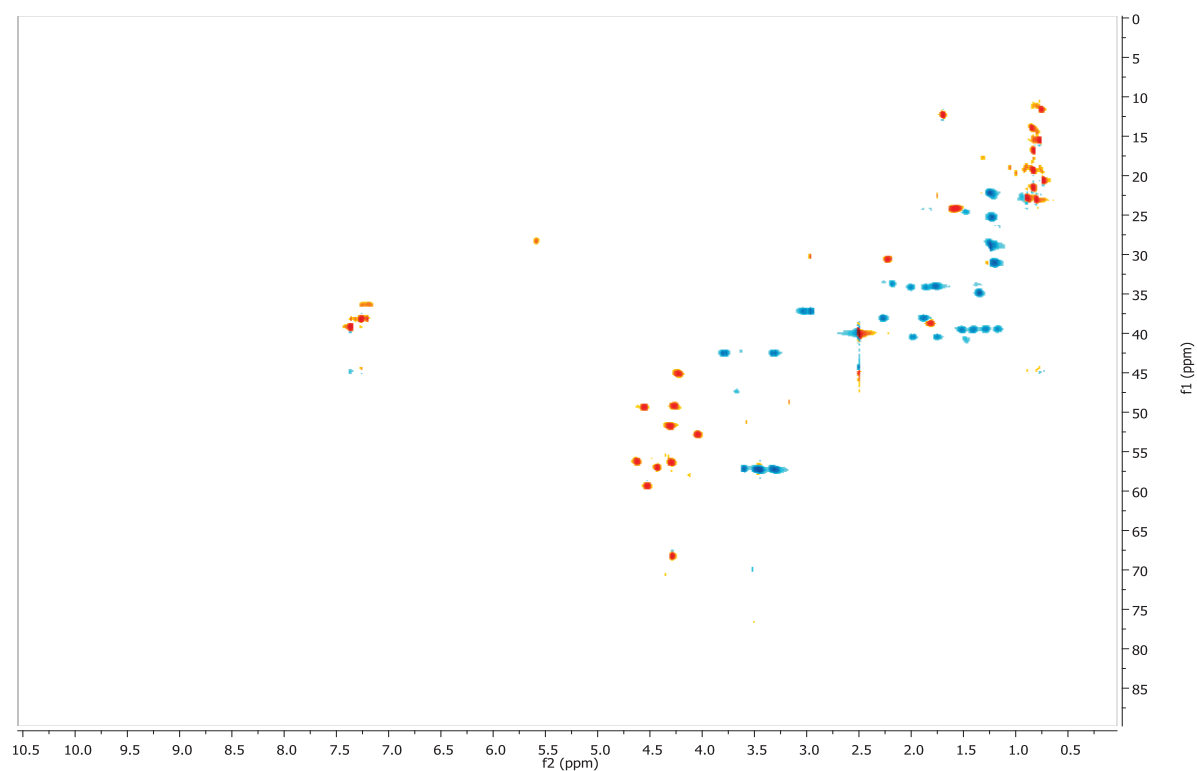
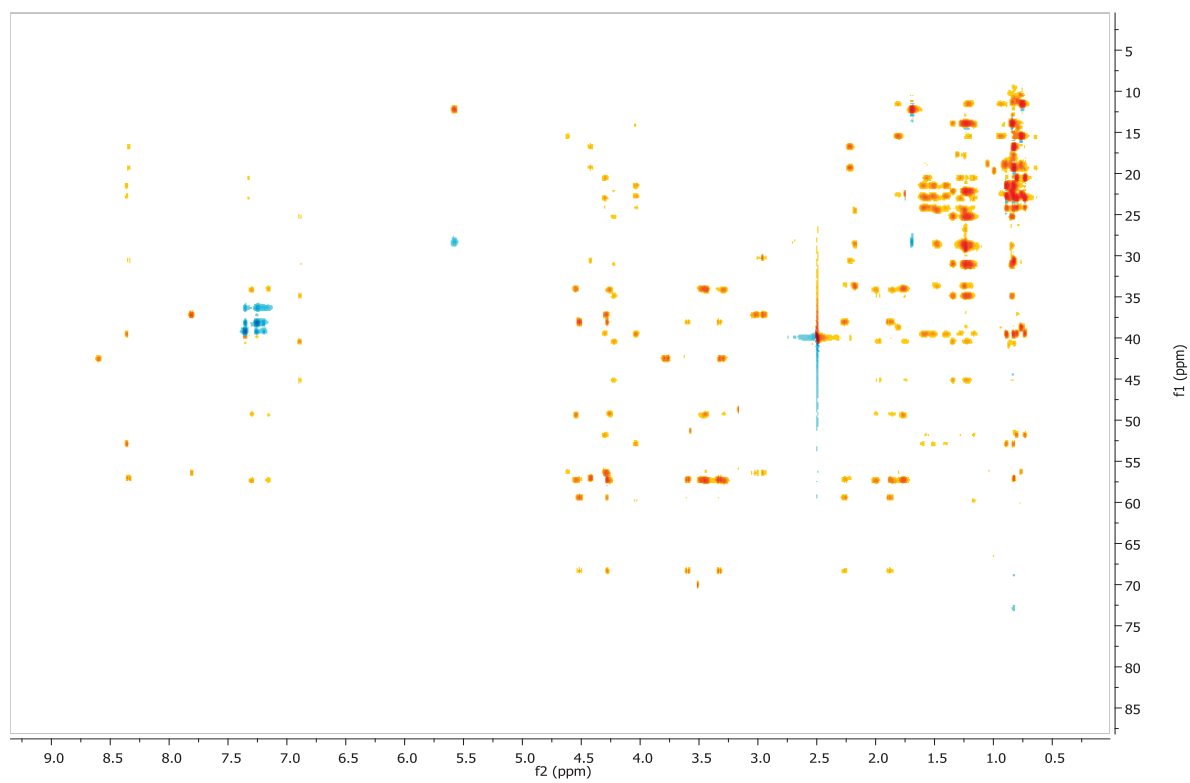
Figure S4. 38. TOCSY spectrum of [D-Val⁹]laxaphycin A (**5**) in DMSO (303K)**Figure S4. 39.** HSQC-TOCSY spectrum of [D-Val⁹]laxaphycin A (**5**) in DMSO (303K)

Figure S4. 40. HMBC spectrum of [D-Val⁹]laxaphycin A (**5**) in DMSO (303K)

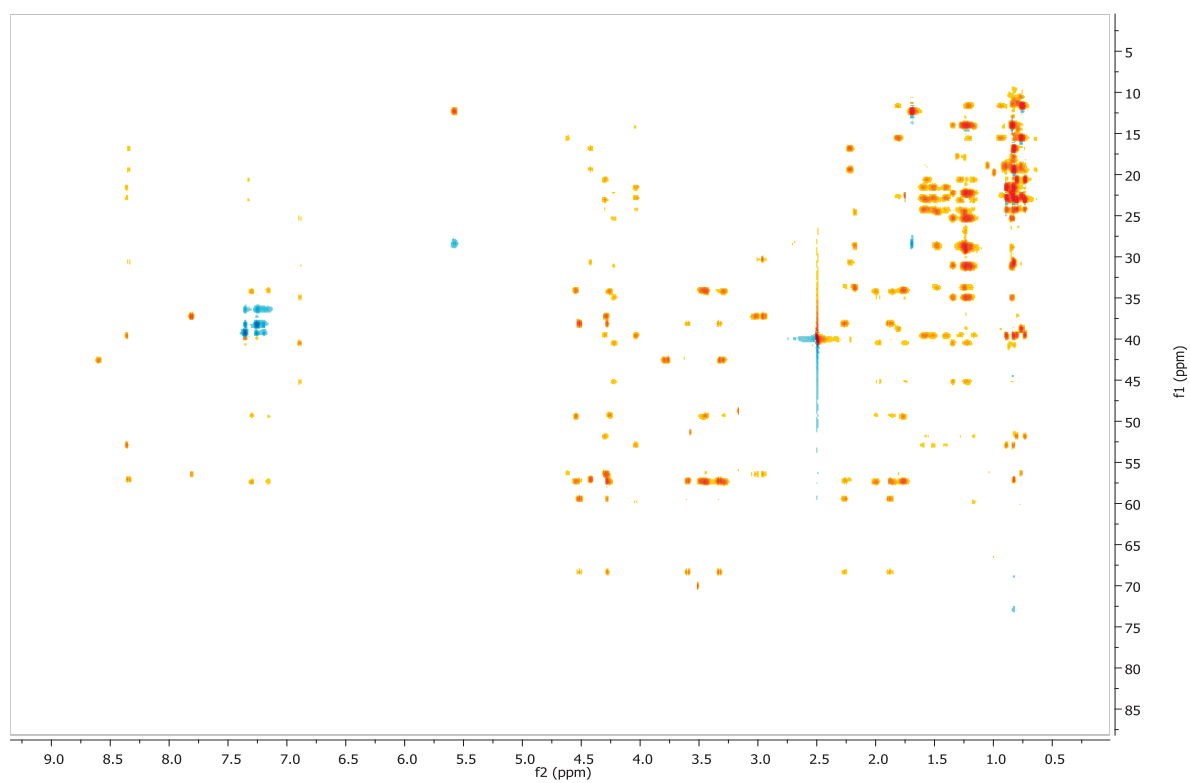


Figure S4. 41. ROESY spectrum of [D-Val⁹]laxaphycin A (**5**) in DMSO (303K)

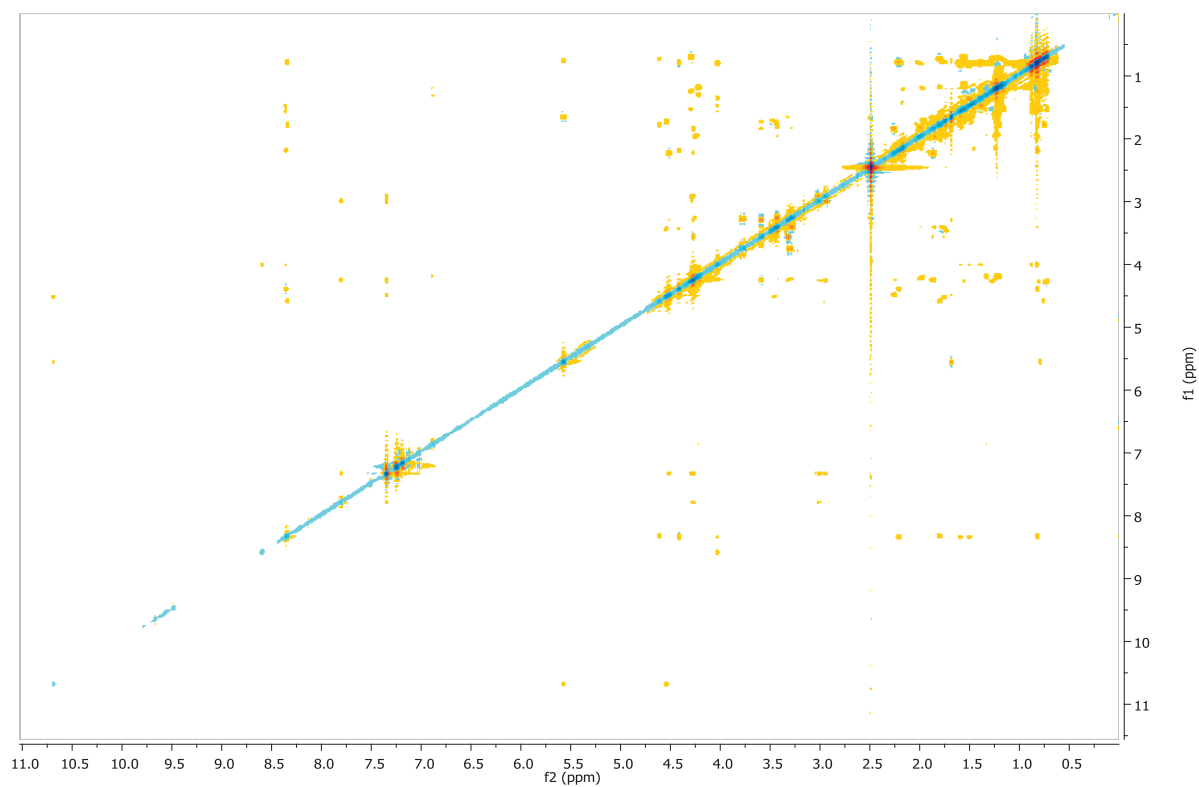
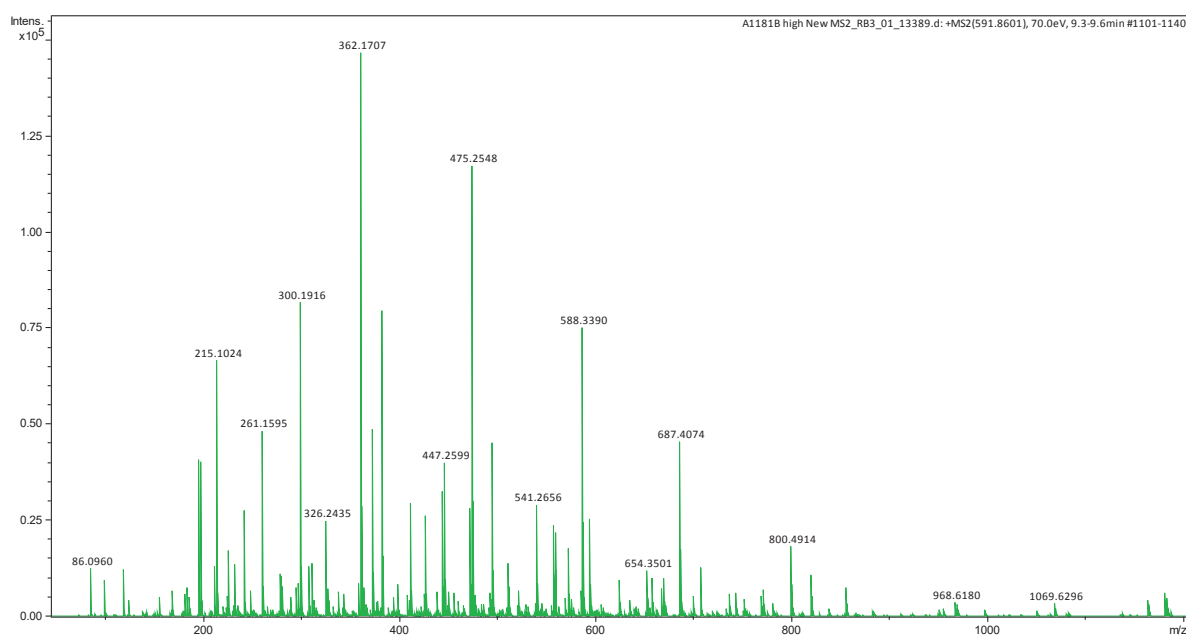


Figure S4. 42. Orbitrap MS/MS spectrum of [D-Val⁹]Iaxaphycin A (5)**Figure S4. 43.** Advanced Marfey's analysis of [D-Val⁹]Iaxaphycin A (5)

[D-Val⁹]Iaxaphycin A			
		RT for each Marfey's derivatives (min)	Absolute configuration
Aoc	L-FDLA	54,48 (1)	3R
	D-FDLA	43,02 (1)	
Hse	L-FDLA	35,38 (2)	2x L (2S)
	D-FDLA	37,98 (2)	
Hyp	L-FDLA	30,38 (2)	L (2S)
	D-FDLA	29,37 (2)	
Phe	L-FDLA	65,13 (2)	D (2R)
	D-FDLA	53,90 (2)	
Leu	L-FDLA	66,33(2)	D (2R)
	D-FDLA	52,67 (2)	
Ile	L-FDLA	51,83 (2)	(2R,3S)
	D-FDLA	65,42 (2)	
Val	L-FDLA	40,48 (1)	D (2R)
	D-FDLA	28,35 (1)	
Leu	L-FDLA	52,58 (2)	L (2S)
	D-FDLA	66,32 (2)	

(1) Gradient 1: from 20% CH₃CN-..% 0.01 M formic acid to 60% CH₃CN-40% 0.01 M formic acid at 0.3 mL/min over 70 min

(2) Gradient 2: from 10% CH₃CN-..% 0.01 M formic acid to 50% CH₃CN-50% 0.01 M formic acid at 0.3 mL/min over 70 min, then to 80% CH₃CN-20% over 10 min

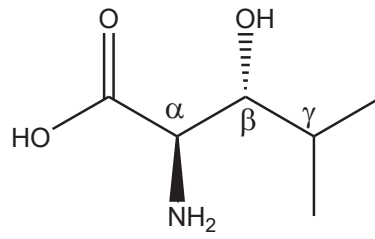
Figure S4. 44. Advanced Marfey's analysis of acyclolaxaphycin B (**6**) and acyclolaxaphycin B3 (**7**) and Newman projections analysis of hydroxyleucine residue

Acyclolaxaphycin B				Acyclolaxaphycin B3			
		RT for each Marfey's derivatives (min)	Absolute configuration			RT for each Marfey's derivatives (min)	Absolute configuration
Ade	L-FDLA	65,78 (1)	3R	Ade	L-FDLA	65,80 (1)	3R
	D-FDLA	54,08 (1)			D-FDLA	54,11 (1)	
Val	L-FDLA	28,37 (1)	L (2S)	Val	L-FDLA	28,40 (1)	L (2S)
	D-FDLA	40,46 (1)			D-FDLA	40,47 (1)	
Hle	L-FDLA	57,45 (2)	2x D (2R)	Hle	L-FDLA	57,49 (2)	2x D (2R)
	D-FDLA	40,60 (2)			D-FDLA	40,66 (2)	
Ala	L-FDLA	23,04 (1)	L (2S)	Ala	L-FDLA	23,03 (1)	L (2S)
	D-FDLA	29,49 (1)			D-FDLA	29,52 (1)	
Gln	L-FDLA	38,74 (2)	L (2S)	Gln	L-FDLA	38,77 (2)	L (2S)
	D-FDLA	41,61 (2)			D-FDLA	41,62 (2)	
N-Melle	L-FDLA	28,69 (1)	L (2S)	N-Melle	L-FDLA	28,73 (1)	L (2S)
	D-FDLA	46,71 (1)			D-FDLA	46,72 (1)	
Hasn	L-FDLA	28,37 (2)	(2R)	Hasn	L-FDLA	28,34 (2)	(2R)
	D-FDLA	33,39 (2)			D-FDLA	33,41 (2)	
Thr	L-FDLA	34,50 (2)	2x (2S,3R)	Thr	L-FDLA	34,47 (2)	2x (2S,3R)
	D-FDLA	43,78 (2)			D-FDLA	43,75 (2)	
Pro	L-FDLA	41,24 (2)	L (2S)	Hyp	L-FDLA	30,42 (2)	L (2S)
	D-FDLA	46,64 (2)			D-FDLA	29,46 (2)	
Leu	L-FDLA	66,33 (2)	D (2R)	Leu	L-FDLA	66,36 (2)	D (2R)
	D-FDLA	52,64 (2)			D-FDLA	52,61(2)	

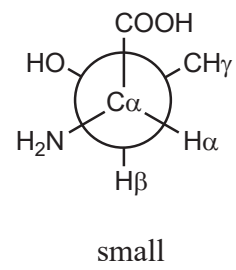
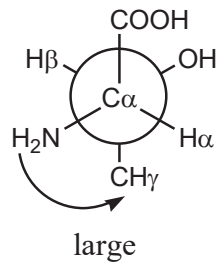
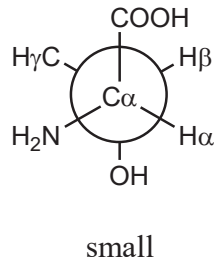
RT of Marfey's derivatives for each standard threonines (min)							
(2R,3R)-Thr	D-Thr	(2R,3S)-Thr	D-Allo-Thr	(2S,3R)-Thr	L-Allo-Thr	(2S,3S)-Thr	L-Thr
L-FDLA	43,73	L-FDLA	39,68	L-FDLA	36,38	L-FDLA	34,44
D-FDLA	34,48	D-FDLA	36,54	D-FDLA	39,57	D-FDLA	43,73

(1) Gradient 1: from 20% CH₃CN-..% 0.01 M formic acid to 60% CH₃CN-40% 0.01 M formic acid at 0.3 mL/min over 70 min

(2) Gradient 2: from 10% CH₃CN-..% 0.01 M formic acid to 50% CH₃CN-40% 0.01 M formic acid at 0.3 mL/min over 70 min, then to 80% CH₃CN-20% over 10 min



Hydroxyisoleucine (2R,3S)



Supporting information - Chapter 5

Figure S5. 1. ^1H -NMR spectrum of Laxaphycin B1195 in DMSO (303K)

Figure S5. 2. ^{13}C -NMR spectrum of Laxaphycin B1195 in DMSO (303K)

Figure S5. 3. HSQC spectrum of Laxaphycin B1195 in DMSO (303K)

Figure S5. 4. TOCSY spectrum of Laxaphycin B1195 in DMSO (303K)

Figure S5. 5. HMBC spectrum of Laxaphycin B1195 in DMSO (303K)

Figure S5. 6. ROESY spectrum of Laxaphycin B1195 in DMSO (303K)

Figure S5. 7. ^1H -NMR spectrum of Laxaphycin B1211 in DMSO (303K)

Figure S5. 8. ^{13}C -NMR spectrum of Laxaphycin B1211 in DMSO (303K)

Figure S5. 9. HSQC spectrum of Laxaphycin B1211 in DMSO (303K)

Figure S5. 10. TOCSY spectrum of Laxaphycin B1211 in DMSO (303K)

Figure S5. 11. HMBC spectrum of Laxaphycin B1211 in DMSO (303K)

Figure S5. 12. ROESY spectrum of Laxaphycin B1211 in DMSO (303K)

Figure S5. 13. ^1H -NMR spectrum of Laxaphycin B1212 in DMSO (303K)

Figure S5. 14. ^{13}C -NMR spectrum of Laxaphycin B1212 in DMSO (303K)

Figure S5. 15. HSQC spectrum of Laxaphycin B1212 in DMSO (303K)

Figure S5. 16. TOCSY spectrum of Laxaphycin B1212 in DMSO (303K)

Figure S5. 17. ^1H -NMR spectrum of Laxaphycin B1228 in DMSO (303K)

Figure S5. 18. ^{13}C -NMR spectrum of Laxaphycin B1228 in DMSO (303K)

Figure S5. 19. HSQC spectrum of Laxaphycin B1228 in DMSO (303K)

Figure S5. 20. TOCSY spectrum of Laxaphycin B1228 in DMSO (303K)

Figure S5. 21. Monitoring of the conversion of Laxaphycins B1212 and B1228 into Laxaphycins B1195 and B1211 in methanol

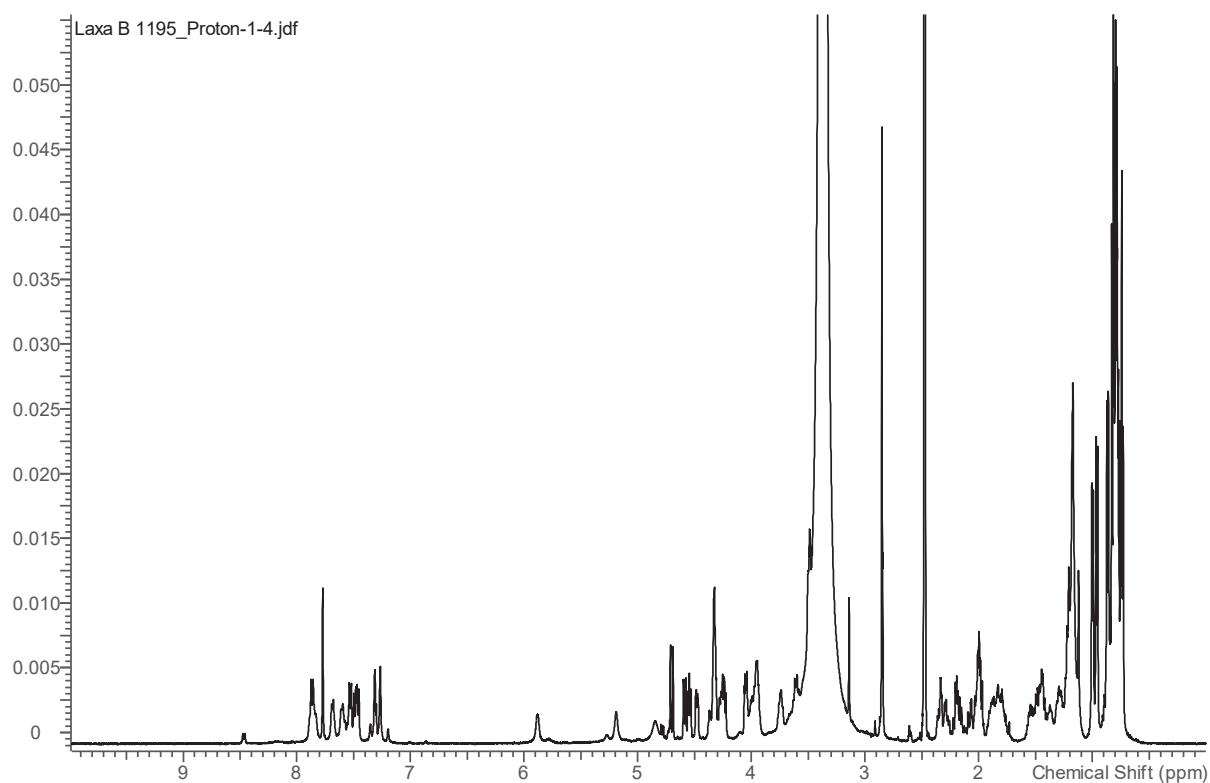
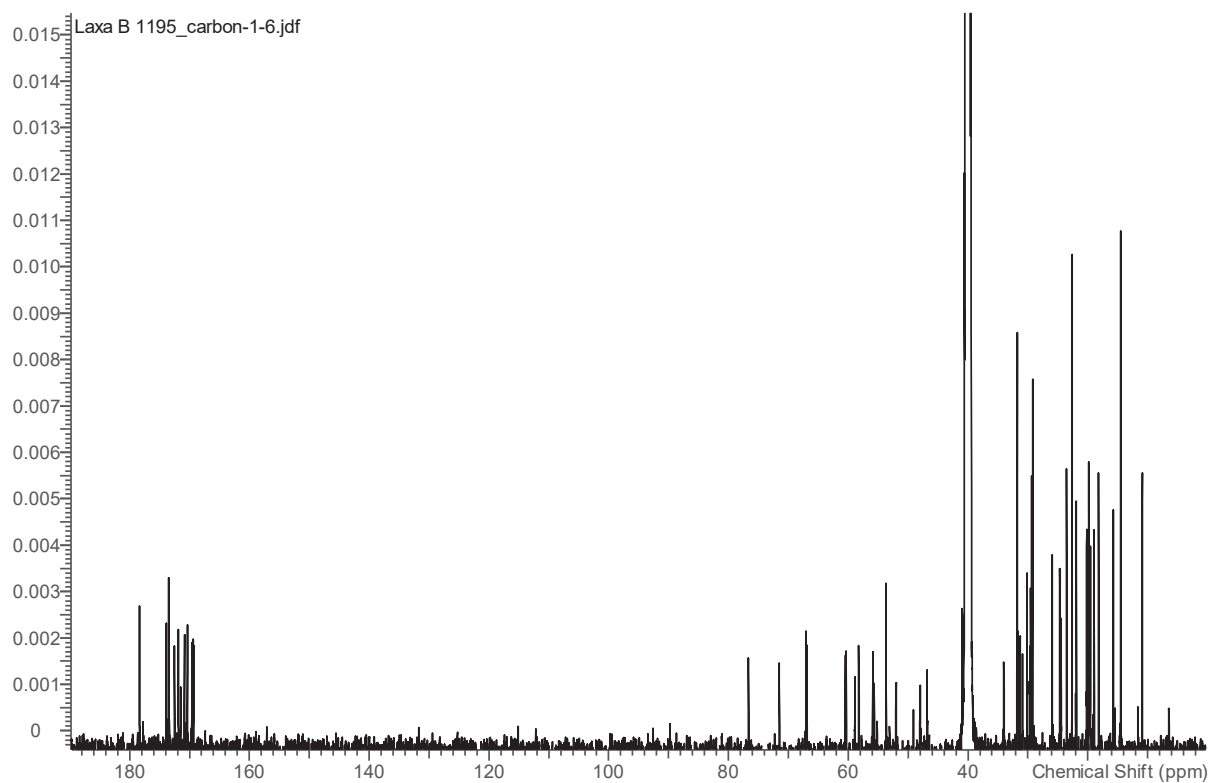
Figure S5. 1. $^1\text{H-NMR}$ spectrum of Laxaphycin B1211 in DMSO (303K)**Figure S5. 2.** $^{13}\text{C-NMR}$ spectrum of Laxaphycin B1211 in DMSO (303K)

Figure S5. 3. HSQC spectrum of Laxaphycin B1211 in DMSO (303K)

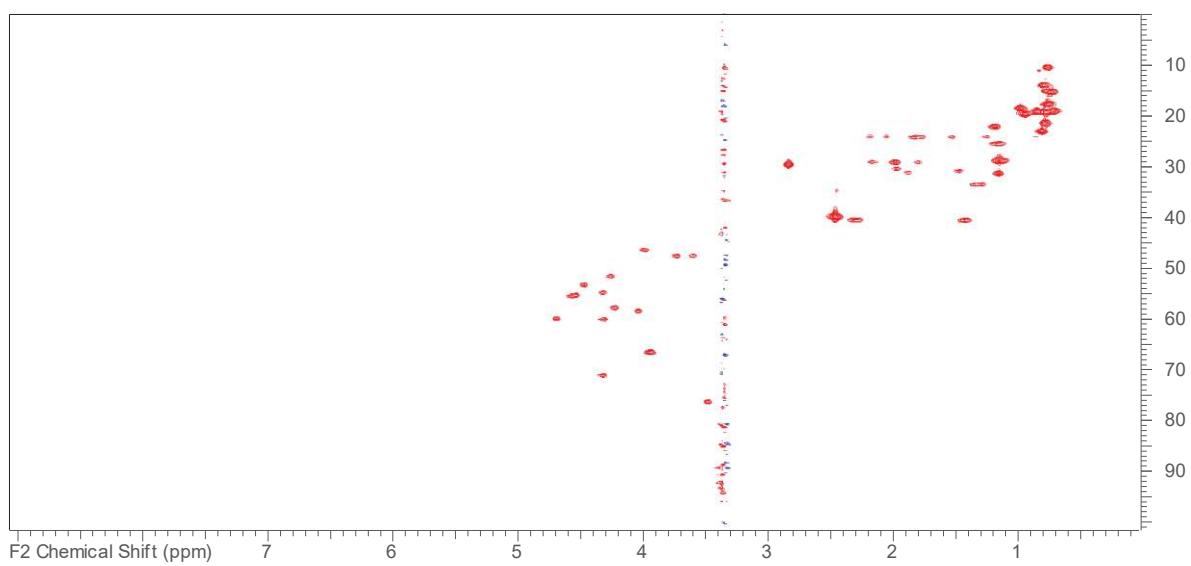


Figure S5. 4. TOCSY spectrum of Laxaphycin B1211 in DMSO (303K)

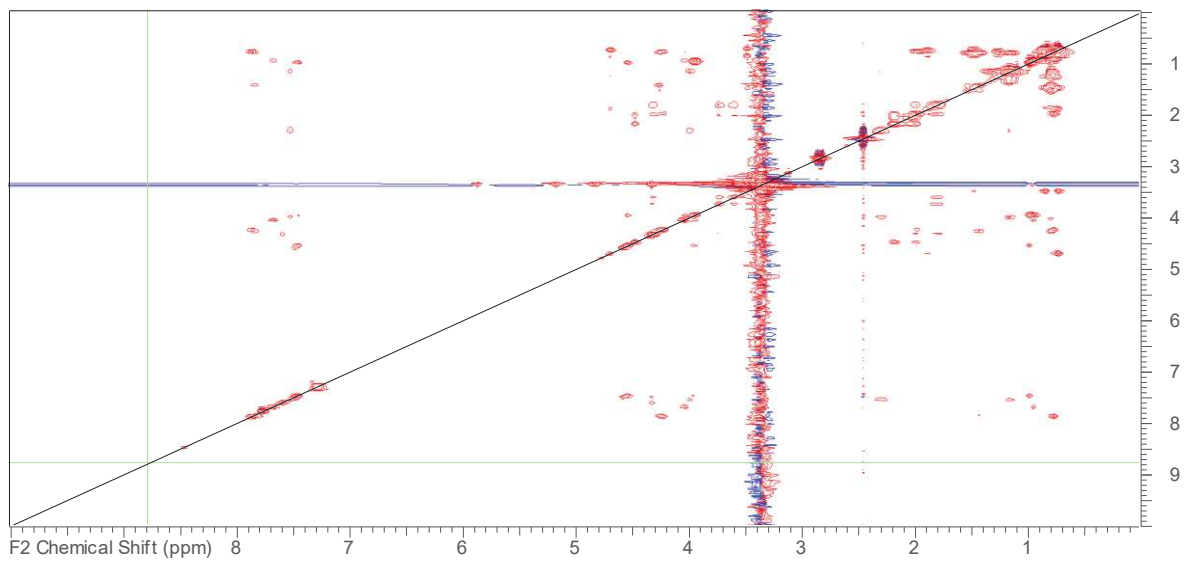


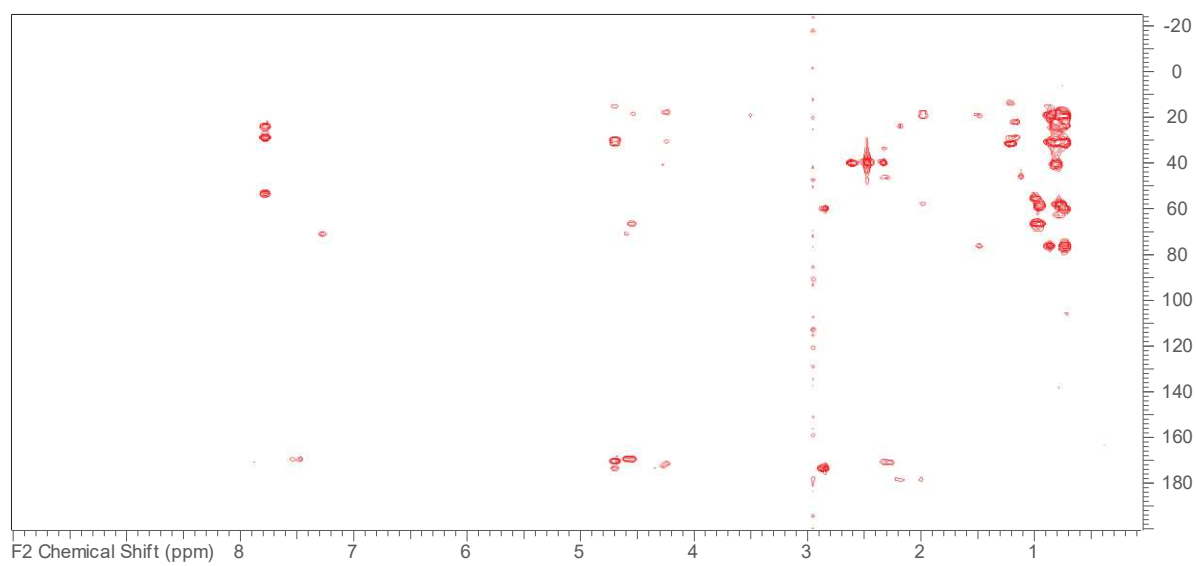
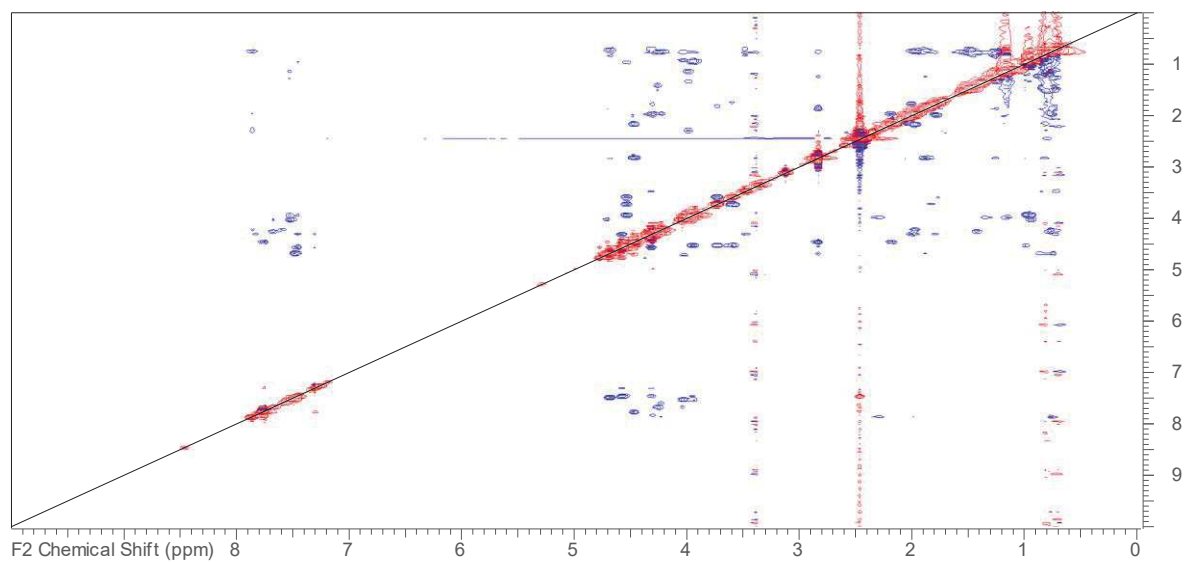
Figure S5. 5. HMBC spectrum of Laxaphycin B1211 in DMSO (303K)**Figure S5. 6.** ROESY spectrum of Laxaphycin B1211 in DMSO (303K)

Figure S5. 7. ^1H -NMR spectrum of Laxaphycin B1211 in DMSO (303K)

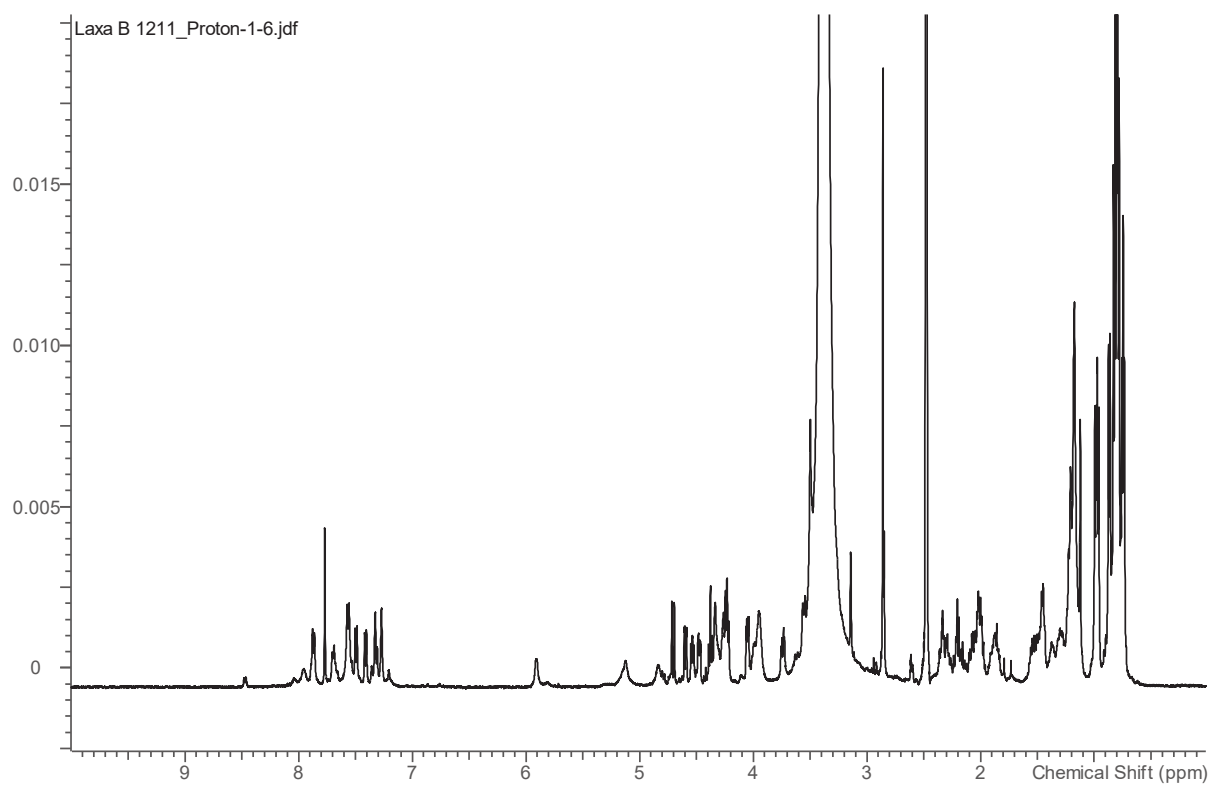


Figure S5. 8. ^{13}C -NMR spectrum of Laxaphycin B1211 in DMSO (303K)

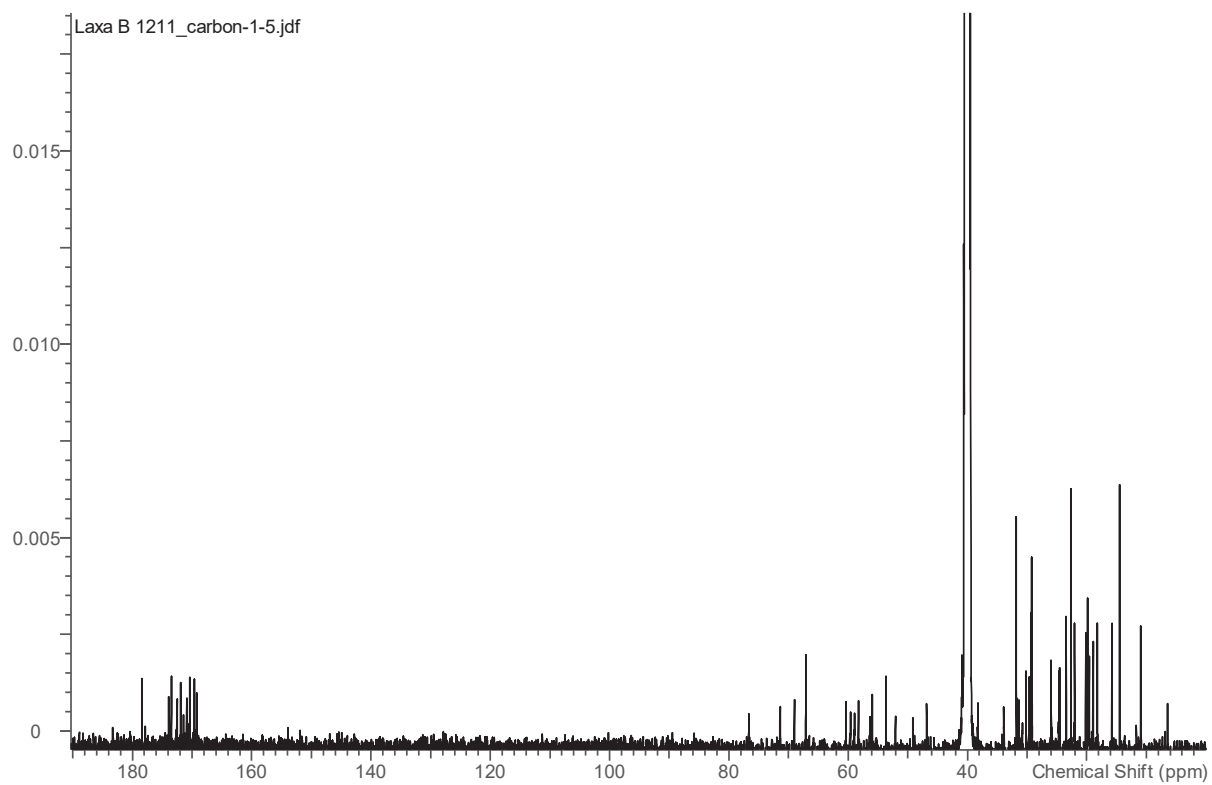


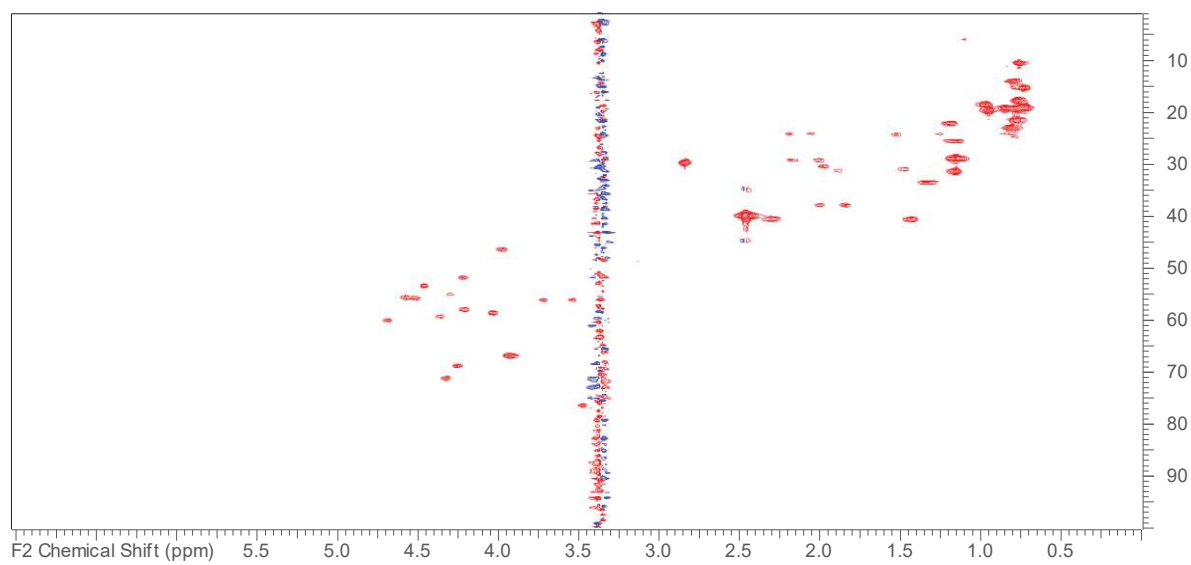
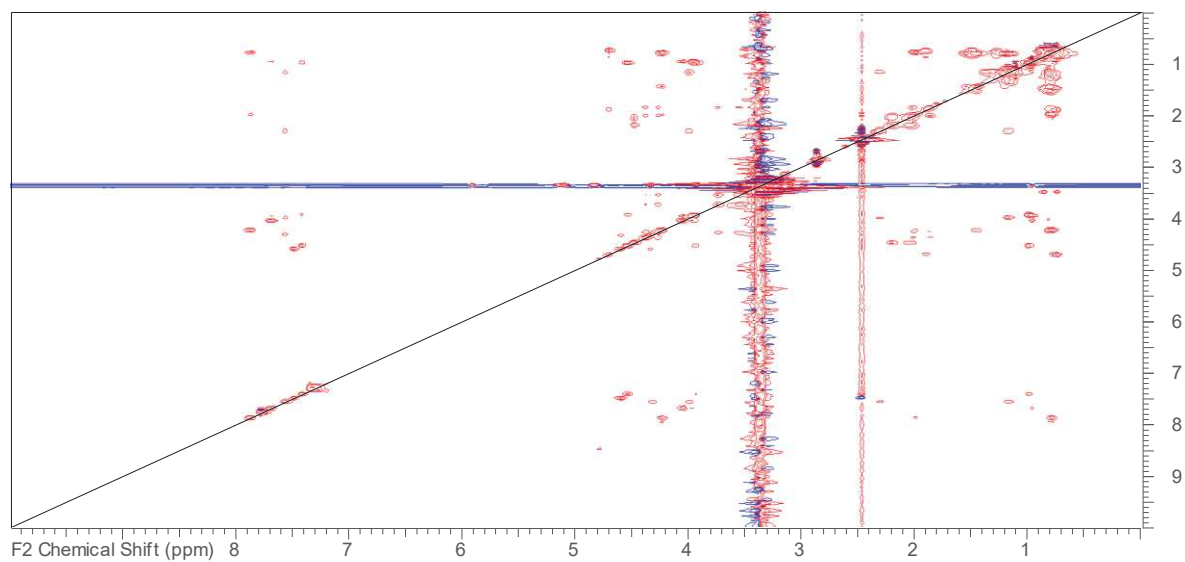
Figure S5. 9. HSQC spectrum of Laxaphycin B1211 in DMSO (303K)**Figure S5. 10.** TOCSY spectrum of Laxaphycin B1211 in DMSO (303K)

Figure S5. 11. HMBC spectrum of Laxaphycin B1211 in DMSO (303K)

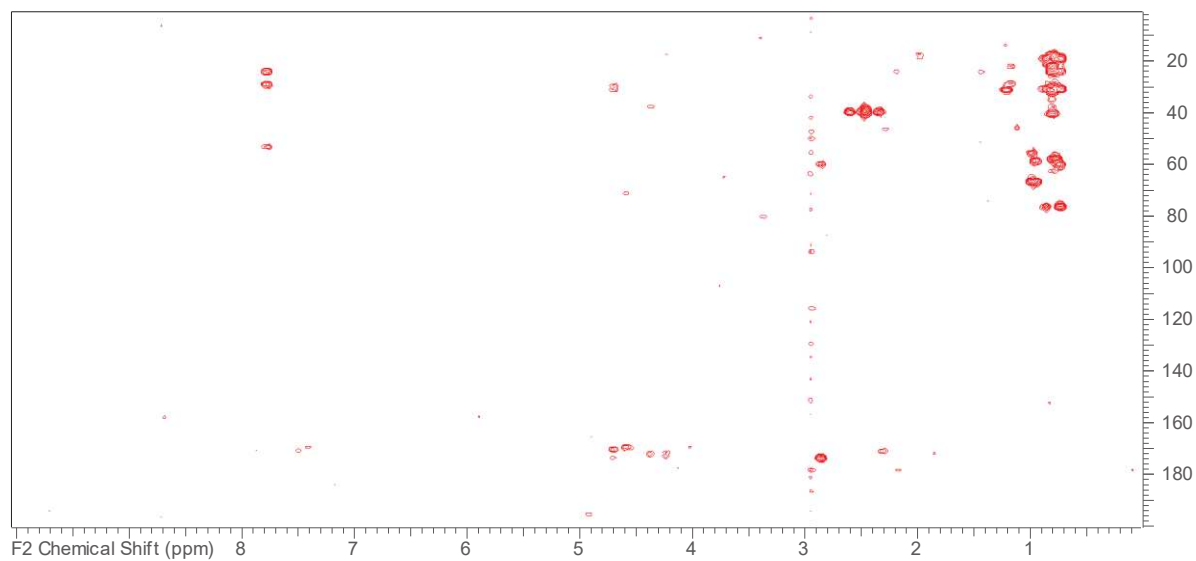


Figure S5. 12. ROESY spectrum of Laxaphycin B1211 in DMSO (303K)

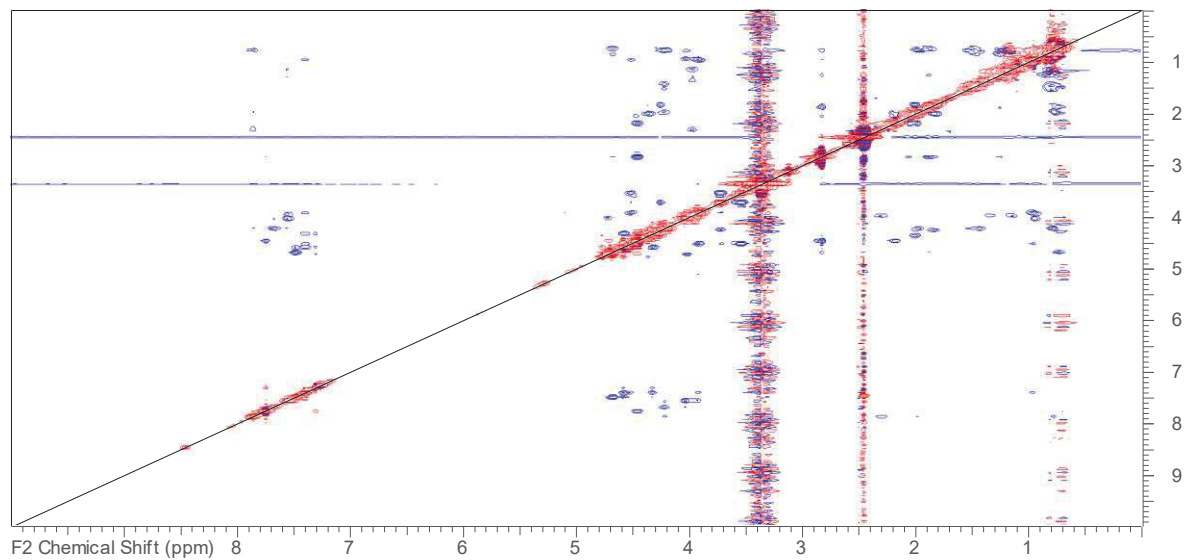


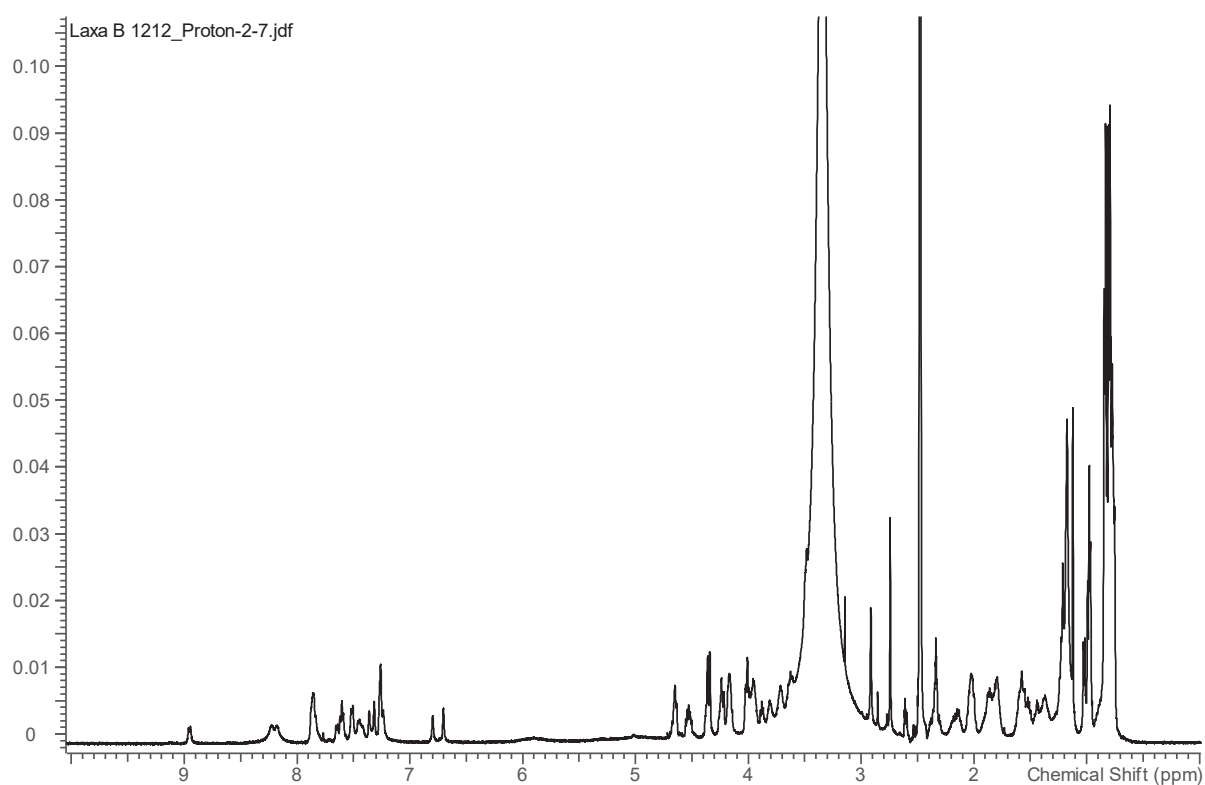
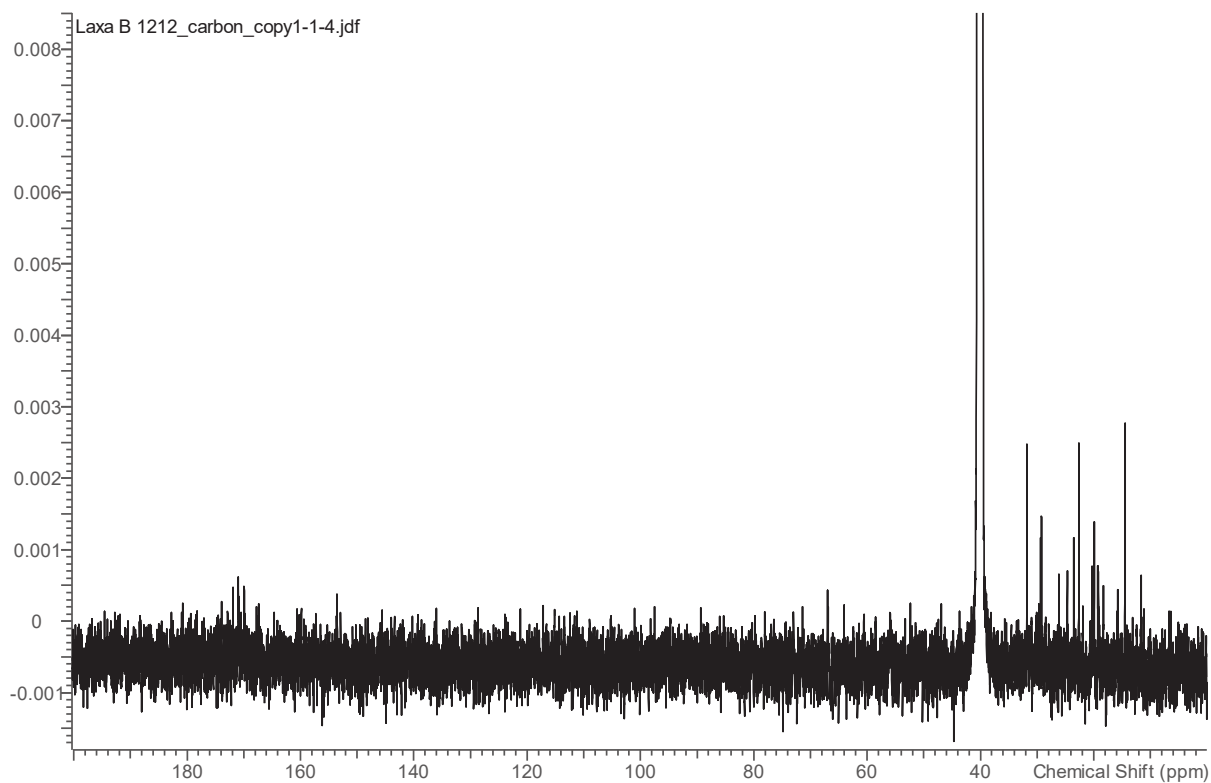
Figure S5. 13. ^1H -NMR spectrum of Laxaphycin B1212 in DMSO (303K)**Figure S5. 14.** ^{13}C -NMR spectrum of Laxaphycin B1212 in DMSO (303K)

Figure S5. 15. HSQC spectrum of Laxaphycin B1212 in DMSO (303K)

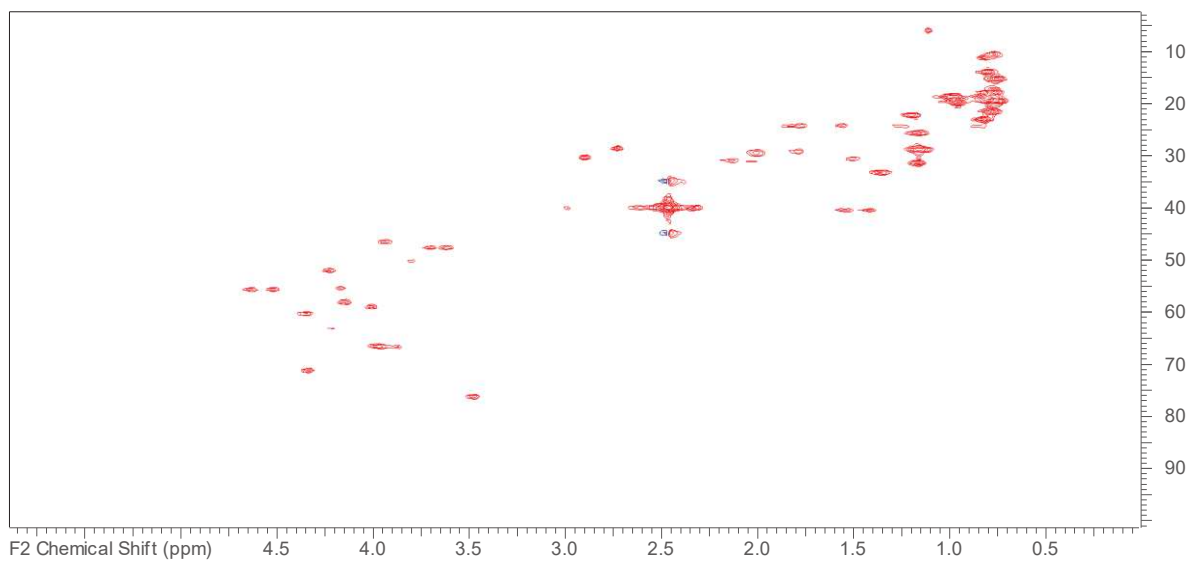


Figure S5. 16. TOCSY spectrum of Laxaphycin B1212 in DMSO (303K)

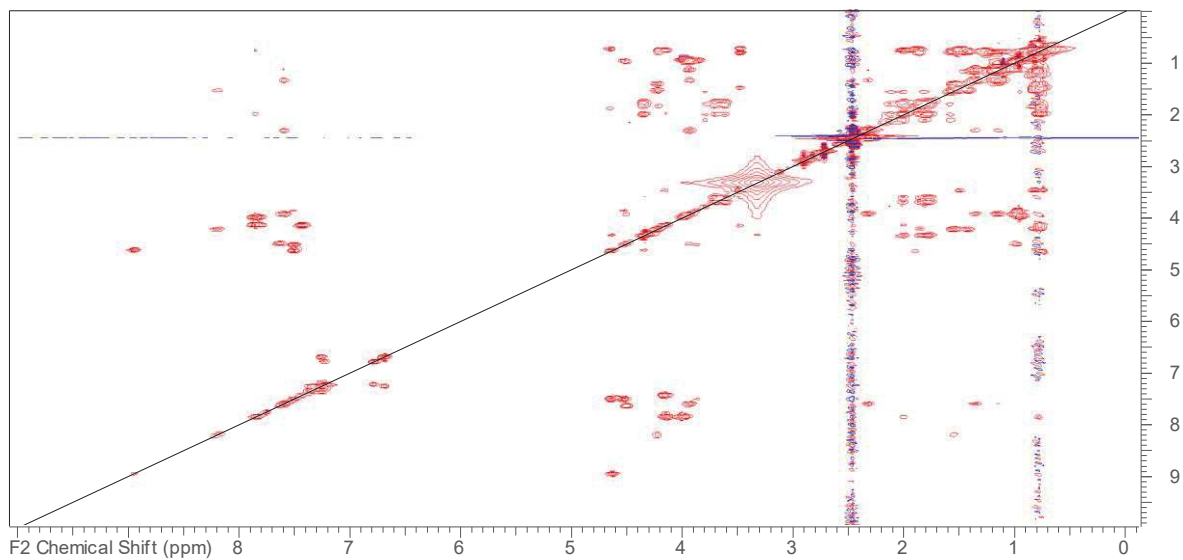


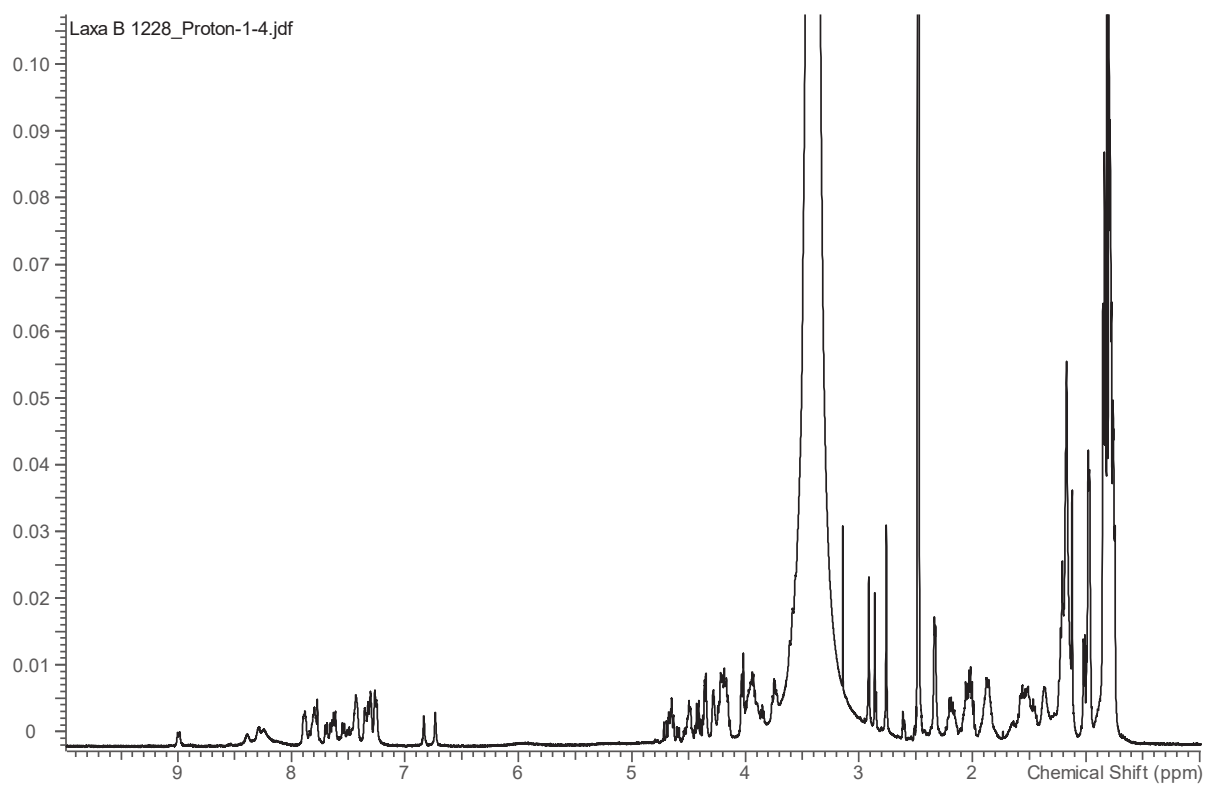
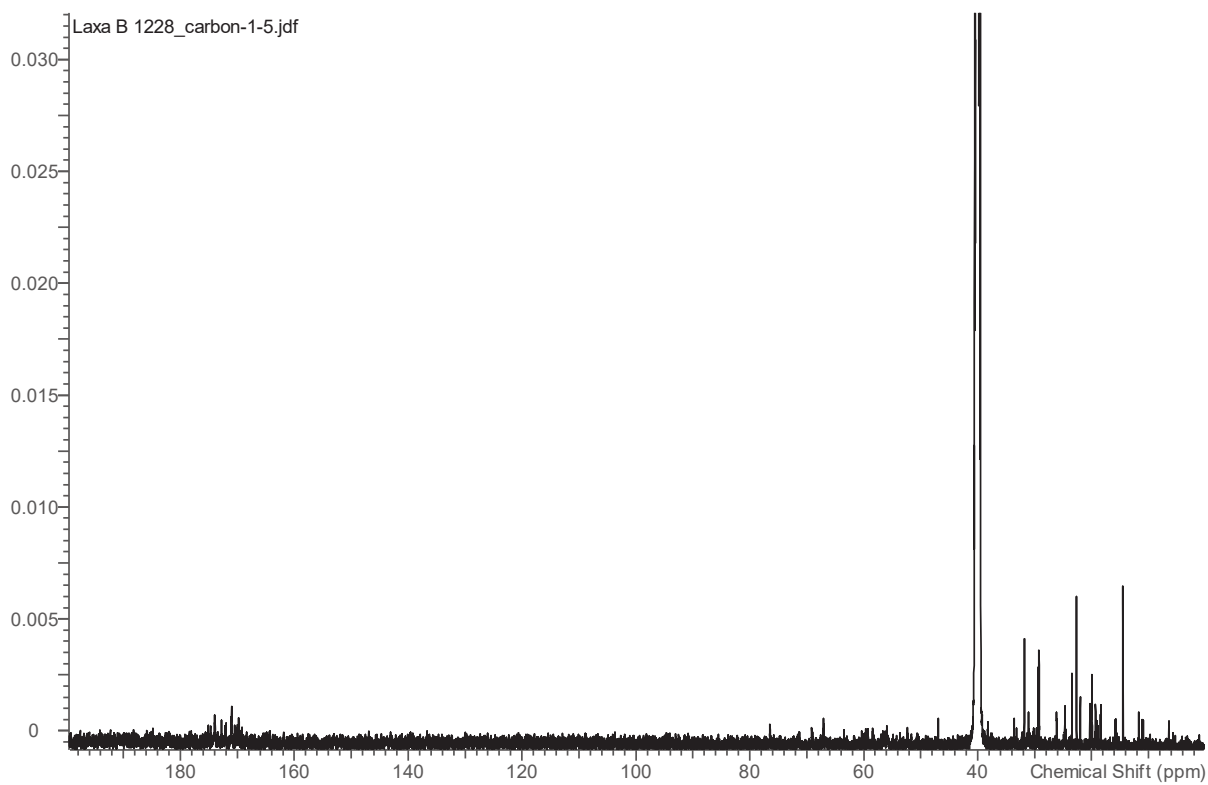
Figure S5. 17. ^1H -NMR spectrum of Laxaphycin B1228 in DMSO (303K)**Figure S5. 18.** ^{13}C -NMR spectrum of Laxaphycin B1228 in DMSO (303K)

Figure S5. 19. HSQC spectrum of Laxaphycin B1228 in DMSO (303K)

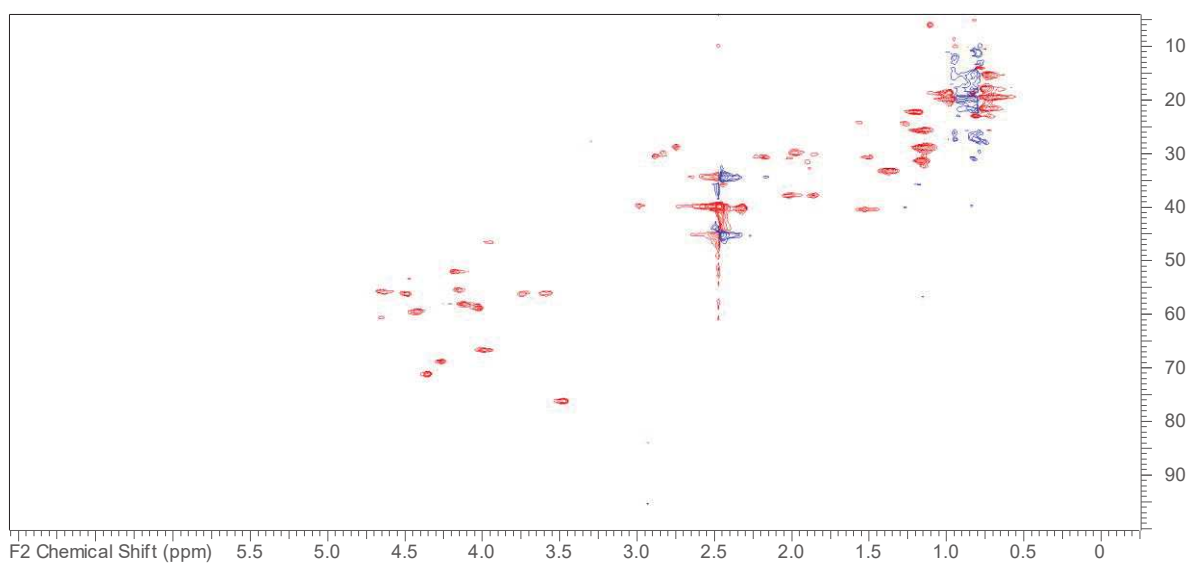


Figure S5. 20. TOCSY spectrum of Laxaphycin B1228 in DMSO (303K)

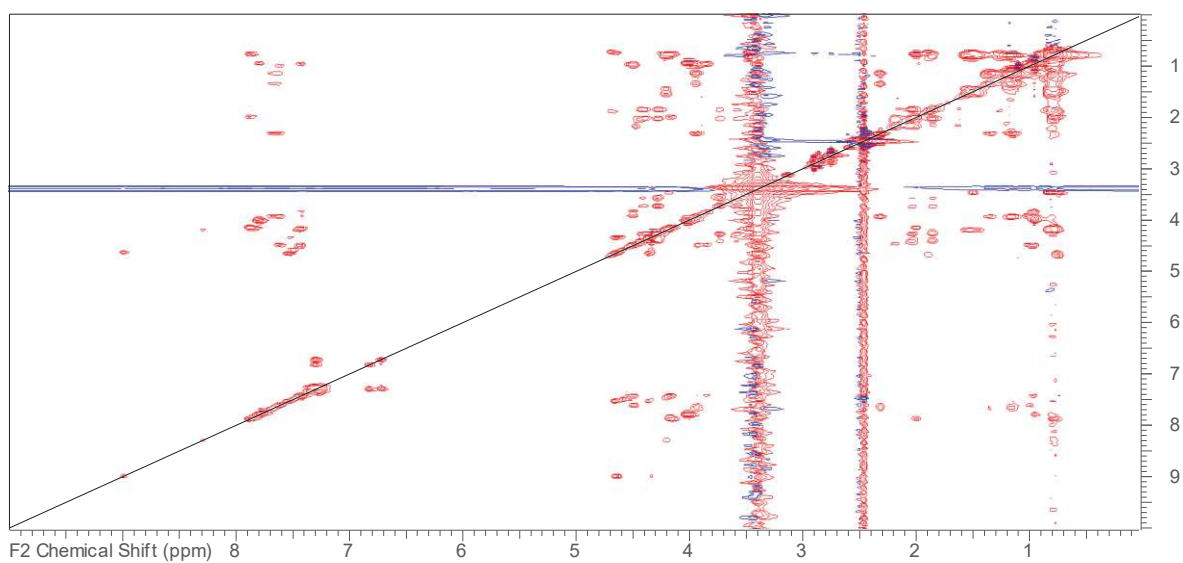
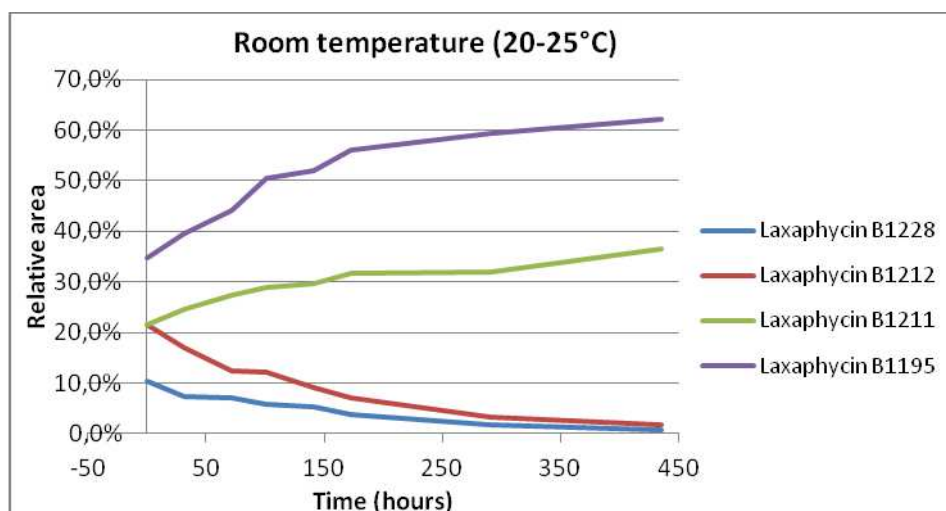
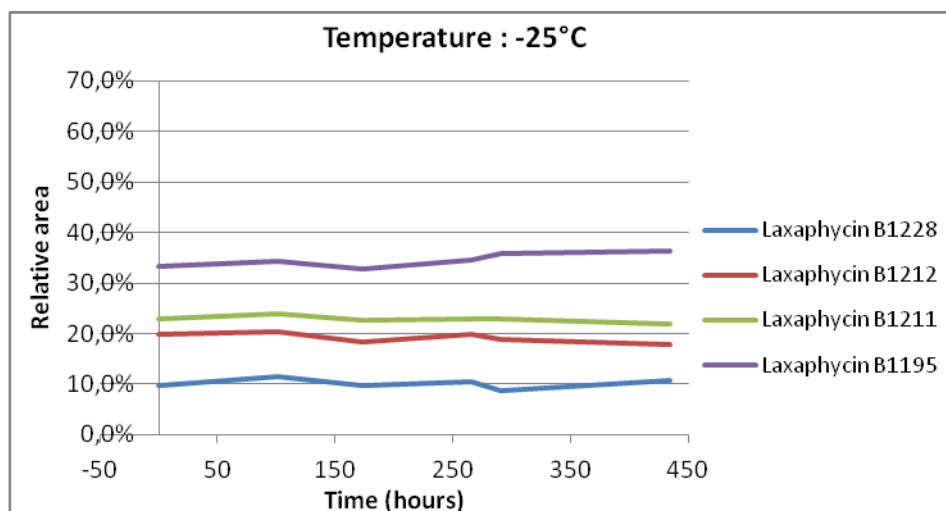


Figure S5. 21. Monitoring of the conversion of Laxaphycins B1212 and B1228 into Laxaphycins B1195 and B1211 in methanol

Peak area of each of the four compounds were reported to laxphycin A peak area to obtain relative area.



The conversion occurred at room temperature in methanol



The conversion did not occur at -25°C in methanol

Résumé général

1. Introduction

L'écologie chimique marine est une science interdisciplinaire dont les premières études datent de seulement quelques dizaines d'années. Du fait de l'apparition des premières formes de vie sur dans l'océan, le monde marin présente une exceptionnelle biodiversité marine qui a donné lieu à une diversité considérable de composés chimiques. En effet, des organismes tels que les éponges, les algues, les bryozoaires, les ascidies ou les cyanobactéries sont des sources importantes de métabolites secondaires. L'originalité de ces molécules a commencé à susciter l'intérêt des chimistes pour lesquels elles constituaient un intérêt pour la recherche de nouveaux médicaments. Puis, les chimistes se sont intéressés à comprendre le rôle de ces métabolites secondaires pour l'espèce productrice. D'autre part, les écologistes ont toujours étudié des interactions entre et parmi les espèces, qui semblent être régies par des médiateurs chimiques. Puis, chimistes et écologistes ont commencé à travailler ensemble pour étudier la médiation chimique dans les écosystèmes et ont identifié des métabolites secondaires responsables d'interactions complexes. Il a ainsi été démontré que les médiateurs chimiques régissent certains comportements des espèces, étudiés par les écologistes, tel que l'accouplement, la métamorphose des larves ou la sélection de proie. Actuellement, il a été prouvé que les métabolites secondaires sont impliqués dans la défense contre les pathogènes ou les prédateurs généralistes, l'accouplement et des interactions plus complexes qui affectent des communautés et des écosystèmes en impliquant plus de deux espèces.

Les cyanobactéries sont un groupe monophylétique parmi les bactéries et représentent un vaste groupe d'organismes procaryote autotrophe. Elles sont photosynthétiques, sont parfois capable de fixer le diazote atmosphérique, et montre une adaptabilité remarquable en occupant des habitats aux conditions variées et parfois extrêmes. Cette polyvalence est d'ailleurs un atout considérable pour rentrer en compétition avec les algues eucaryotes et les coraux.

Dans le lagon de Moorea, en Polynésie Française, *Lyngbya majuscula* et *Anabaena* cf. *torulosa* sont deux espèces de cyanobactéries filamenteuses marines qui prolifèrent sur de vastes zones jusqu'à épiphyter les coraux. Comme beaucoup de cyanobactéries, *L. majuscula* et *A. cf. torulosa* sont des producteurs importants de métabolites secondaires, principalement des lipopeptides cycliques, qui peuvent être toxiques ou répulsifs. *L. majuscula* est répandue dans les latitudes tropicales et sub-tropicales et forme des efflorescences qui posent notamment des problèmes de dermatite et d'intoxication chez les humains ainsi que des problèmes de santé animale. La grande majorité des molécules isolées de cyanobactéries proviennent de *L. majuscula*, bien qu'une révision de la taxonomie

semble nécessaire au sein du genre *Lyngbya* puisque des espèces phylogénétiquement différentes peuvent être morphologiquement similaires menant ainsi à des mauvaises identifications. A Moorea, *L. majuscula* exprime principalement les tiahuramides A-C, tandis que les trungapeptins A-C et les sérinols 4a et 4b ont également été détectés. Les tiahuramides et trungapeptins sont des lipodepsipeptides cycliques contenant un cycle de six résidus et font partie d'une sous famille des kulolides comprenant les antanapeptins A-D, la radamamide B, les hantupeptins A-C, les veraguamides A-J, la naoeptin et les kulomo'Opunalides 1-2 isolées des cyanobactéries *L. majuscula*, *Symploca cf hydnoïdes*, *Oscillatoria margaritifera*, *Moorea* sp. et du mollusque *Philinopsis speciosa*. D'autre part, *A. cf torulosa* produit principalement les laxaphycines A, B et B3 qui appartiennent à une famille de lipopeptides cycliques. Cette famille comprend la sous-famille des laxaphycines A avec un cycle à 11 acides aminés et la sous-famille des laxaphycines B contenant 12 acides aminés. Parmi la première sous-famille, la laxaphycin A, l'hormothamnin A, la laxaphycin E, la lobocyclamide A, la scytonemamide A et les trichormamides A and D sont produites par les cyanobactéries *Anabaena cf torulosa*, *Anabaena laxa*, *Hormothamnion enteromorphoides*, *Lyngbya confervoides*, *Scytonema hofmanni*, *Trichormus* sp. et *Oscillatoria* sp. Les laxaphycines B, B2, B3, et D, les lobocyclamides B et C, les trichormamides B et C et les lyngbyacyclamides A et B forment la sous famille des laxaphycines B et sont produites par les cyanobactéries *Anabaena laxa*, *A. torulosa*, *Lyngbya confervoides*, *Trichormus* sp., *Oscillatoria* sp. et *Lyngbya* sp..

Malgré les potentielles activités répulsives de leurs métabolites secondaires, les deux cyanobactéries sont consommées par plusieurs mollusques gastéropodes herbivores. En effet, le cephalaspidea *Bulla orientalis* ainsi que l'anaspidea (ou lièvre de mer) *Stylocheilus striatus* consomment la cyanobactérie *L. majuscula*. Bien qu'il soit considéré comme un spécialiste de *L. majuscula*, le lièvre de mer *S. striatus* a également été observé sur la cyanobactérie *A. cf torulosa*, accompagné de *S. longicauda*. Il est important de remarquer que le nudibranche *Gymnodoris ceylonica*, prédateur vorace de *S. striatus*, ainsi que le crabe *Thalamita coerulipes*, prédateur des trois espèces de mollusques, sont seulement observés sur *L. majuscula*.

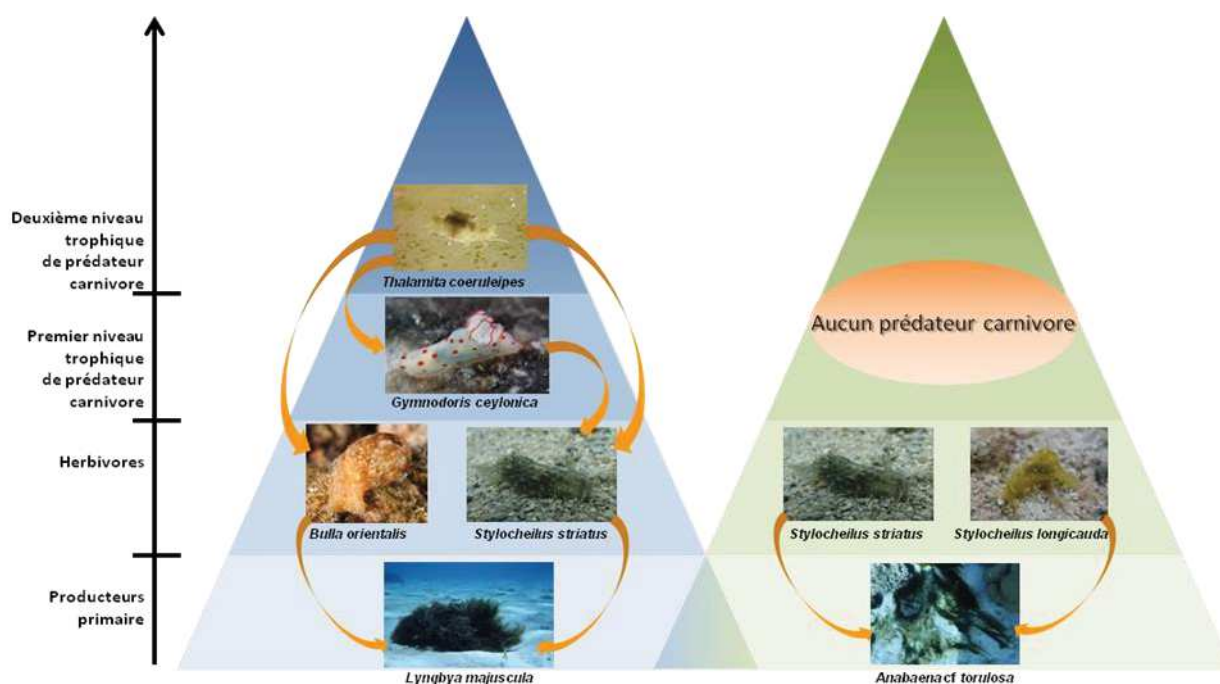


Figure R. 1. Interactions entre les producteurs primaires, les herbivores et les prédateurs carnivores

Dans cet écosystème modèle, l'objectif de cette thèse est d'étudier les effets des médiateurs chimiques dans ces interactions multi-trophiques, de la séquestration et la biotransformation des métabolites secondaires issues des cyanobactéries aux interactions inter-spécifiques grâce à des mécanismes de reconnaissances des médiateurs chimiques. Dans un premier temps, il est primordial de définir les profils métaboliques des deux espèces de cyanobactéries.

Cette thèse est organisée de la façon suivante :

-Le chapitre 2 est une approche bibliographique concernant le rôle des métabolites secondaires dans les interactions entre les gastéropodes marins et leur proie. Ce chapitre est une review soumise au journal *Natural Product Reports*. Il est résumé dans la partie 2.

-Dans les chapitres 3 et 4, la caractérisation de nouvelles laxaphycines cycliques et acycliques provenant d'*A. cf. torulosa* est décrite. Le chapitre 3 constitue une portion du travail publié dans le journal *Marine Drugs*. Le chapitre 4 sera soumis prochainement après intégration des activités biologiques. Ces deux chapitres sont résumés dans la partie 3.

-Enfin, le chapitre 5 est consacré sur l'étude de la séquestration et la biotransformation des métabolites secondaires au long du réseau trophique, leur rôle pour les différentes espèces présentes dans cet écosystème : tant sur la détection à distance des signatures chimiques des producteurs primaires que sur le rôle des métabolites secondaires dans le choix de nourriture. Ce chapitre est résumé dans la partie 4.

2. Médiation chimique dans les interactions entre les gastéropodes marins et leur proie

La médiation chimique est facteur essentiel dans la structuration des écosystèmes. La sélection naturelle imposée par les prédateurs a entraîné l'évolution des défenses chimiques pour parer les attaques des prédateurs et supprimer les virus, les bactéries, les champignons ou les compétiteurs. Ces métabolites secondaires sont également utilisés dans des interactions plus complexes tels que dans la détection à distance des proies, la recherche d'un partenaire sexuel ou les interactions avec les conspécifiques. Les gastéropodes marins sont des organismes benthiques lents, possédant une vision réduite, parfois sans coquille et ont dû développer des mécanismes de défense originaux pour augmenter leur chance de survie. Ce chapitre est consacré aux interactions entre les gastéropodes marins et leur proie influencées par les métabolites secondaires.

2.1. Séquestration des métabolites secondaires

Les organismes sessiles tels que les éponges, les coraux, les ascidies, ou les bryozoaires ainsi que les producteurs primaires comme les algues ou les cyanobactéries produisent une multitude de métabolites secondaires leur servant de défense chimique envers les prédateurs potentiels. Certains gastéropodes marins ont développé des mécanismes de tolérance leur permettant de consommer ces espèces toxiques ou répulsives et peuvent utiliser ces armes chimiques pour leur propre protection en les séquestrant et les concentrant dans différentes parties de leur corps. Parmi les gastéropodes, les hétérobranches ont particulièrement développé ces mécanismes de séquestration en y associant soit des couleurs similaires cryptiques ou au contraire des couleurs mimétiques. La différence entre les espèces cryptiques et les espèces mimétiques a été introduite par Vane-Wright qui explique que les espèces mimétiques émettent des informations qui ne sont pas intéressantes pour leurs prédateurs ce qui les rends difficiles à repérer et leur permet d'accroître leur chance de survie. Par exemple, les espèces qui possèdent les mêmes couleurs que leur proie sont dites cryptiques. Les espèces mimétiques émettent des informations qui peuvent être intercepté par les prédateurs et qui sont soit répulsives soit attractives. On distingue cependant plusieurs mimétismes : Le mimétisme mullérien consiste à associé des couleurs très visible à une toxicité et peut être associé à l'aposématisme. Le mimétisme batésien consiste à ressembler à des espèces toxiques en présentant des couleurs similaires par exemple, tout en ne présentant aucune toxicité.

Les sacoglossans (Gastropoda, Heterobranchia, Euthyneura, Nudipleura, Euheterobranchia, Panpulmonata) sont un groupe de mollusque vivant en eau relativement peu profonde possédant une répartition géographique importante principalement. Considérés comme des herbivores spécialistes consommant des algues vertes, ils sont généralement cryptiques et certains sont protégés par une coquille fragile et séquestrent

des métabolites secondaires issus de leur nourriture pour accroître leur défense en les concentrant dans les parties externes de leur corps tel que le manteau. Parmi les espèces qui ne possèdent pas de coquille, certaines sont également capable de telles adaptations tandis que d'autres biosynthétisent *de novo* des molécules de défenses et arborent des couleurs aposématiques. Ainsi, les sacoglossans sont capables de séquestrer des molécules très diverses tel que des terpenoïdes, des molécules bromées, des alcaloïdes, des terpènes, des acides gras ou des depsipeptides dans leur manteau. L'excrétion de ces molécules par le biais du mucus leur permet également d'accroître leur défense.

Au contraire des sacoglossans, les nudibranches (Gastropoda, Heterobranchia, Euthyneura, Nudipleura) sont un groupe de mollusques carnivores se nourrissant d'éponges, d'ascidies, de bryozoaires ou d'autres mollusques. Ils ne possèdent de coquilles qu'au stade larvaire et présentent des couleurs aposématiques. Dans bien des cas, ils acquièrent leur toxicité en séquestrant les métabolites secondaires de leur proie. Ces composés toxiques se retrouvent à des concentrations élevées dans des tissus externes, notamment dans des glandes spécialisées nommées MDF (Mantle Dermal Formation) et qui se situe sur les zones les plus exposées des limaces de mer. En cas d'attaque de prédateur, ces glandes relarguent ainsi des concentrations élevées de molécules toxiques ou répulsives envers les offenseurs. Enfin, il convient de souligner que certains nudibranches deviennent toxiques en biosynthétisant *de novo* des composés.

Les Anaspidea (Gastropoda, Heterobranchia, Euthyneura, Nudipleura), ou lièvres de mer, sont des mollusques herbivores cryptiques qui se nourrissent d'algues ou de cyanobactéries. Les espèces du genre *Aplysia* sont considérés comme des herbivores généralistes consommant des algues rouges tandis ce que certaines espèces, notamment *Stylocheilus striatus* se nourrissant de la cyanobactérie *Lyngbya majuscula*, sont considérés comme des spécialistes. Les lièvres de mer sont reconnus pour libérer une encre qui agit comme un mécanisme de défense contre les prédateurs. Cette encre est notamment constituée de l'aplysiovinoline et de la phycoerythrobiline, deux molécules dérivées d'un pigment algal. Ces gastéropodes séquestrent des métabolites secondaires issus de leur proie bien que leur rôle de défense chimique puisse être mis en doute. En effet, la séquestration se produisant dans un organe interne, la glande digestive ou hépatopancréas, ne peut pas être associé à un rôle de défense car les composés toxiques ne peuvent pas être libérés dans l'environnement proche. De tels processus peuvent toutefois être associés à un des mécanismes de tolérance et constituer un avantage non négligeable permettant d'échapper à la prédation en profitant de l'espace protégé chimiquement par l'algue ou la cyanobactérie. Enfin, il convient de noter que certains composés sont cependant retrouvés à faible concentration dans le manteau et le mucus de certaines espèces, n'excluant pas totalement un rôle putatif de défense.

Si les exemples de séquestration sont nombreux parmi les sacoglossans, les nudibranches et les lièvres de mers, il existe également d'autres groupes de gastéropodes, tel que les Cephalaspidea et les Umbraculoidea qui utilisent des mécanismes similaires. Si les espèces de ces deux ordres possèdent des coquilles, elles demeurent cependant fragiles et la bioaccumulation de composés issus de leur proie leur permet d'accroître leur chance de survie.

2.2. Mécanismes générales de biotransformation et excrétion des métabolites secondaires

Certains métabolites secondaires produits par les proies sont ainsi toxiques pour des espèces tout en étant tolérés par d'autres consommateurs soit par une absence de toxicité soit par le biais de détoxification. Il existe un mécanisme général pour le traitement des xénobiotiques, commun aux espèces terrestres et marines, qui inclut quatre paramètres : Absorption, Distribution, Métabolisation (biotransformation ou détoxification) et Excrétion. La métabolisation et l'excrétion sont divisées en trois phases, faisant intervenir plusieurs classes d'enzymes, les deux premières phases constituant la détoxification ou biotransformation et la dernière l'excrétion. Durant la première phase, diverses réactions permettent l'introduction d'un groupe fonctionnel qui s'associe généralement avec une faible réduction de la lipophilicité de la molécule. Ces réactions impliquent une variété d'enzymes capables de réaliser différentes biotransformation tel que des hydroxylations, des hydrolyses, des réductions, des oxydations, des déhalogénations, déhydrogénations, déalkylations sur hétéroatome, des déaminations ou encore des époxydations. Parmi ces enzymes, les cytochrome P450 monooxygénases (CYPs) forment un groupe important permettant l'introduction d'un groupe polaire tel qu'un hydroxyl. Ces enzymes sont impliqués dans la métabolisation des molécules endogènes et la biosynthèse de molécules signales tels que les stéroïdes mais sont également capable de fonctionnaliser des xénobiotiques. Bien que les CYPs soient capables de détoxifier un large éventail de molécules lipophiles, certaines réactions mènent parfois à des composés plus toxiques, cancérogènes ou mutagènes.

Les réactions réalisées durant la phase II interviennent soit sur le groupe fonctionnel introduit dans la phase précédente, soit sur un groupe déjà présent sur la molécule d'origine. Ces réactions résultent généralement d'une réduction significative de la lipophilicité avec des glucurono-conjugaisons, des sulfonations, des acétylations, méthylations, des conjugaisons avec des acides aminés ou avec la glutathione. Ainsi, les glutathione S-transférases (GST) sont un groupe important d'enzymes situés dans le cytosol des cellules et les microsomes qui jouent un rôle essentiel chez les espèces marines en étant impliqués dans la biotransformation des métabolites secondaires issues des proies. Il est important de souligner que ces enzymes sont également impliqués dans la séquestration de certains composés toxiques dans le cytosol en formant des complexes. Ainsi, la molécule

toxique est gardée à distance des protéines nucléaires cibles ce qui permet de désactiver son effet sur l'organisme.

Enfin, les transporteurs ABC sont des enzymes intervenant durant la phase III, permettant donc l'excrétion des molécules. Il est important de noter que certains de ces enzymes sont capables de prendre en charge les molécules biotransformées durant la phase II.

La plupart des études concernant les mécanismes ADME ont été menées sur les humains, les mammifères ou les insectes. Seulement quelques études ont identifié l'action de tels enzymes chez les gastéropodes marins. Les enzymes des trois phases ont notamment été identifiées dans une espèce généraliste de l'ordre Littorinomorpha. D'autres espèces des ordres Sorbeoconcha et Vetigastropoda utilisent également des GSTs et les biotransformations réalisées par ces espèces généralistes à coquille semblent intervenir dans un but de détoxification. À l'inverse, les exemples de biotransformations réalisés par les sacoglossans et les nudibranches sont opérés pour augmenter les défenses chimiques des mollusques. En effet, les molécules biotransformées, plus répulsives ou plus toxiques, sont séquestrées dans des tissus externes des organismes. En revanche, concernant les lièvres de mer, la concentration des molécules biotransformées dans la glande digestive associée à une perte de toxicité semble indiquer un but de détoxification.

La consommation des proies protégées chimiquement influe sur le choix de nourriture des herbivores généralistes. En effet, l'hypothèse de la limite de détoxification prédit que les herbivores généralistes préfèrent une nourriture variée avec des métabolites secondaires différents pour éviter la saturation des enzymes dans les mécanismes de détoxification. De plus, certaines proies sont capables d'activer leur défense et d'accroître les concentrations de métabolites secondaires en réponse à l'herbivorie. D'autres paramètres rentrent également en compte dans la sélection comme les valeurs nutritives des proies.

2.3. Influence des métabolites secondaires dans les interactions entre espèces

Les métabolites secondaires jouent un rôle important dans l'organisation des communautés et la structuration des écosystèmes, que ce soit dans les communications inter et intra spécifiques. Si, par des mécanismes de tolérance, les gastéropodes marins sont capables de consommer des proies protégées chimiquement, ils utilisent également les métabolites secondaires de leur proie dans le choix de nourriture, la métamorphose ou la recherche de nourriture. En effet, la consommation de cyanobactérie par le lièvre de mer *Stylocheilus striatus* est stimulée par certains métabolites secondaires produits par le producteur primaire et qui montrent pourtant une toxicité chez d'autres espèces d'herbivores. La détection à distance de molécules dissoutes dans l'eau par chémoréception est un phénomène important pour les gastéropodes, notamment pour la métamorphose. En effet, les mollusques gastéropodes possèdent différents organes sensoriels leur permettant

de détecter des signaux chimiques à relativement longue distance. Les espèces marines passent par un stade larvaire pélagique qui leur permet de coloniser des zones éloignées avant de se métamorphoser en juvénile. Cette transformation peut être soit spontanée soit induite par des facteurs exogènes physiques, biologiques ou chimiques. Plusieurs études démontrent ainsi que la signature chimique de leur proie préférentielle induit la métamorphose des hétérobranches. Les sacoglossans et les nudibranches, souvent considérés comme des spécialistes, se métamorphosent généralement en présence de leur proie spécifique. Cependant, il convient de souligner que certaines espèces produisent des larves qui peuvent se métamorphoser à la fois spontanément ainsi que sous l'induction des molécules de la source nourriture. La métamorphose des lièvres de mers est généralement induite par différentes espèces d'algues ou de cyanobactéries même si une préférence pour une espèce en particulier est souvent observée. Les gastropodes à coquilles, qui possèdent une mobilité réduite, ont un taux important de métamorphose induite chimiquement par les proies ou les conspécifiques. Si peu de composés chimiques responsables de la métamorphose ont été clairement identifiés, il semble néanmoins que ces composés soient solubles dans l'eau avec des masses moléculaires variées. Le fait que la métamorphose puisse également être déclenchée spontanément indique que la sélection pour la nourriture se produit également aux stades juvénile et adulte.

En effet, pour de nombreuses espèces de gastéropodes, la détection à distance de la signature chimique d'une proie se produit également après métamorphose dans la recherche de nourriture. Cependant, comme dans le cas de la métamorphose, seulement quelques études ont identifié les molécules impliquées dans ces interactions. C'est le cas dans certaines interactions entre sacoglossans et leurs algues spécifiques ainsi qu'entre nudibranches et leur proie. D'autres études relatent l'importance de la chémoréception pour les gastéropodes dans la recherche de leurs proies bien que les molécules ne soient pas clairement identifiées. Enfin, des métabolites secondaires issus de proies ont également été retrouvés dans les traces de mucus indiquant éventuellement une communication intraspécifique.

3. Caractérisations des laxaphycines

Les organismes marins sont une source importante de métabolites secondaires pouvant jouer un rôle écologique, en étant toxiques ou répulsifs, et possédant des activités biologiques diverses tels qu'antibactériennes, anti-tumorales ou anti-fongiques. Les cyanobactéries sont reconnues pour produire principalement des lipopeptides cycliques, contenant des acides aminés non-protéinogéniques. Les voies de biosynthèses de ce type de composé font intervenir différentes classes d'enzymes multifonctionnelles, tels que les NRPS (Non Ribosomal Peptide Synthetases) responsables de la modification d'acide aminé en configuration D, N-méthylé, β -hydroxylé ou déshydratés. Les PKS (PolyKetide Synthetases), parfois associé avec d'autres enzymes tels que les FAAL (Fatty Acyl-AMP ligase), sont impliqués dans la formation et l'insertion d'acide gras β -aminé. Les laxaphycines sont une famille de lipopeptides cycliques contenant des acides aminés non-protéinogéniques tel qu'un acide gras β -aminé et des acides aminés D et sont divisé en deux sous familles. Les peptides de la sous familles des laxaphycines A contiennent onze acides aminés tandis que les laxaphycines B présentent un cycle à douze résidues. Ces métabolites secondaires sont produits par différentes espèces de cyanobactéries telles que *Anabaena cf torulosa*, *Anabaena laxa*, *Hormothamnion enteromorphoides*, *Lyngbya confervoides*, *Scytonema hofmanni*, *Trichormus* sp. et *Oscillatoria* sp.

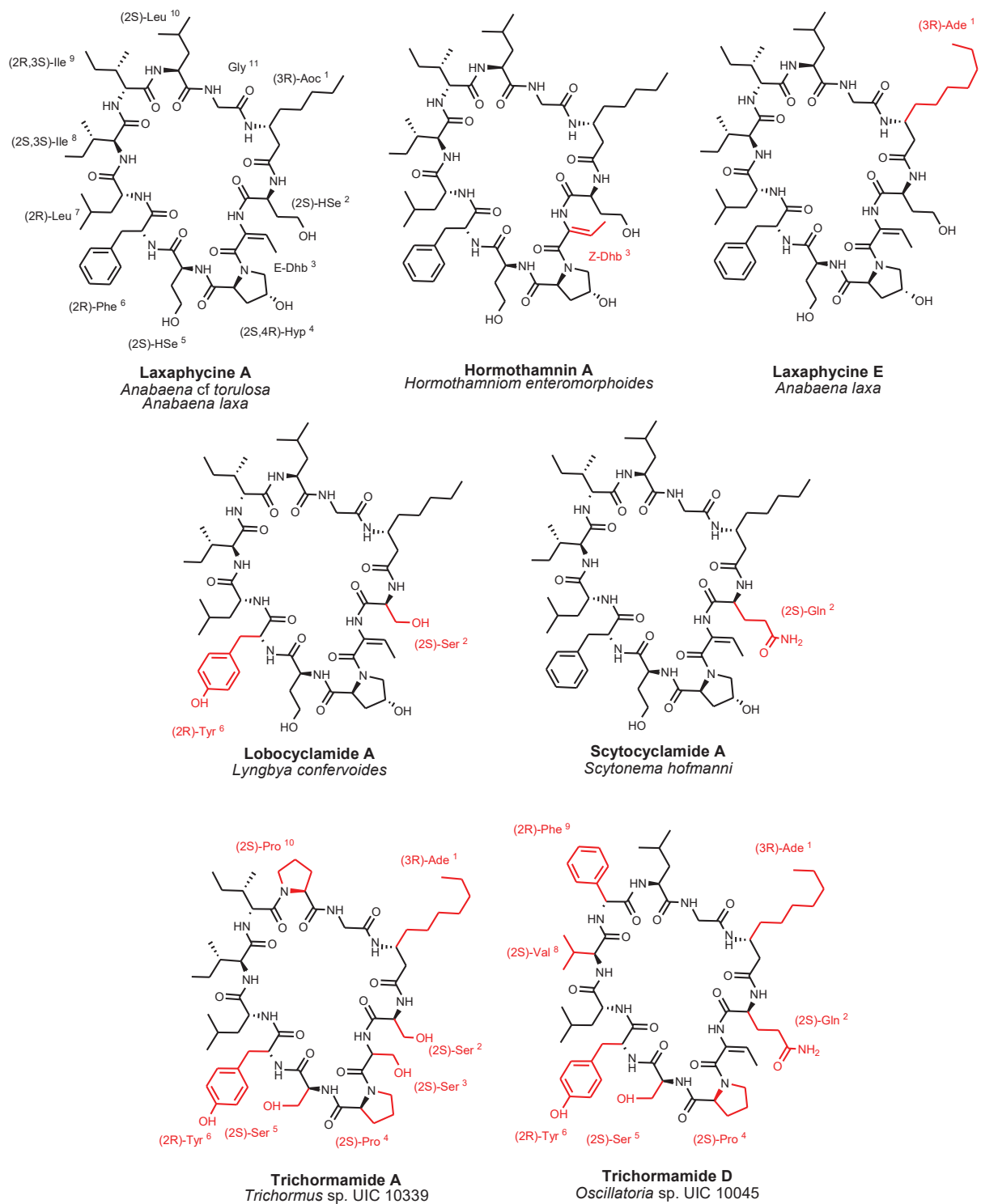


Figure R. 2. Laxaphycines A et E, et les analogues hormothamnin A, lobocyclamide A, scytocyclamide A et trichormamides A et D. Les modifications des acides amines par rapport à la laxaphycine A sont indiquées en rouge.

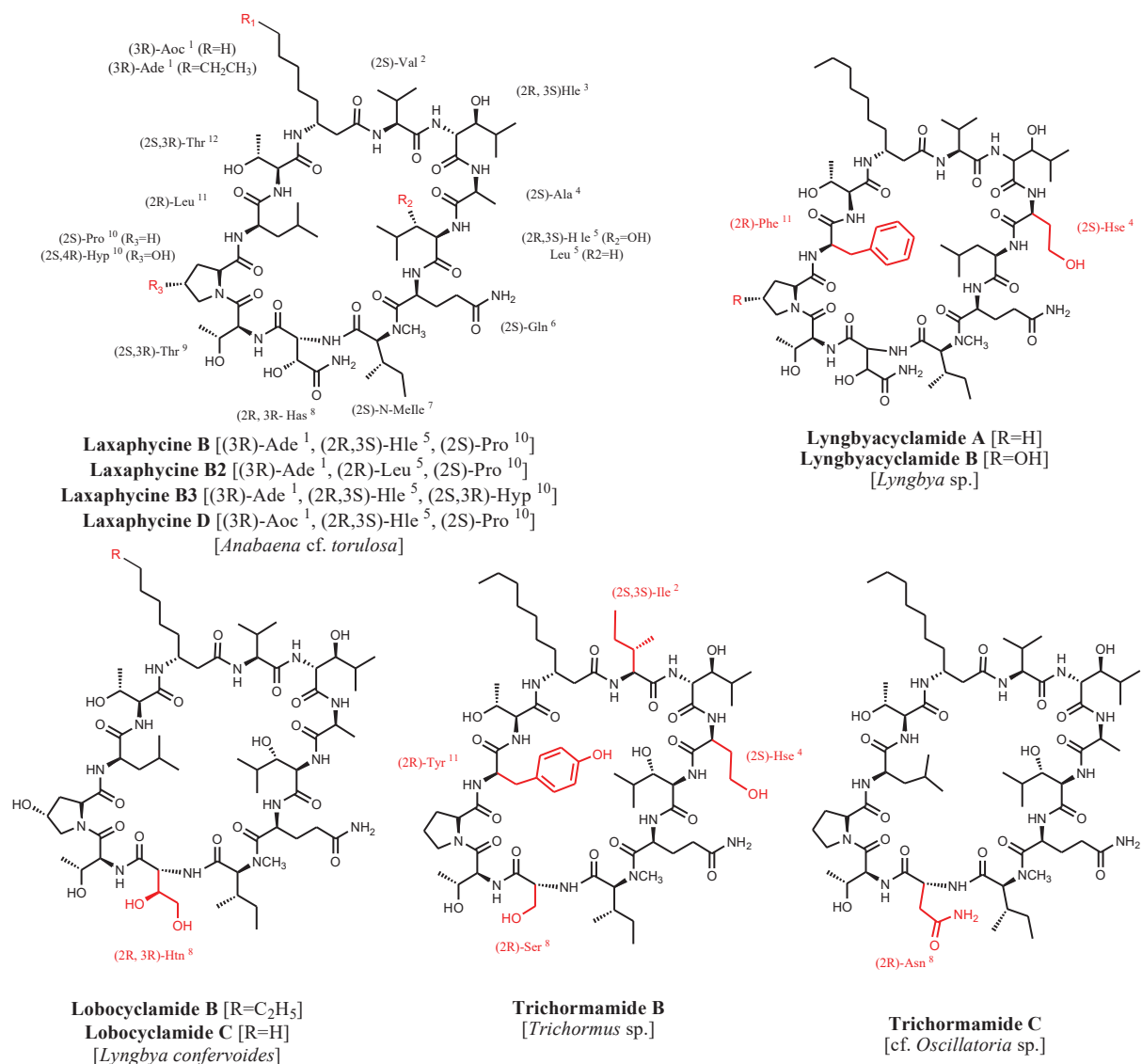


Figure R. 3. Laxaphycines A, B, B2, B3 et D, et les analogues lynbyacyclamides At et B, lobocyclamides B et C, et trichormamides B et C. Les modifications des acides aminés par rapport à la laxaphycine B sont indiquées en rouge.

Ces travaux de thèse ont permis de caractériser cinq analogues acycliques des laxaphycines A et B ainsi que deux analogues cycliques de la laxaphycine A.

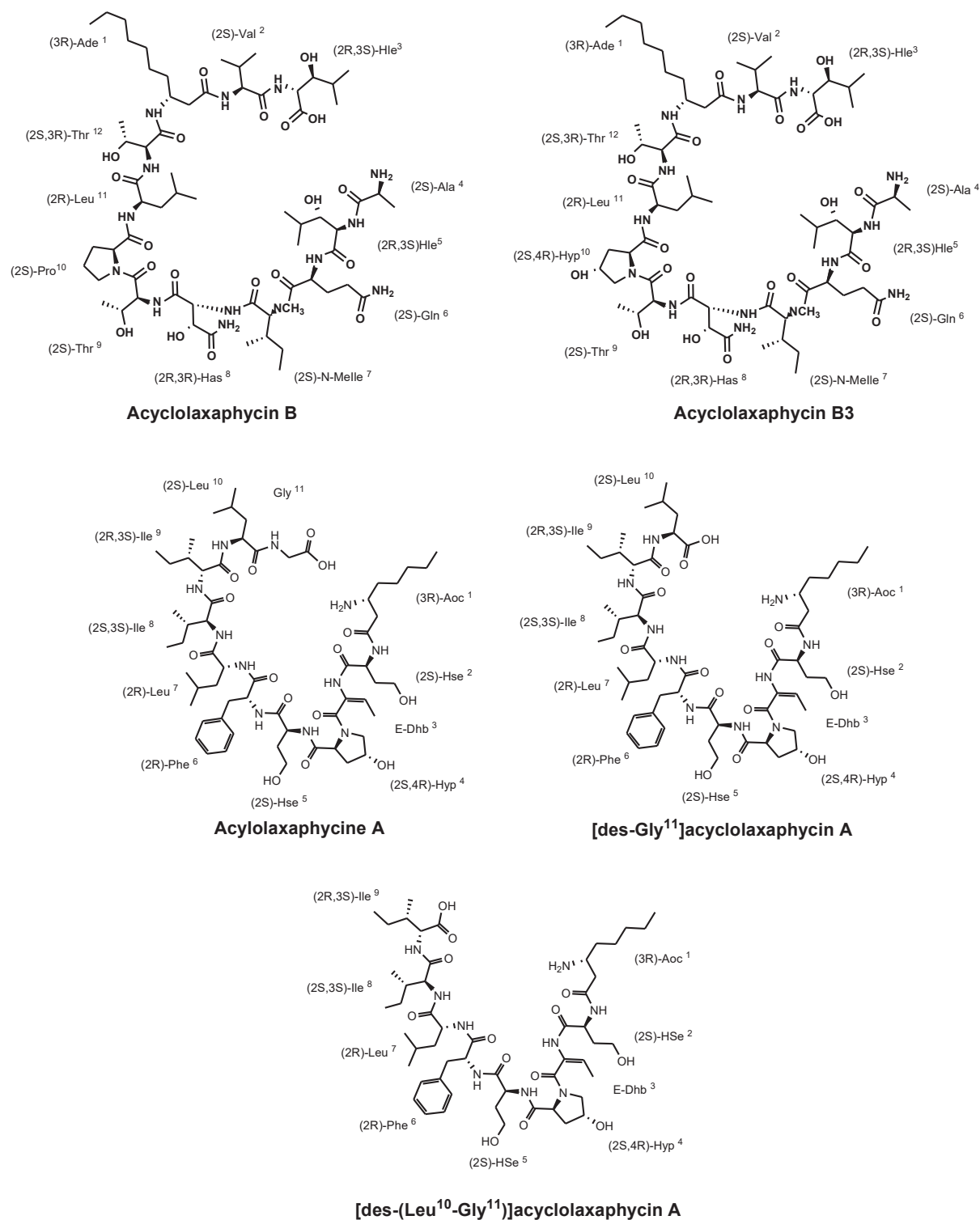


Figure R. 4. Acyclolaxaphycines B, B3, A et [des-Gly¹¹]acyclolaxaphycin A et [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A

Les expériences RMN 1D 1H et 13C, RMN 2D (TOCSY, ROESY, HSQC, HMBC, HSQC-TOCSY) ainsi que la spectrométrie de masse haute résolution et l'étude de la fragmentation

ont permis la caractérisation structurale de ces molécules. Ainsi, l'acyclolaxaphycine B et l'acylaxaphycine B3 sont les équivalents acycliques des laxaphycines B et B3 avec un peptide ouvert entre l'hydroxyleucine en position 3 (HLe) et l'alanine (Ala) en position 4. Concernant l'acyclolaxaphycine A, équivalent acyclique de la laxaphycine A, la séquence est interrompue entre la glycine (Gly) en position 11 et l'acide octanoïque β -aminé (β -Aoc) en position 1. La [des-Gly¹¹]acyclolaxaphycin A et la [des-(Leu¹⁰-Gly¹¹)]acyclolaxaphycin A diffèrent de la dernière par l'absence de la glycine (Gly¹¹) ou de la glycine (Gly¹¹) et de la leucine (Leu¹⁰), respectivement. La [L-Val⁸]laxaphycine A et la [D-Val⁹]laxaphycine A sont des analogues cycliques de la laxaphycine A qui diffèrent par la présence d'un valine en position 8 ou 9 à la place d'un isoleucine. La configuration de chaque acide aminé a été déterminé par la méthode de Marfey pour chaque peptide, à l'exception de l'acyclolaxaphycine A pour laquelle la quantité était insuffisante.

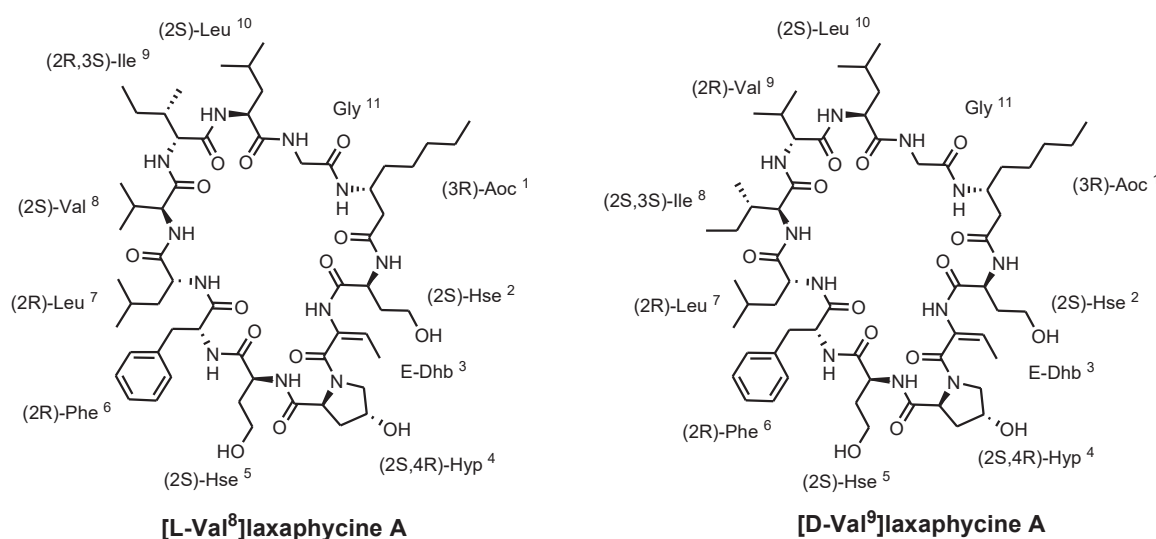


Figure R. 5. [L-Val⁸]laxaphycine A et [D-Val⁹]laxaphycine A

Il est intéressant de constater que la configuration des carbones α de chaque résidue ne montre aucune variation. La biosynthèse de ce genre de composé implique la cyclisation lors de la dernière étape et la présence de laxaphycines acycliques et cycliques dans le même extrait pourrait indiquer une voie de biosynthèse similaire. Ainsi, deux voies de biosynthèses pourraient être mises en évidence avec une cyclisation intervenant soit sur l'amine de l'acide β -aminé pour laxaphycine A soit entre l'hydroxyleucine et l'alanine pour les laxaphycines B. Cependant, il convient de considérer que des dégradations enzymatiques peuvent intervenir dans le cadre de mécanismes de résistances par des espèces compétitrices. En effet, certaines enzymes permettent notamment l'ouverture de depsipeptides cycliques au niveau d'une liaison ester. Cependant, aucune liaison ester n'est présente dans les cycles des laxaphycines les rendant potentiellement plus robustes et moins enclins à une dégradation enzymatique.

4. Rôles des métabolites secondaires dans la fonctionnement et la structuration d'un écosystème corallien

L'écosystème identifié est formé par deux producteurs primaires, les deux cyanobactéries *Lyngbya majuscula* et *Anabaena cf torulosa*, un premier niveau trophique de mollusque herbivores consommant les cyanobactéries ainsi que des prédateurs carnivores. Sur *L. majuscula* sont observés les mollusques herbivores *Stylocheilus striatus* et *Bulla orientalis*, ainsi que le nudibranche *Gymnodoris ceylonica*, prédateur vorace de *S. striatus*, ainsi que le crabe prédateur *Thalamita coerulipes*, consommant les trois mollusques présents. Le lièvre de mer *S. striatus* a également été observé sur *A. cf torulosa*, accompagné du lièvre de mer *S. longicauda* mais aucun prédateur carnivore n'a été observé sur cette cyanobactérie. Les cyanobactéries sont reconnues pour produire un large éventail de métabolites secondaires qui peuvent être toxiques, répulsives ou attractantes. L'objectif global de ce chapitre était de déterminer le rôle des métabolites secondaires produits par les cyanobactéries dans le fonctionnement et la structuration de cet écosystème. Par une expérience originale dans laquelle étaient proposés deux eaux différentes, conditionnées ou non avec l'une ou l'autre des cyanobactéries, nous avons démontré que les herbivores *S. striatus* et *B. orientalis* étaient capable de détecter à distance les signatures chimiques des cyanobactéries. En effet, les herbivores remontent préférentiellement la piste chimique de la cyanobactérie sur laquelle ils ont été prélevés. La présence des laxaphycines et, à plus faible concentration, des tiahuramides dans l'eau conditionnée avec les cyanobactéries, permet de suggérer leur rôle dans l'attraction des herbivores. Afin de cibler les molécules qui pourraient être impliqués dans cette attraction chimiques, une seconde série d'expériences a été réalisés avec un dispositif dans lequel deux choix étaient offerts à *S. striatus* prélevé sur *L. majuscula*. En effet, deux boules de coton, disposées sur les côtés opposés d'un aquarium, étaient précédemment trempées dans l'eau conditionnée avec les cyanobactéries ou imprégnées de différents extraits : extrait organique, extrait hydroalcoolique ou les deux extraits. Les expériences ou étaient offert un coton traité et un coton témoin n'ont pas permis d'identifier dans quelle extrait se situait la molécule ou le bouquet de molécules responsable de l'attraction. En effet, tous les cotons traités étaient préférentiellement colonisés quels que soit la cyanobactérie ou l'extrait. Cependant, les expériences ont permis de démontrer que les métabolites secondaires présents dans l'extrait organique étaient déterminants dans la distinction entre les deux cyanobactéries. Les familles de peptides, spécifiques à chaque espèce de cyanobactérie, sont présentes dans les extraits organiques tandis que les extraits hydroalcooliques pourraient contenir des molécules non spécifiques produites par les deux cyanobactéries.

Les expériences de choix de nourriture, réalisés avec une nourriture artificielle imprégnée ou non d'extrait brut cyanobactéries, a cependant montrer que *S. striatus* et *B. orientalis* préfèrent consommer les métabolites secondaires de la cyanobactérie qu'ils n'ont pas

consommé précédemment. Ces résultats pourraient être cohérents avec l'hypothèse de limite de détoxification (Detoxification Limit Hypothesis) et indiquerait ainsi un comportement généraliste des deux espèces d'herbivore. En effet, la consommation de métabolites secondaires permettrait de ne pas saturer les voies de détoxification et les enzymes en impliquant différentes voies et enzymes. Parmi les enzymes de détoxification, les GSTs (glutathione S-transférases) sont également impliqués dans la séquestration des métabolites secondaires.

Des concentrations élevées de métabolites secondaires des cyanobactéries ont ainsi été retrouvées dans les herbivores. En effet, *S. striatus* and *B. orientalis* nourri avec *L. majuscula* séquestrent les tiahuramides, les trungapeptines ainsi que les sérinols. Ces composés sont particulièrement concentrés dans l'hépatopancréas, un organe interne, ce qui suggère que la bioaccumulation n'intervient pas dans un rôle de défense mais plutôt comme un mécanisme de tolérance. Il est intéressant de remarquer que le facteur de bioaccumulation du sérinol 4b est sept fois plus important que celui des tiahuramides.

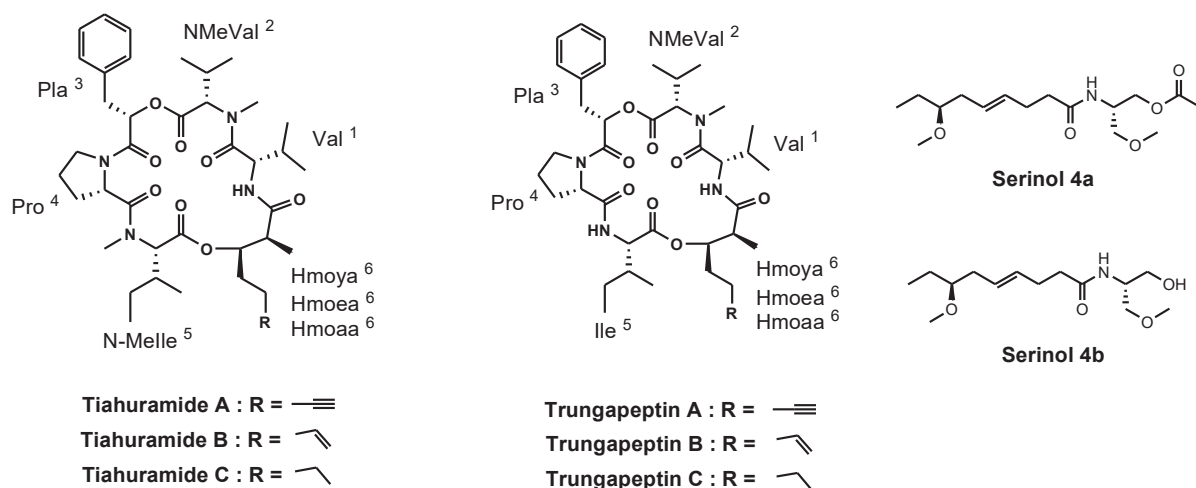


Figure R. 6. Tiahuramides A-C, trungapeptins A-C et sérinols 4a et 4b

Concernant les herbivores prélevés sur *A. cf torulosa*, la laxaphycine A est séquestrée par *S. striatus* et *S. longicauda* mais les laxaphycines B et B3 sont biotransformées et donnent laxaphycines B1212, B1228, B1195 et B1211 par les deux herbivores. Les exemples de biotransformations réalisées par *S. striatus* sont des acétylations au niveau d'un groupement de métabolites secondaire. Dans notre étude, ces réactions sont originales car elles impliquent l'hydrolyse des peptides cycliques, potentiellement par les enzymes de phase I de type peptidases, avec perte de l'hydroxyleucine et l'alanine pour donner les laxaphycines B1212et B1228. Ensuite, la cyclisation de la glutamine N-terminal en pyroglutamate donne les laxaphycines B1195 et B1211. Cette cyclisation pourrait aussi bien être prise en charge par des enzymes de type glutaminy-peptide cyclotransférase que se produire spontanément

comme pour certains anticorps monoclonaux humains. Nous n'avons pas testé et comparé les activités des laxaphycines biotransformées aux laxaphycines d'origine, ni disséqué les lièvres de mers provenant de *A. cf torulosa* mais ces biotransformations pourraient être réalisées dans un but de détoxification, la laxaphycine B étant antifongique et cytotoxique sur lignée tumorale humaine. Les encre de lièvres de mers, qui sont excrétées en cas d'attaque d'un prédateur, ne contiennent pas les métabolites secondaires des cyanobactéries, ce qui conforte l'hypothèse du mécanisme de tolérance plutôt que de défense chimique.

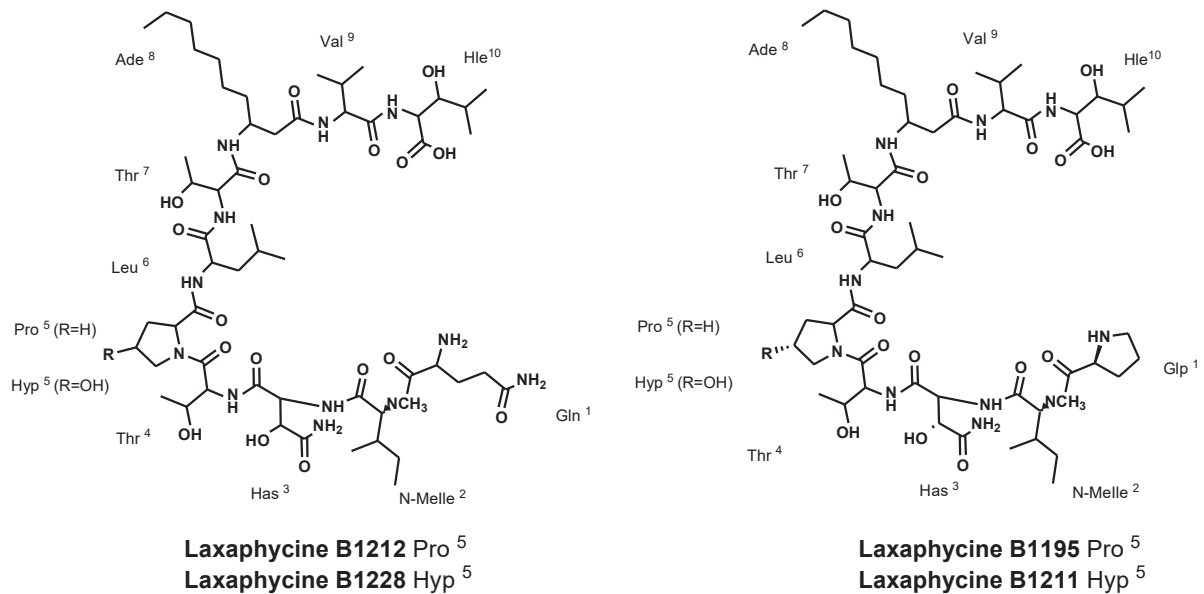


Figure R. 7. Laxaphycines B1212, B1228, B1195 et B1211 issues des laxaphycines B et B3

Le nudibranche *G. ceylonica* ainsi que le crabe *T. coerulipes* sont observés seulement sur la cyanobactérie *L. majuscula* mais ne séquestrent pas les métabolites secondaires de la cyanobactérie. La présence *G. ceylonica* est bénéfique pour la cyanobactérie *L. majuscula* car il consomme son prédateur, le lièvre de mer *S. striatus*, formant ainsi une relation tri-trophique. Il serait intéressant de démontrer si le nudibranche est attiré par les métabolites secondaires de *L. majuscula* ou si les molécules d'*A. cf torulosa* sont repulsives ou toxiques envers lui.

5. Conclusion

L'écologie chimique marine a émergé des collaborations relativement récentes entre écologistes et chimistes. Avec 8 molécules approuvées par le FDA (Food and Drug Administration) et l'EMA (European Medicine Agency) parmi les 28 000 isolées, la recherche de nouveaux médicaments issues d'organismes marins peut s'apparenter à chercher une aiguille dans une motte de foin. C'est pourquoi la recherche de molécules marines originales dans le but d'explorer à la fois leurs activités pharmacologiques et écologiques semble être une stratégie judicieuse. Comme toute discipline émergente, elle fait face à des défis tels que la conception des expériences adaptées aux différentes espèces, la difficulté de trouver suffisamment de matériel biologique ainsi que le défi de comprendre des mécanismes écologiques relativement complexes.

La diversité et la complexité des interactions écologiques marines peuvent s'expliquer par l'exceptionnelle diversité des métabolites secondaires, avec des activités qui varient d'une espèce à l'autre. Ces molécules peuvent ainsi être répulsives envers la plupart des espèces généralistes tout en étant attractante pour une espèce spécialiste. Parmi les gastéropodes marins, on retrouve des espèces spécialistes et généralistes qui ont continuellement évolués en interaction avec des proies défendues chimiquement. Ils ont ainsi développé des stratégies diverses pour consommer ces proies toxiques, profiter de l'hôte chimiquement protégé et voler ses défenses chimiques pour améliorer ses chances de survie. Une architecture enzymatique organisée semble être responsable des mécanismes de tolérance et de biotransformation permettant l'amélioration des défenses chimiques. D'autre part, la chémoréception parmi les gastéropodes est un phénomène important chez les gastéropodes qui permet de détecter à distance les signatures chimiques de leur proie et demeure prépondérant dans la métamorphose et la recherche de leur proie.

Les cyanobactéries sont une source importante de molécules originales qui peuvent être toxiques, répulsives, appétentes ou attractantes envers différentes espèces. L'objectif global de cette thèse était de déterminer si la structuration de notre écosystème modèle, présenté précédemment, était régie par des médiateurs chimiques. Dans un premier temps nous avons caractérisé les métabolites secondaires des producteurs primaires et notamment sept nouvelles laxaphycines. La caractérisation de laxaphycines ouvertes n'a jamais été publiée et ces analogues pourraient être aussi bien des produits de dégradation enzymatique que des intermédiaires de biosynthèse permettant ainsi de proposer deux voies de biosynthèse pour les deux familles de laxaphycine.

La laxaphycine A est décrite comme étant répulsive envers plusieurs espèces mais notre étude montre qu'elle est tolérée par les lièvres de mer *S. striatus* et *S. longicauda*. L'étude de tels composés, permet d'évaluer leur devenir le long du réseau trophique en

étant séquestrés ou biotransformés, mais également de déterminer leurs activités tant pharmacologiques qu'écologiques.

Bien que considéré comme un spécialiste dans la littérature, *S. striatus* consomme différentes cyanobactéries et semble se comporter comme les lièvres de mer généralistes du genre *Aplysia*. *B. orientalis*, collecté sur *L. majuscula*, ne montre pas non plus un comportement généraliste en consommant sans contraintes les métabolites secondaires de la cyanobactérie *A. cf torulosa* dans les expériences de choix de nourriture. La façon dont *S. striatus* manipule une variété de métabolites secondaires en les séquestrant et les biotransformant montre qu'il est capable de s'adapter à des sources de nourriture variées. Si la laxaphycin A n'a pas d'effet visible sur les lièvres de mers, les laxaphycines B et B3, qui sont cytotoxiques sur lignée tumorales humaines, sont biotransformées par les deux lièvres de mer, ce qui pourrait suggérer un mécanisme de détoxification. L'intérêt de séquestrer des composés issus de l'alimentation dans un organe interne reste toujours un indéterminé bien qu'il semble que ce soit plus un mécanisme de tolérance que de défense.

De manière générale, et d'après la littérature, aucune interaction entre cyanobactérie et herbivores spécialistes n'a été observé, l'association de plusieurs espèces dans les efflorescences et leur éphémérité pouvant en être la raison. Cependant nous pouvons suggérer que les espèces généralistes, tel que *S. striatus*, peuvent s'habituer à consommer une proie, éduquer leur sens de perceptions et ainsi être capable de repérer à distance cette source de nourriture qui peut également lui fournir un abri. D'autre part, une espèce généraliste peut montrer une préférence pour une source de nourriture et se rabattre sur une nourriture alternative en cas d'absence de la première. Dans notre modèle, *A. cf torulosa* pourrait être une nourriture de substitution pour *S. striatus* mais peut également être un avantage adaptatif pour *S. striatus* afin d'éviter la prédation de *G. ceylonica* et *T. coeruleipes*.

Sur certains producteurs primaires sont aussi observé des prédateurs carnivores qui prédatent sur les espèces herbivores formant ainsi des interactions multi-trophiques. L'absence de ses prédateurs sur d'autres producteurs primaires suggère que les métabolites secondaires régissent des interactions plus complexes. Dans notre modèle, la cyanobactérie pourrait répondre à la pression de l'herbivorie en émettant une molécule que le nudibranche a pris habitude d'associer à la présence des herbivores.

L'écologie chimique marine est encore a ses débuts mais la médiation chimique dans les écosystèmes marins est souvent sous estimée. Des molécules clés peuvent en effet régir des communautés écologiques à grande échelle et doit être pris en compte dans la modélisation des écosystèmes et la gestion des pêches. Enfin, il est nécessaire de comprendre ses interactions afin d'évaluer leurs évolutions dans un contexte de changement climatique avec notamment le réchauffement climatique et l'acidification des océans.

Abstract

In the lagoon of Moorea in French Polynesia, we have identified a relatively simple tropical marine ecosystem consisting of two primary producers (two filamentous cyanobacteria, *Lyngbya majuscula* and *Anabaena cf. torulosa*), three herbivorous molluscs (*Stylocheilus striatus*, *S. longicauda* and *Bulla orientalis*), a carnivorous nudibranch (*Gymnodoris ceylonica*) and a carnivorous crab (*Thalamita coerulipes*). *L. majuscula* and *A. cf. torulosa*, that bloom ephemerally across wide sandy areas and even on corals, are prolific producers of secondary metabolites, mainly cyclic lipopeptides, which may either be toxic or act as feeding deterrents to potential consumers. However, these compounds do not prevent the sea hare *S. striatus*, feeding on cyanobacteria. *S. striatus*, considered as *L. majuscula* specialist, is known to sequester and transform some secondary metabolites produced by *L. majuscula*. However we found also *S. striatus* feeding on *A. cf. torulosa* and in this case it was less susceptible to predation by the nudibranch *G. ceylonica* than when it fed on *L. majuscula*. In the study of this model ecosystem, we combine cyanobacterial metabolome profiling and ecological bioassays in order to study the cascading effects of chemical mediators in multi-trophic relationships; we completed the metabolic profile characterization of the two cyanobacteria, we studied vertical and horizontal transmissions of the cyanobacterial secondary metabolites along the trophic web, and studied the role of these compounds in predator-prey relationships. Focusing our attention on *A. cf. torulosa* we isolated seven new lipopeptides, derived from the known laxaphycins, and characterized them using extensive NMR experiments (1D and 2D NMR: COSY, TOCSY, HSQC, HMBC, NOESY), mass spectrometry (HR-MS and fragmentation by MSⁿ) and Marfey's advanced method. It is the first time that acyclic analogs of laxaphycins have been described. Although the peptides from *L. majuscula* are found intact in herbivores, some lipopeptides from *A. cf. torulosa* are biotransformed by sea hares into four new compounds we characterized. The sequestration and biotransformation by the herbivores may be considered as a tolerance mechanism rather than a defense mechanism. We demonstrate also that the herbivores use cyanobacterial compounds as chemical cues for cyanobacteria tracking and feeding choice. Our experiments suggest that *S. striatus* and *B. orientalis* are generalist consumers, although the influence of cyanobacterial chemical cues on their foraging preferences may suggest an adaptive behavior enabling the mollusc to track their host of origin.

Résumé

Dans le lagon de Moorea, en Polynésie Française, nous avons identifié un écosystème constitué de deux producteurs primaires (les cyanobactéries filamenteuses *Lyngbya majuscula* et *Anabaena cf. torulosa*), trois mollusques herbivores (*Stylocheilus striatus*, *S. longicauda*, et *Bulla orientalis*), un nudibranche carnivore (*Gymnodoris ceylonica*) et un crabe carnivore (*Thalamita coerulipes*). *L. majuscula* et *A. cf. torulosa* prolifèrent sur de vastes zones jusqu'à épiphyter les coraux ; elles sont des producteurs importants de métabolites secondaires, principalement des lipopeptides cycliques, qui peuvent être toxiques ou répulsifs. Cependant, ces composés n'empêchent pas le lièvre de mer *S. striatus* de consommer les cyanobactéries. *S. striatus*, décrit comme un prédateur spécialiste de *L. majuscula*, est connu pour séquestrer et/ou biotransformer les métabolites secondaires de *L. majuscula*. Cependant nous avons également observé *S. striatus*, sur *A. cf. torulosa* où il semble moins exposé à la prédation du nudibranche *G. ceylonica* que quand il est sur *L. majuscula*. Dans cet écosystème modèle, nous avons combiné le profilage des métabolomes des deux cyanobactéries et des expériences en écologie dans le but d'étudier le rôle des médiateurs chimiques dans la structuration de cet écosystème ; nous avons complété la caractérisation des profils métaboliques des deux cyanobactéries, étudié les transmissions verticale et horizontale des métabolites secondaires produits par les cyanobactéries le long de la chaîne trophique, et étudié le rôle de ces composés dans les relations prédateurs-proies. De *A. cf. torulosa*, nous avons isolé cinq analogues acyliques et deux analogues cycliques des laxaphycines que nous avons caractérisés par RMN (1D et 2D RMN : COSY, TOCSY, HSQC, HMBC, NOESY), spectrométrie de masse (spectrométrie de masse à haute résolution et fragmentation en MSⁿ), ainsi que par dégradation chimique avec la méthode de Marfey. La présence de laxaphycines acycliques n'a jamais été décrite auparavant. Nous avons montré que les peptides de *L. majuscula* sont séquestrés sans biotransformation par les herbivores, alors que les herbivores présents sur *A. cf. torulosa* biotransforment deux laxaphycines en quatre composés nouveaux que nous avons caractérisés. Il ne semble pas que la séquestration et la biotransformation soient opérées dans le but d'améliorer les défenses chimiques des herbivores mais plutôt comme un mécanisme de tolérance. Nous avons également montré que les mollusques herbivores utilisent les composés produits par les cyanobactéries comme signaux chimiques pour détecter à distance les cyanobactéries et pour le choix de leur nourriture. Ces expériences de choix semblent indiquer que *S. striatus* et *B. orientalis* sont des herbivores généralistes bien que l'influence des molécules des cyanobactéries suggère un comportement adaptatif permettant au mollusque de retrouver l'hôte sur lequel il a été prélevé.