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Energetic processes driving potential peptide protometabolisms at the origin of living systems

Ghinwa Ajram

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Chimie Organique

École doctorale 459
Sciences Chimiques Balard

Unité de recherche Institut des Biomolécules Max Mousseron (IBMM),
UMR 5247 CNRS, Université de Montpellier, ENSCM

Energetic processes driving potential peptide protometabolisms at the origin of living systems

Présentée par Ghinwa AJRAM
Le 29 Novembre 2018

Sous la direction de Robert PASCAL
et Jean-Christophe ROSSI

Devant le jury composé de :

M. Hervé COTTIN , Professeur,	Université de Paris-Est Créteil	Rapporteur
M. Joseph MORAN , Professeur,	Université de Strasbourg	Rapporteur
M. Michael SMIETANA , Professeur,	Université de Montpellier	Président
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M. Jean-Christophe ROSSI , Maître de Conférences,	Université de Montpellier	Co-encadrant de thèse



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Abbreviations

A adenine

a.a. amino acid

aa-AMP aminoacyl adenylates

aaRS aminoacyl-tRNA synthetases

AMP adenosine monophosphate

ATP adenosine triphosphate

DKS dynamic kinetic stability

DNA deoxyribonucleic acid

Ga billion years before present

mRNA messenger ribonucleic acid

NCA α -amino acid *N*-carboxyanhydride

PPi inorganic pyrophosphate

RNA ribonucleic acid

tRNA transfer ribonucleic acid

UV ultraviolet

General Introduction

The origin of life is still one of the most important questions to which science has given no satisfactory explanation. There is a strong interest in the general public about the issue of the origin of life, which has strongly increased within the last decades after the discovery of exoplanets around stars other than the Sun in number reaching several thousand. Space missions or new observation devices are dedicated to answering the main question of astrobiology: is there another form of life in the solar system or elsewhere?

Since organic matter is formed through an abiotic way in the Universe and it could be brought to the Earth surface and also formed locally on it, the subject now is more about studying how chemical systems can self-organize to more complex systems. Researching the nature of complex systems has become a major topic of interest in some relevant scientific fields (such as physics, biology, computer science), while chemistry enhanced “systems” prospect only recently in 2005, 2008 (**Kindermann et al., 2005; Ludlow and Otto, 2008**). After that progress, systems chemistry approaches developed (**for example, Ruiz-Mirazo, 2014**), they are based on the idea that the molecular interactions, reactions, and connections between the chemical systems (peptides, nucleotides, fatty acids) could lead eventually to the emergence of life. These connections and interactions give rise to the dynamic and the whole functionality of the living organisms.

Therefore, researchers should now be oriented towards the identification of self-organization processes, and a theoretical framework has been developed to understand the processes. Chemical systems should be kept in a far from equilibrium environment in order to be capable to further evolutionary changes (**Pross and Pascal, 2013**). This requires a new kind of stability called Dynamic Kinetic Stability (DKS), where the chemical systems replicate exponentially (**Pross, 2012**). Irreversibility should be permanently or repeatedly maintained during all the self-organization process by coupling free energy sources with the chemical systems (**Pross and Pascal, 2013; Pascal et al., 2013**).

The previous conceptual framework to the problem of “origin of life” opposes many earlier hypothesis allocated a key role to an extremely uncertain event. It is impossible to solve the historical issue regarding the “the origin of life,” but now we are aiming to

determine why and how systems sharing at least part of the characteristics of living things could emerge.

Starting from the conceptual framework, the objectives of the thesis comprise studies that begin within the chemical prebiotic processes of amino acids and peptides including their activated forms (NCAs and oxazolones, respectively) that can feed the environment with free energy, a prerequisite related to kinetic irreversibility and evolution. These studies are likely to give empirical foundations for the above approach in a pattern consistent with the chemistry presented in a literature review (**Danger et al. 2012**). The earlier studies in the laboratory are consistent with the fact that amino acids (or *N*-carbamoyl amino acids) can be activated as NCAs (**Taillades et al., 1999; Danger et al., 2006; Liu et al., 2014**). They also demonstrated that the activation of the C-terminal group of *N*-acyl amino acids by cyanamide (prebiotically activating agents) takes place through oxazolones intermediates (**Danger et al., 2013**).

In addition to supporting systems chemistry view of self-organization (irreversible pathways required for the development of metabolism), experimental work on the co-evolution between amino acids and peptide systems and nucleotides systems could also give some help in understanding how translation emerged. Such co-evolution would rely on the formation of adducts involving covalent bonds between peptides and nucleic acids, which would be at the origin of the RNA-protein translation system and the genetic code (**Wong, 1975; Szathmary, 1993; Di Giulio, 1997**). Some elements of the contemporary translation machinery are likely relics of the primitive stage, including aminoacyl adenylates, aa-tRNA, peptidyl-tRNA. Studies in the DSBC (Dynamique des Systèmes Moléculaires Complexes) team in prebiotic chemistry are involved in this approach, they showed that NCAs could also react with nucleotides to give aa-AMPs (**Biron et al., 2005**). These studies pave the way for a better understanding of the emergence of translation, in which the NCAs could play the central role of active intermediates at the crossroads between peptides and nucleotides by allowing an abiotic formation of mixed anhydrides anticipating their essential role in biochemistry.

The following problems will be handled: (1) through which chemical carrier could energy be provided to a chemical system? (2) On which chemical background involving amino acids and nucleotides could translation have evolved ?

Finally, finding answers to the origin of life issue would solve a philosophical interrogation of human beings but may also open new fields of application such as that of partly autonomous chemical systems.

The work presented in this thesis is in the continuity of the themes already developed within the DSBC team. The program of my PhD work was following the main objectives of the ANR PeptiSystems project (ANR-14-CE33-0020): 1) identify prebiotically plausible processes capable of activating peptides and amino acids into 5(*4H*)-oxazolones or *N*-carboxyanhydrides (NCAs) in addition to the already known ones 2) Develop the knowledge about the chemical pathways through which amino acids (aa(s)) or short peptides could have interacted with ribonucleotides monomers and oligomers.

This thesis is thus composed of a bibliographic study (State of Art) and two parts reporting experiments to which I contributed in two different directions. State of the art will be first presented. It introduces current knowledge about the formation of the Earth and its primitive environment. It includes a part on the sources of organic matter with particular emphasis on the presence of different building blocks at the same place (supporting systems chemistry approach). Moreover, It includes the various existing pathways of peptide elongations in primitive conditions, with an emphasis on the prebiotically plausible pathways that takes place through energy-rich intermediates (NCAs, oxazolones). Lastly, it presents general views about the common chemistry of peptide and nucleotides.

The first part deals with the search for efficient prebiotic activation processes of amino acids and peptides as NCAs and oxazolones. **The second part** presents new results in support of a coevolution between RNAs and peptides.

The first part of this thesis is divided into two chapters. **Chapter 1** presents a reassessment of prebiotically relevant chemical agents for the activation of α -amino acids and peptides, which has been submitted for publication in the Journal of Systems Chemistry. **Chapter 2** presents a study on the C-terminus elongation of *N*-carbamoyl amino acids in the scope of the group recent works on carbodiimide promoted C-terminus elongation of peptides in prebiotic chemistry already published in Chemistry – a European Journal.

The second part of this thesis is composed of chapter 3 to 5. As we were particularly interested in studying how peptides and nucleotides may have interacted in an abiotic environment because it could provide an adequate answer to the subject of chemical

aminoacylation of RNA and hence on the origin of translation, we have performed a study on non-enzymatic aminoacylation reagents for the 3'-terminal of RNA (**Chapter 3** already published in a special issue of Synlett on prebiotic chemistry). In a popular view among scientists, it is suggested that the life has emerged from a single kind of polymers, either RNA polymer (RNA world) or peptide polymers (Peptide world). However, as we know that there are limitations that face each of these possibilities (explained in state of the art), we have instead considered the possibility of mixed structures involving amino acid as well as nucleotide monomers bound by phosphoramidate and ester linkages. Experimental reactivity studies supporting the kinetic relevance of such structures as well as the availability of plausible chemical processes allowing their formation are presented in **chapter 4** (submitted for publication). In the last chapter, an experimental attempt performed with the aim of improving the preceding process by facilitating aminoacylation in folded oligomers is described (**Chapter 5**).

Most of the work reported in this thesis is the result of collaborative efforts from which it is difficult to separate my contribution without making the reading uneasy. The experimental work described in chapters 1 and 5 has mostly been carried out by myself. I made a significant experimental contribution to the analysis of the reaction of *N*-carbamoyl amino acids (chapter 2), to the aminoacylation of nucleotides by NCAs and oxazolones (chapter 3 and 4) and to the kinetics of hydrolysis of esters and phosphoramidates including the influence of temperature (chapter 4).

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State of Art

Origins of life and prebiotic chemistry:

The issue of the origin of life includes many questions. When, where and how did it happen? Was it the result of chance and/or necessity? Did it start early and through simple system? Did it occur once only or several times, on the Earth or elsewhere?

The investigation of the origin of life is separable into historical and ahistorical factors, as suggested by scientists (**see for example, Pross et al. 2016**). The historical approach is difficult or even impossible to achieve because the remaining evidence is little or even nothing as, for instance, about the geochemical conditions on the early earth's surface. Although the fossil record is rich, those older than 2.5 billion years (Ga) (Hadean) are scarce, and their significance is debated because of their possible attribution to abiotic self-organization patterns and of further contamination. However, from the ahistorical side, there are possibilities to understand the physicochemical driving forces that could have led to the origin of life. In the light of these concerns, Albert Eschenmoser wrote that "Biogenesis, as a problem of science, is lastly going to be a problem of synthesis. The origin of life cannot be "discovered," it has to be re-invented" (**Eschenmoser, 2007**). As we see, we cannot give precise answers about where or when questions. The exact pathway through which the origin of life happened will very probably remain unknown as well. We can only suggest plausible prebiotic scenarios. Prebiotic chemistry is a branch of chemistry concerned with early chemical systems that preceded the appearance of life. It involves reactions yielding molecules of biological interest according to models describing the conditions of the primitive Earth (chemical composition, pH, temperature). All these chemical reactions occurring in a far from equilibrium environment are then making it possible to form dissipative systems capable of primary evolution.

Birth of prebiotic chemistry: (1920-1953)

Prebiotic chemistry has been developing as the experimental search for the chemistry of life's origin for almost a century now. The conceptual roots of this field can be found in the writings of the Russian biochemist A.I. Oparin who for the first time expressed views on a natural chemical origin of life on Earth. He hypothesized "life would have appeared according to a gradual evolution of primary organic substances" (**Oparin, 1924**). Oparin's hypothesis highlights that chemical reactions were possible in a primitive Earth environment which was very different from today's environment, and explains that it is no longer possible to witness this kind of reactions nowadays because

of the very active molecules (including O₂) generated by our biosphere and which occupy our environment (**Oparin and Fesenkov, 1956; Oparin, 1976**). Miller experiment gave a glow to the Oparin's hypothesis. In 1953, an experiment was performed by Miller as a result of which organic matter including amino acids was formed starting from a mixture of reducing gases (CH₄, H₂, NH₃, H₂O) subjected to electric sparks (**Miller, 1953**).

Developments of prebiotic chemistry: (1953-2000)

After the Miller experiment was published, numerous investigations on abiotic processes leading to the formation of building blocks were initiated. In parallel, the developments of molecular biology led to a new theory towards the origins of life which is the RNA world hypothesis (gene-first hypothesis), where RNA plays both the role of storing the genetic information and a catalytic function (**Gesteland et al., 1999**). Other researchers supported a peptide world hypothesis (metabolism first hypothesis) (**Rode, 1999; Rode et al., 1999**) based on peptides in a way consistent with the possibility of an abiotic formation of amino acids demonstrated by Miller. However, none of these hypotheses is devoid of difficulties. The RNA world can be seen as a "molecular biologist's dream" as well as a "prebiotic chemist's nightmare" (**Joyce and Orgel, 1999**) owing to the many tricky synthetic problems inherent to the abiotic formation of long enough RNA strands. The formation of the phosphodiester bond in abiotic ways is a complicated issue, especially when considering the control of the 3'-5'-linkage regioselectivity between monomers (**Lohrmann and Orgel, 1978**).

On the other hand, a peptide world hypothesis hardly explains how peptide sequences selected by evolution could be stored and replicated without the possibility of template replication and then translated back into nucleic acid sequences. Hypotheses based on a metabolic network barely yield an entity which is characterized as evolvable starting from a system of small molecules, because there will be no possibility of variation in the system (**Vasas et al., 2012**). On the contrary, RNA-based genetic replicators could undergo replacement of bases constituting mutations that might allow for the selection of improved abilities. So if we want to talk about evolutionary processes, a purely metabolic system is highly improbable.

The modern chemical evolution approaches: (2000- present)

Modern approaches were developed to overcome the drawbacks mentioned above for both the RNA or peptide world hypotheses. Systems Chemistry field is now an emerging field (**Ludlow and Otto, 2008**). It mainly focuses on the interactions between different subsystems (peptides, nucleic acids, lipid membranes) that could lead to complex systems (**Ruiz-Mirazo et al., 2014**). It takes into account that a high diversity of precursor components should have been available on the primitive Earth. These components could progressively transform, or be incorporated into, primordial metabolic, self-replicating, and membrane-isolated subsystems by different pathways. Then the cooperation between these subsystems (peptides, nucleic acids, fatty acids) might have led to the emergence of new properties.

The importance of systems chemistry is not only about the combination of the different subsystems (peptides, nucleic acids, lipids) leading to the emergence of complex systems. The experimental investigation of chemical processes is indeed likely to provide information on how evolution could take place. For example, the cooperation between peptides and nucleic acids can give us information on how the evolution of the translation process could have occurred (**Biron et al. 2005**).

Formation of the Early Earth:

With the aim of verifying the precedent hypotheses, many scientists have undertaken experimental investigations. Astronomical observations and geological investigations have therefore been carried out to determine the state of the primitive Earth, vital prerequisite to know more about the prebiotic environment.

The Earth formed soon after a nebula collapsed into the solar system 4.57 Ga ago. It is generally accepted that rather than being a slow and continuous accretion process, the formation of our planet occurred through the collision of planetesimals. The heat released during the accretion of these small bodies would have led them to a melted state in which most of the volatile components would have been lost into space. Then, the Earth evolved as a result of different processes.

After the formation of the Earth, a global differentiation happened, where the core comprised of 85-90% iron and nickel was formed. Its properties led to the advent of a magnetic field responsible for protecting the atmosphere from being blown away by the solar wind. Therefore it was essential to protect the constituents of the surface (**Sotin, 2005; Cockell, 2015**).

Subsequently, the Earth underwent slower geophysical transformation leading to the separation of a continental crust floating on a denser mantle and then of wide areas of emerged lands. This process was associated with the emergence of plate tectonics and subduction that played a significant part in the formation of the atmosphere and the recycling of its components. Most water vapor and gases could have been lost during the formation of the Earth, which could then have originally been dry and without a significant atmosphere that formed later.

Early Earth Environment:

A better understanding of the composition of the early environment is important to account for the evolution of life. The constitution of the soil and the atmosphere has great importance in the formation of living systems. The composition and nature of the atmosphere, the temperature of the ocean and on the surfaces, or the pH of the water on the surface are essential data, and some of them are still unknown and subject to debate.

- ✓ **The composition of atmosphere:** The early earth atmosphere was different from the current atmosphere. Urey considered that the atmosphere was identical to that of giant planets that conserved the gas composition of the pre-solar nebula (H_2 , H_2O , NH_3 , CH_4) rich in H_2 . Actually that hypothesis turned out to be inaccurate. As mentioned above, the formation of rocky planets included different stages at which volatile elements (and especially H_2) were degassed. At the time being, prevailing views acclaim that the atmosphere was weakly rather than strongly reducing where it was mainly composed of CO_2 and N_2 (**Kasting, 1993**) with saturated water vapor in equilibrium with a liquid ocean (**Wilde et al., 2001**). Today the partial pressure of N_2 is 0.8 bar, and usually, the researchers assumed that it was stable for 4.4 Ga (**Tolstikhin et al., 1998**). But now a substantial amount of nitrogen is found in the continental crust; it originates from the trapped organic matter of the degraded dead living beings. After this observation, there is uncertainty about the content of N_2 in the primitive atmosphere; there is a suggestion that it is more likely 0.5 bar or more by double or triple (**Som et al., 2016**). Methane could also be present as a minor component, but there is uncertainty on the amount that could have been present between 4.4 and 4.0 Ga (**Gargaud et al., 2012**). The current amount of oxygen (21%) is entirely different from the one that was present on early earth (5 orders

of magnitude below the present atmospheric level) (**Holland, 2006**), it started to rise 2.5 billion years (Ga) ago due to the activity of some living things capable of oxygenic photosynthesis (**Kasting et al., 2002**).

- ✓ **pH:** Cooling the surface of the earth allowed the condensation of water vapor, which led to oceans. It is reasonable to consider that the initial pH of the primitive ocean was weakly acidic (4.8-6.5) due to dissolved CO₂ at equilibrium with higher partial pressures in the atmosphere (**Kasting, 1993; Zahnle, 1998; Cockell, 2015**). While the today's pH is ~8 (current partial pressure of CO₂ 4 x 10⁻⁴ bar).
- ✓ **The temperature of the early Earth:** Isotopic measurements made on silica indicated that the temperature of the early ocean could be as high as 70°C (**Robert and Chaussidon, 2006**). This statement seems to be in contradiction with the fact that the young-sun luminosity was 27 % lesser than today (**Gargaud et al., 2012**). Under these conditions, most water on the surface of the earth would have been frozen (glaciation). Therefore, in this case, there is a need for a robust greenhouse effect for the compensation of the temperature and to avoid a global glaciation. Moreover, high temperatures in the ocean have more recently been challenged by geochemical investigations (**Marin-Carbonne et al., 2014**). In the end, no definitive conclusion can be made before the composition of the atmosphere between 4.4 Ga and 2.5 Ga is better known by scientists. Therefore, the temperature on the early earth still constitutes a subject of debate.

Sources of organic matter:

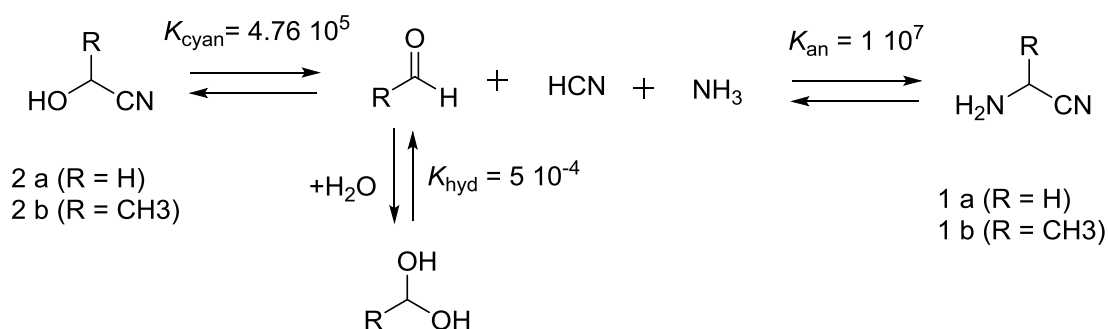
The organic molecules present on the primitive Earth can be divided into different categories according to their origins: the ones that were delivered by extraterrestrial bodies, the ones that were synthesized on Earth from inorganic carbon and sources of energy (UV, electric discharges). We are specifically interested here in the formation of amino acids and nucleotides as building blocks for the chemistry we aim to study here.

- ✓ **Extraterrestrial sources:** It is well proven that the meteorites and the comets that crashed on Earth carried a massive quantity of molecules of biological interests (**Mullie and Reisse, 1987**). It has been taken into consideration that an extended meteorite flux on the primitive Earth introduced a significant

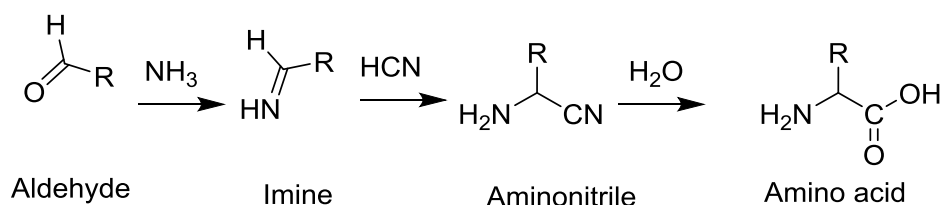
quantity of organic matter, such as α -amino acids. A considerable amount of amino acids was discovered in meteorites belonging to the chondrite family, which have suffered much less transformation during the formation and the evolution of the solar system (**Pizzarello and Shock, 2010**). Further, comets could be another extraterrestrial source of organic matter including amino acids. This was confirmed by experimental simulations on cometary ice analogs yielding a mixture of amino acids within the laboratory (**Meinert et al., 2012**). Moreover, organic matter was made artificially by simulating space conditions prevailing in molecular clouds (**Danger et al., 2016**). Particles made of nearly 50 percent organic matter in mass were discovered indeed during the Rosetta mission to the comet 67P/Churyumov-Gerasimenko (67P), (**Bardyn et al., 2017**). Moreover, potential prebiotic activating agents like cyanamide and cyanoacetylene were found in the interstellar medium (**Thaddeus, 2006**).

- ✓ **Endogenous sources:** Nonetheless, those molecules should have also been formed “in situ” from inorganic precursors on the surface of the Earth. Miller and Urey performed their experiment in 1953. They hypothesized that the condition of the early Earth atmosphere was strongly reducing. Their experiment represents a closed system containing heated water and a mixture of gases (H_2 , CH_4 , NH_4 , and H_2O). Then the mixture of gases was sparked with electricity. After two weeks, they found that organic molecules were formed including especially amino acids. A variety of experiments were performed under a wide range of conditions leading to the formation of organic building blocks starting from inorganic precursors. However, the strongly reducing (rich in H_2) composition of the atmosphere selected by Miller was considered later as non-realistic (**Kasting, 1993**). Though results indicating that atmospheres based on CO_2 and N_2 are much less productive have been published (**Schlesinger and Miller, 1983**), the issue was reconsidered in further experiments, which showed that the formation of amino acids was possible even by using less reducing conditions (**Cleaves et al., 2008**). Moreover, since Miller’s experiment, many reactions were performed in the field of prebiotic chemistry over the years. All the organic building blocks related to life have been formed including nucleotides, fatty acids, and amino acids (including their derivatives). Many prebiotic processes for the formation of amino acids and *N*-carbamoyl amino

acids are proposed, especially the Strecker and Bucherer-Bergs reactions, in which the DSBC group have had significant contribution in understanding their mechanisms since 1980 (**Rousset et al., 1980**). Taillades et al. reported that the system of three components (HCHO/NH₃/HCN) leads to an equilibrium between hydroxylacetonitrile (>99.9%) and aminonitrile (<0.1%) in the assumed prebiotic conditions ($T = 25\text{ }^{\circ}\text{C}$, $\text{pH} = 8$) (**Scheme 1, Taillades et al. 1998**). Aminonitrile formed through Strecker pathway (**Scheme 2**).



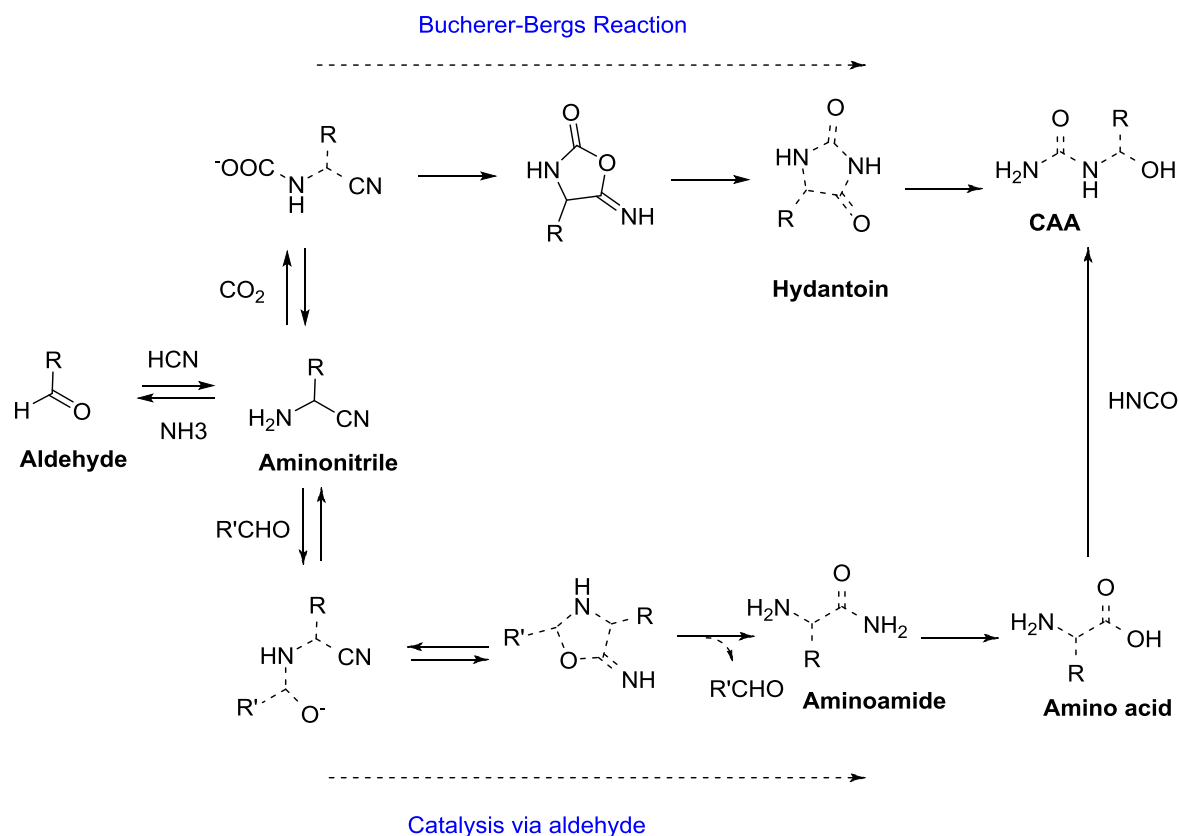
Scheme 1. Initial equilibria (1) of the system (RCHO/HCN/NH₃) in diluted aqueous solutions at $T = 25\text{ }^{\circ}\text{C}$ and $\text{pH} = 8$. K_{cya} , K_{an} and K_{hyd} are given for $\text{R} = \text{H}$.



Scheme 2. Strecker Reaction: synthesis of amino acid through aminonitrile precursor.

Moreover, they have shown that the reaction of the aminonitrile with prebiotic reagents (formaldehyde or carbon dioxide) could shift the equilibrium toward the formation of aminonitrile according to the ‘‘Le Chatelier’s’’ principle. Aminoamides or hydantoin are produced first after the reactions of aminonitrile with prebiotic reagents (formaldehyde or carbon dioxide), respectively (**Scheme 3**). The formation of hydantoin via the Bucherer Bergs reaction is easier than the formation of the aminoamide since carbon dioxide was more readily available from the primitive hydrosphere than formaldehyde. The hydrolysis of hydantoin leads to the formation of *N*-carbamoyl amino acids while the hydrolysis of aminoamide leads to the formation of amino acid (**Scheme 3**).

Moreover, in 2001, they have shown that *N*-carbamoyl amino acids could also be formed easily by the reaction of amino acids and cyanate (**Taillades et al. 2001**). The abiotic synthesis of nucleotides is much more difficult. However, Powner et al. successfully established a plausible prebiotic synthesis of pyrimidine ribonucleotides by successive steps starting from a 2-aminooxazole intermediate (**Powner et al., 2009**). Moreover, in 2015, the group of John Sutherland showed that precursors of amino acids, two (of four) ribonucleotides, as well as glycerol-1-phosphate, a precursor of lipids, could be formed through a single process starting from hydrogen cyanide (HCN) and hydrogen sulfide (H₂S) under ultraviolet irradiation (UV) in the presence of inorganic catalysts (**Patel et al., 2015**).



Scheme 3. Reaction scheme of the formation *N*-carbamoyl amino acids (CAA) via the hydrolysis of aminonitriles promoted by CO₂ (Bucherer-Bergs Reaction) or by an aldehyde.

From all of the above-mentioned reasons, we can derive that different organic molecules of biological interest could be formed through abiotic pathways. The diversity of molecules that could be formed in the same location is fascinating, which supports the system chemistry approach. John Sutherland in his paper "studies on the

origin of life - the end of the beginning” gives his opinion and suggests that the first phase (the beginning) may have nearly finished (**Sutherland, 2017**). He means by the first phase that a diversity of organic molecules becomes available and now we should focus on the second phase corresponding to the construction of complex biochemical systems. Indeed, the presence of organic matter alone does not infer that life must have emerged, there are other requirements for the presence of life.

Sources of Energy:

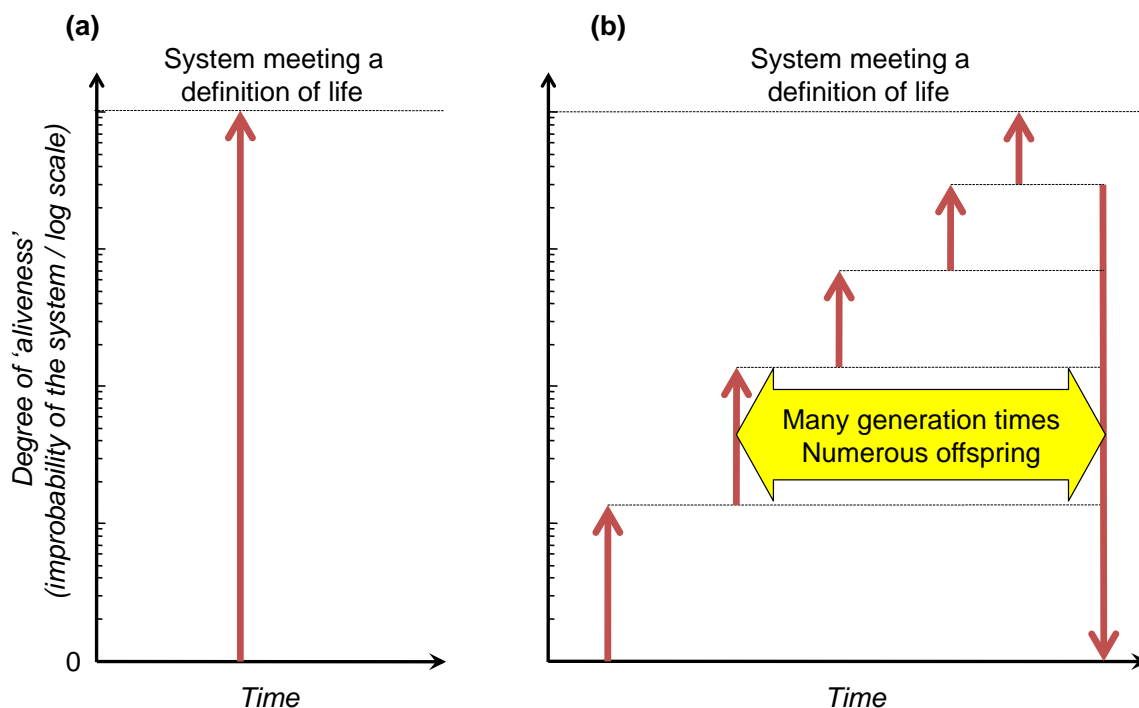
We can reasonably assume that the energy sources present on the primitive Earth are the same as the current ones (**Deamer and Weber, 2010**).

- ✓ Solar radiation: visible, UV (absorbed by the atmosphere)
- ✓ Cosmic radiations
- ✓ Geological sources: (volcanism, hydrothermal vents)

In contemporary life, most of the organic matter present on the Earth is produced by photosynthesis. The pigments capture visible light, and a complex system, involving redox processes, proton translocation and enzymatic complexes, makes use of it by producing chemical energy under the form of ATP while, at the same time, oxidizing water and generating reducing power that subsequently converts inorganic carbon into organic matter. Other metabolisms, such as respiration or fermentation, have also been identified to generate chemical energy used by the metabolism. However, until now, no plausible prebiotic way has been discovered for gathering energy in a prebiotic environment and coupling this process with the development of the protometabolism of a self-organizing entity. Consequently, a critical question to be answered in the investigations to understand the origin of life is how is it possible to convert these sources into a form that can contribute to the metabolism of an early life form?

Complexification starting from chemical systems: (the driving force towards complexity)

Theoretical studies (kinetic and thermodynamic analyses) strongly suggest that life has not emerged from an abrupt transition (**Scheme 4a**), it could probably have emerged in a gradual process (**Scheme 4b**). In scheme 4b, the development of the life should take place via a series of consecutive entities that could be represented as “partially alive” (**Bruylants et al., 2011; Pascal et al., 2013**).



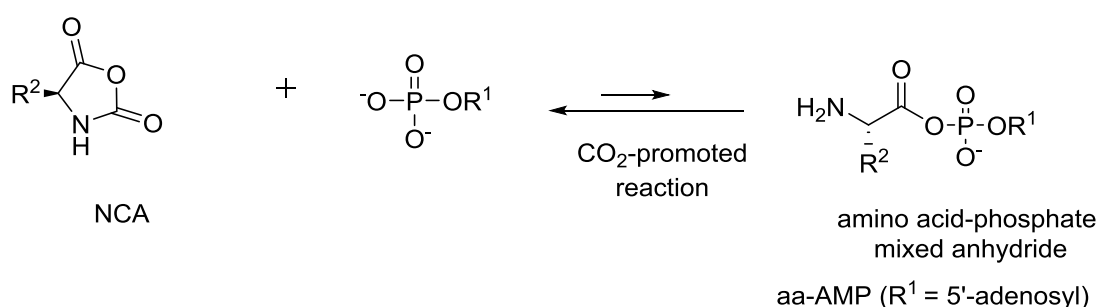
Scheme 4. The emergence of life considered as a transition to a highly improbable system. **a)** Abrupt transition induced by a highly improbable random event in contradiction with the 2nd Law of thermodynamics; **b)** Stepwise process in which intermediate steps (there is in principle no limitation to the number of steps) allow further evolution towards greater degrees of organization on the basis of entities that are capable of reproducing themselves and, therefore, that exhibit a significant persistence before reverting to the unorganized state (right arrow) (**Pascal et al., 2013**).

These populations of entities should be stable in far from an equilibrium state to undergo further evolutionary changes (**Pross and Pascal, 2013**). The stability of these entities is of a particular kind, called dynamic kinetic stability DKS; It is specific to entities that can replicate and thus grow exponentially (**Pross 2009, 2011, 2016**). Maintaining DKS maximization as a driving force in simple chemical systems (*i.e.* replicating entities, autocatalysts) requires a kinetic environment able to supply energy

and in this way the system will be held in a far from equilibrium state; As a conclusion, reproduction of the entities must be via an irreversible cycle (**Pascal 2012, 2013; Pross and Pascal 2017**). A cost of irreversibility should be paid to hold the systems in a far from equilibrium state and allow them evolving toward the living state; this must be made by dissipating energy in the medium (**Pascal and Boiteau, 2011; Pascal, 2012, 2013; Pross and Pascal, 2017**). This approach shows that coupling free energy to the system is not only needed for the synthesis and the polymerization of building blocks but that the most of it should merely be dissipated to ensure the prerequisite for kinetic irreversibility and that robust activation processes should direct the process of self-organization. Our group has been involved in a long-term investigation of prebiotic processes for the chemical activation of α -amino acids and peptides as *N*-carboxyanhydrides (NCAs) and oxazolones, respectively (**Danger et al., 2012**). The role of these high-energy intermediates (NCAs and oxazolones) is worth to be emphasized in the context of strongly activated energy carriers.

Activated energy carriers in the chemistry of α -amino acids:

NCAs are energy-rich intermediates which are formed by the strong activation of amino acids. Their role in the prebiotic chemistry of amino acids is supported by a body of evidence including the abiotic formation of random peptides (**Leman et al., 2004; Pascal et al., 2005**). Additionally, by reaction with phosphate esters (5'-AMP) or inorganic phosphates (**Scheme 5**), they spontaneously yield adenylates (aa-AMPs, one of the crucial intermediates in the contemporary peptide biosynthesis) or other mixed anhydrides (**Biron and Pascal 2004; Biron et al., 2005; Leman et al., 2006**).



Scheme 5. Formation of mixed anhydrides including adenylates (aa-AMP) from the NCAs.

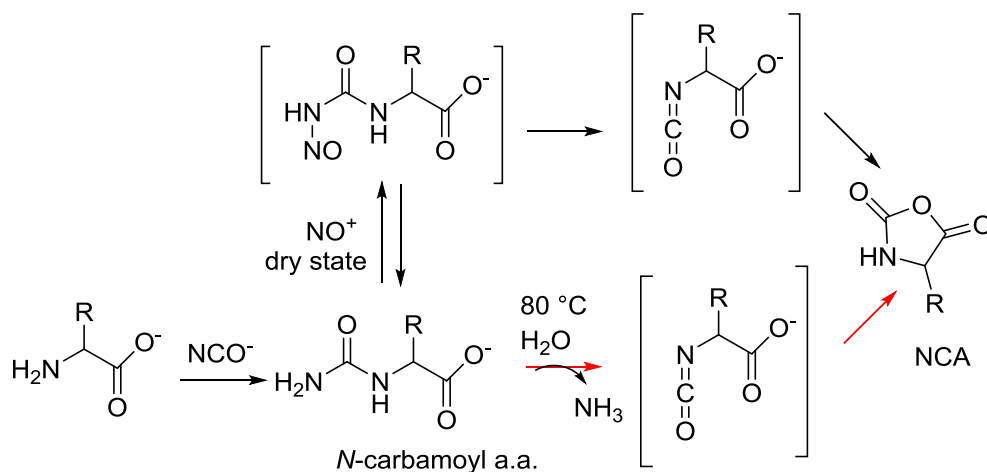
aa-AMPs possess free energies of hydrolysis values ($\Delta G^\circ = \text{ca. } -70 \text{ kJ mol}^{-1}$) among the highest of the metabolites present in the cells (**Wells and Fersht, 1986; Pascal et**

al. 2005). More recently, similar reactions of 5(4*H*)-oxazolones (considered as strongly activated peptides) have been described (**Liu et al., 2014, 2016**). The main processes for the prebiotic formation of NCAs and oxazolones described in the literature are listed below.

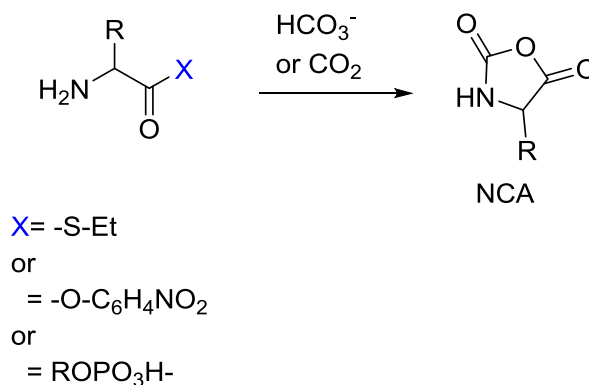
✓ **Formation of *N*-carboxyanhydride intermediates (NCAs):**

NCAs can be formed in different ways compatible with the presumed prebiotic conditions.

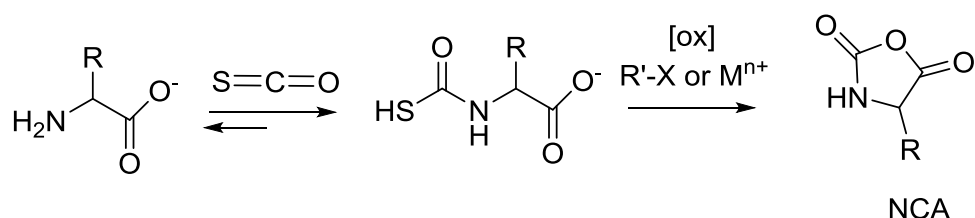
- 1) From *N*-carbamoyl amino acids by nitrosation with nitric oxide (NO) and oxygen in a dry state (**Taillades et al., 1999**). It is worth noting that heating neutral aqueous solutions of *N*-carbamoyl amino acids at 80°C also yield peptides *via* NCAs (**Danger et al., 2006**).



- 2) Activated amino acids such as thioesters, *p*-nitrophenyl esters or mixed phosphoric carboxylic anhydrides in the presence of CO₂ are readily converted into NCAs: (**Brack et al., 1987; Liu et al., 2014**).

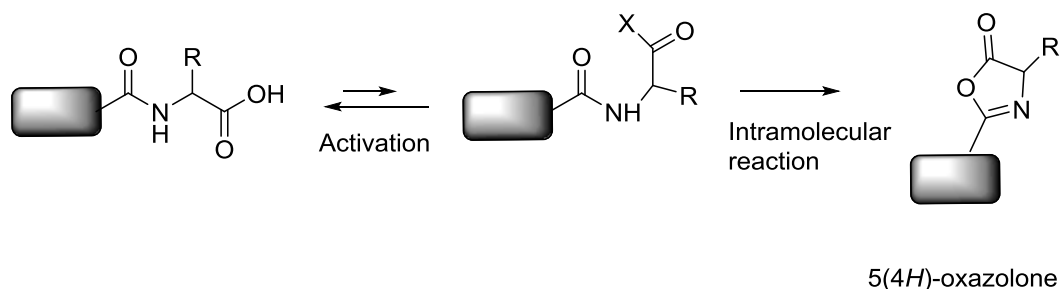


- 3) They could be formed by the reaction of the amino acids with carbonyl sulfide reagent (COS) in the presence of oxidizing or alkylating reagents and transition metal ions (**Leman et al., 2004, 2006**). It is noteworthy to mention that COS has been identified in volcanic gases.



✓ **Activation of the C-terminus in peptides: (formation of the 5(4H)-oxazolone intermediates)**

Another way for the abiotic formation of peptides is through 5(4H)-oxazolone intermediates. In aqueous solutions, the oxazolone intermediates can be formed by the activating the C-terminus group of peptides (free dipeptide or *N*-protected ones) by strong activating agents (**Scheme 6**) (**Danger et al., 2012; Beaufils et al., 2014, 2016**).



Scheme 6. Formation of 5(4H)-oxazolones.

Formation of peptides via physical pathways:

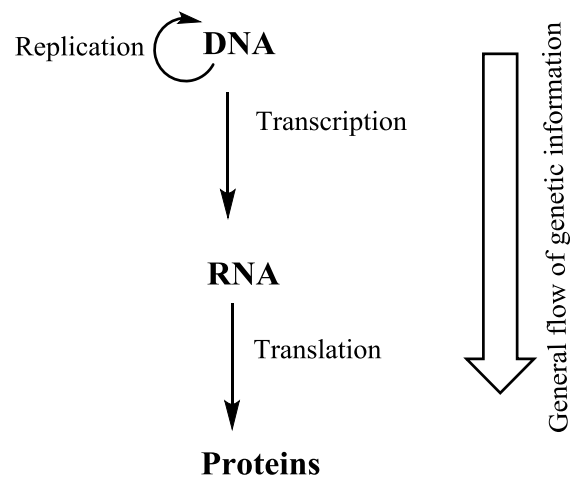
However, there are other ways for the formation of peptides. It takes place through plausible physical ways of activation (drying, adsorption, heat). It has been shown that peptide could be formed by dehydration or by the adsorption of amino acids on clays or other minerals. (**Rohlfing, 1976; Lahav et al., 1978; Rimola et al., 2007; Lambert, 2008**). Moreover, elongation of oligopeptides by heating aqueous solutions of amino

acids to temperatures of 200–250 °C was demonstrated in experiments that simulate a submarine hydrothermal system (**Imai et al., 1999**). A team of researchers showed that through mild wetting/drying cycles of a mixture of amino acids and hydroxy acids, longer peptides could be formed under plausible prebiotic conditions. The peptide bonds were formed via an ester intermediate undergoing a further ester-amide exchange (**Forsythe et al., 2015; Yu et al., 2017**). However, even though these processes yield peptides, they cannot be the basis of energetic metabolism or the basis of the chemistry leading to the development of translation, which requires a process leading to energy-rich adenylates.

The common chemistry of amino acids and nucleotides – Connecting the activation processes:

✓ Modern biology – the Central dogma of molecular biology:

Despite the complexness of life, molecular biology describes life in a quite simple way through its central dogma, which was proposed by Crick (**Scheme 7; Crick, 1970**).



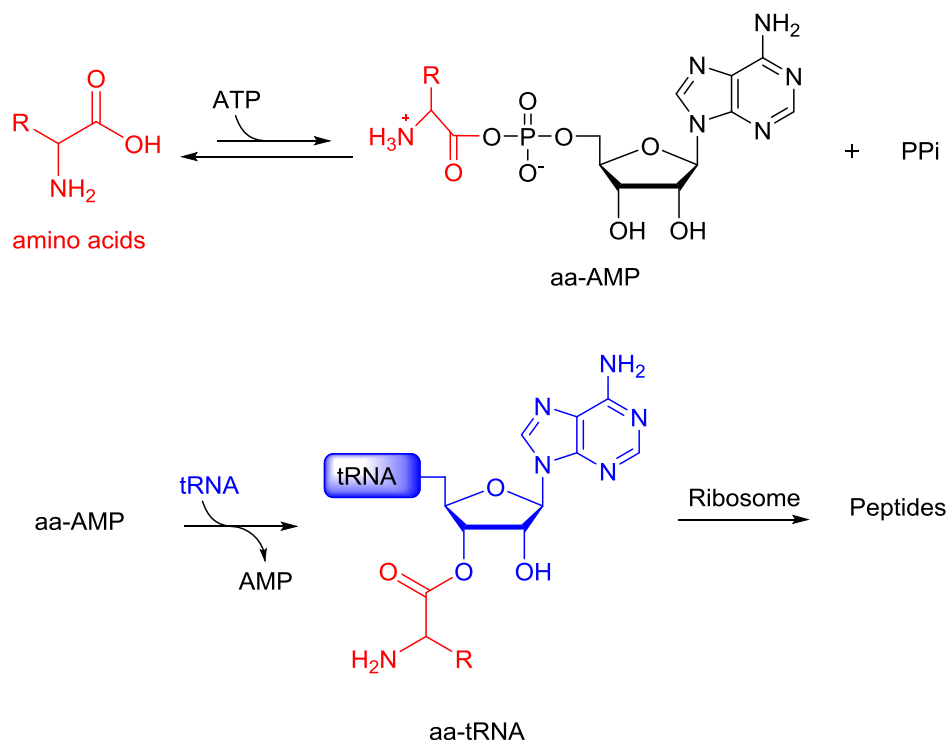
Scheme 7. The central dogma of molecular biology.

The central dogma explains the flow of genetic information. Once it is translated into proteins, it could not go back towards nucleic acids. In all living beings, the genetic information is stored in DNA. The genetic data in the DNA copied by replication process. Then DNA information can be copied into mRNA in a process called transcription.

Moreover, then using the information in mRNA, proteins can be synthesized at the ribosome in a process called translation. The translation step is very important making it possible for the functional expression of most of the information carried by genes. Even though the central dogma is simple it is the result of the catalytic activity of complex enzymes and more importantly of one of the more complex molecular machinery found in the cell, the ribosome and the aminoacyl-tRNA synthetases (aaRS) responsible for the genetic code and the accuracy of the translation process.

✓ **Activation of the amino acids during the biochemical synthesis of peptides:**

Protein biosynthesis starts with the activation of amino acids by ATP, which leads to adenylates (aa-AMPs) that are subsequently converted into esters of tRNA (aa-tRNA) (**Scheme 8**). In this process, the aa-AMPs are formed by reaction of amino acids and ATP in the presence of a family of enzymes, aminoacyl-tRNA synthetases (aaRSs).



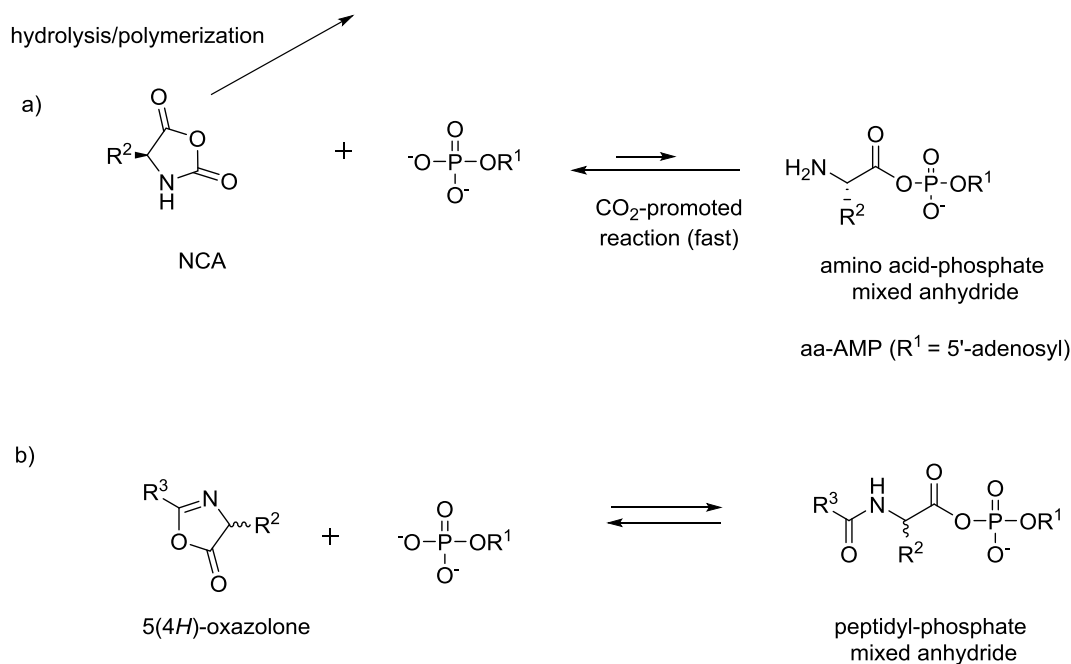
Scheme 8. Activation of the amino acids during the biochemical synthesis of peptides.

At equilibrium, aa-AMPs are formed in negligible quantities from ATP and amino acids (**Wells and Fersht, 1986**). However, aa-AMPs usually remain sequestered in the aaRS enzyme where they are stabilized and can be

processed further. Moreover, the reverse reaction, although being thermodynamically favorable, is not spontaneous (requires catalysis). These observations rule out the possibility that both thermodynamic and catalytic role of aaRSs could have emerged together by the gradual improvement of an abiotic process starting from ATP.

✓ **Alternative prebiotic pathways for the formation of adenylates or other mixed anhydrides:**

Alternative pathways involving strongly activated intermediates (NCAs and oxazolones) for the formation of mixed anhydrides (including aa-AMPs) have been suggested (**Pascal et al., 2005; Pascal and Boiteau, 2011**). In diluted neutral aqueous solution, mixed anhydrides (including aa-AMPs) can be formed from the spontaneous reactions of inorganic phosphates and phosphate esters with NCAs and 5(4*H*)-oxazolones (**Scheme 9**) (**Biron and Pascal 2004; Biron et al., 2005; Leman et al., 2006, Liu et al., 2014**). In a study of the behavior of mixed anhydrides in neutral aqueous solutions, Liu et al. demonstrated that in the presence of CO₂ (even in low amounts), aa-AMPs hydrolyzed fastly through the formation of NCAs (**Scheme 9a**) (**Liu et al., 2014**). It is important to mention that the presence of CO₂ not only increases the rates of hydrolysis but also the yield of peptides because of the easy polymerization of NCAs. Therefore, the peptides are mainly formed by the polymerization of NCAs and not by direct polymerization of aa-AMPs. They concluded that mixed anhydrides (aa-AMPs) could hardly be involved in abiotic peptide formation (**Liu et al., 2014**).



Scheme 9. Potential pathways for the abiotic formation of mixed anhydrides of α -amino acids and peptides. **a)** Formation of mixed anhydrides from NCAs; **b)** Formation of mixed anhydrides from oxazolones.

This observation raises a paradox about the evolution of translation: How could adenylates be selected as peptide precursors despite their unlikely prebiotic role? Moreover, the facility of the reversible conversion of adenylates into NCA must be considered as an indication of the importance of strong activation processes for the origin of life and for the development of combined chemistry of amino acids and nucleotides. These unresolved issues have been addressed during the work reported in this thesis.

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Part 1

**Towards efficient activation processes of α -amino acids
and peptides as NCAs and 5(4*H*)-oxazolones**

Chapter 1. A reassessment of prebiotically relevant chemical agents for the activation of α -amino acids and peptides

Provisionally accepted at *Journal of Systems Chemistry*

Overview:

Living organisms are made up of essential building blocks (amino acids, nucleotides, fatty acids). The oligomerization of these building blocks yields biopolymers (peptides, nucleic acids, lipids), which are responsible for the primary functions of the living cells. These different subsystems (biopolymers) interact to a higher degree of complexity constituting living organisms (**Ruiz-Mirazo et al., 2014**). The self-organization of this high level of functional integration is the major issue that must be understood to account for the origin of life. Self-organization in chemical systems (including the formation of biopolymers) must take place through the emergence of dissipative processes, requiring efficient activation pathways. This work is based on an approach of the origin of life through stages of increasing complexity taking advantage of the dynamic kinetic stability (DKS) that can be expressed by replicating entities (**Pascal et al. 2013; Pross, 2016**). The replication process must work irreversibly for selection to work and, therefore, an evolutionary process to be efficient, which means that activation pathways must fuel the reproduction loop and that the corresponding energy must be dissipated for the loop to become irreversible (**Pascal and Boiteau 2011; Pascal 2012, 2013; Pascal et al. 2013; Pross and Pascal 2017**).

Pathways for the formation *N*-carboxyanhydrides (activated forms of amino acids) in an origin of life perspective have been proposed in earlier studies (**Taillades et al. 1999; Leman et al., 2004; Danger et al., 2006**) (**developed more deeply in the state of art**). While, regarding the formation of 5(4*H*)-oxazolones (activated form of peptides) the situation is less clear since carbodiimides (EDC) are not likely reagents in an origin of life perspective justifying an additional study of more realistic substitutes (**Danger et al., 2013**).

Therefore, we have investigated different prebiotic activating agents, which were found in the literature as active in the formation of peptide bonds (**Hulshof and Ponnampereuma, 1976**). We carried out a series of experiments under conditions reproducing that of prebiotic environments: reagents at mM levels in aqueous solution,

pH range 5-7. We have used a system capable of detecting strong activation by observing the epimerization associated with the conversion of free or acylated dipeptides into 5(4*H*)-oxazolones (**Beaufils et al., 2014**). The measurement of the ratio of epimerization of the starting material after being subjected to potentially activating conditions constitutes a way to monitor the activation process that involves the chirally instable 5(4*H*)-oxazolone.

Our survey shows that none of the readily available reagent or system of activation selected in the literature could be able to promote strong activation by direct interaction with free carboxyl groups and thus to feed a dissipative process. However, this must not be considered as a definitive conclusion, and the identification of activation processes remains a crucial goal in this field to offer alternatives to the already well-established possibility of formation of amino acid *N*-carboxyanhydrides.

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Article 1.

A reassessment of prebiotically relevant chemical agents for the activation of α -amino acids and peptides

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Supporting information for this article will be found online on the journal website.

Abstract

In an origin of life perspective, processes able to provide energy to feed self-organizing systems are required for the formation of their own components but also to maintain a far from equilibrium state essential for the system to remain under kinetic control. α -Amino acid *N*-carboxyanhydrides (NCAs) and 5(4*H*)-oxazolones represent activated intermediates of the chemistry of peptides that may be involved in the self-organization of life and in the evolution of translation. An experimental survey of previously published activating agents has been carried out using the epimerization associated with the transient formation of 5(4*H*)-oxazolones as a tool to monitor their potential to promote strong activation. None of reagents investigated in this survey proved to be totally satisfactory demonstrating that the identification of activation processes remains a crucial goal in this field.

Keywords

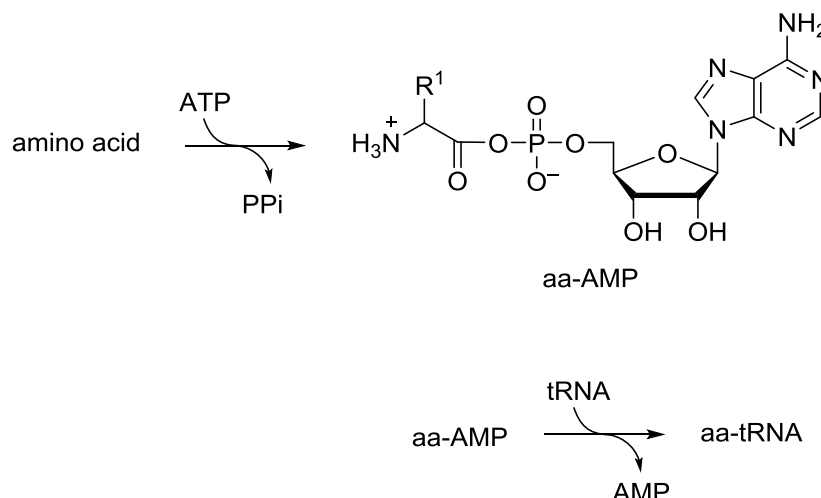
peptides; activating agents; self-organization; origin of life.

Introduction

Life's complexity arose from a limited number of building blocks, including α -amino acids, nucleotides, or fatty acids. The abiotic polymerization or self-assembly of these monomers or their derivatives gave birth to the three subsystems (peptides, nucleic acids, and lipid membranes) that are responsible for the main functions observed in living cells. Complex systems could therefore be formed from their interactions, which resulted eventually into the self-organization of the first living organisms (Ruiz-Mirazo et al., 2014). In addition to the requirement for building blocks, polymers and supramolecular assemblies as material components for building the living entities, the emergence and evolution of life correspond to a state in which change is driven by a continuous research for increased Dynamic Kinetic Stability (DKS) (Pross, 2016). This behaviour is expressed by entities able to reproduce themselves and thus to grow exponentially when resources are not limited. Maintaining a drive towards higher DKS requires an environment able to provide energy and thus to keep the system in a far from equilibrium situation. An analysis of the kinetic requirements for the occurrence of DKS as a driving force in simple chemical systems (*i.e.* replicating entities, autocatalysts) led to the conclusion that the entities must be reproduced through a cycle that proceeds irreversibly (Pascal 2012, 2013; Pross and Pascal 2017). As a result, chemical systems capable of evolving toward the living state would have to spend a cost of irreversibility by dissipating energy and making the reverse reaction (associated with high activation barriers) slow enough compared to the time scale of the reproduction cycle (Pascal and Boiteau 2011 a; Pascal 2012, 2013; Pross and Pascal 2017). This approach suggests that energy must not only be provided to the system to allow for the synthesis of biomolecules or the polymerization of building blocks but must, for the most part, be dissipated to ensure this requirement for kinetic irreversibility and that strong activation processes should be involved in the process of self-organization.

For that reason, sources of free energy are required to constrain self-organization as far as the scope of the chemical perception of the origin of life is concerned. This analysis is supported by considering peptide biosynthesis, shared with minute differences by all extant living organisms, which reveals one of the paradox of the life's emergence and early evolution. Among the intermediates of the process, aa-AMPs, known as aminoacyladenylates (Scheme 1), are produced by a reaction of ATP with

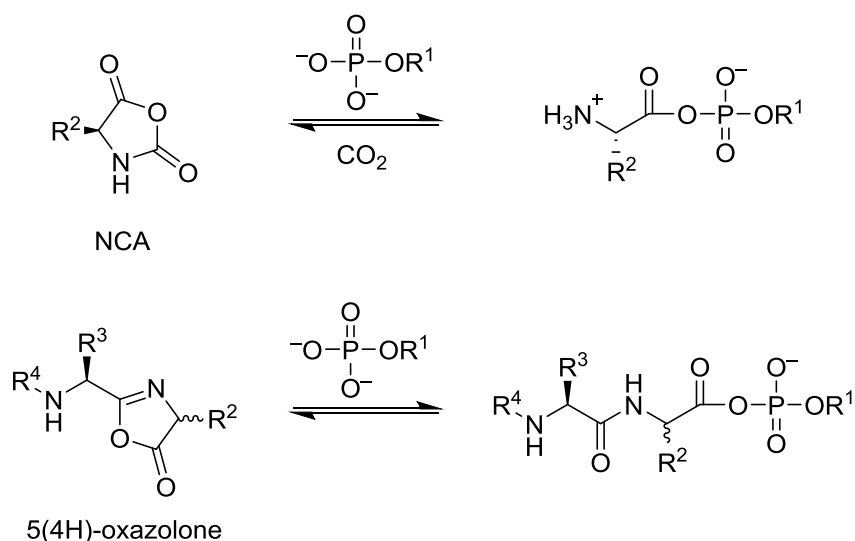
amino acids in the presence of a set of aminoacyl-tRNA-synthetases (aaRSs) the enzymes responsible for the reaction.



Scheme 1. The biochemical pathway of activation of amino acids. tRNA aminoacylation by the two step reaction of α -amino acids via aminoacyl adenylate (aa-AMP) intermediates.

aa-AMPs possess free energies of hydrolysis values (ΔG° = ca. -70 kJ mol^{-1}) among the highest of the metabolites present in the cells (Wells and Fersht, 1986). The aaRS enzymes not only increase the rate of formation of adenylates from ATP and free amino acids owing to their kinetic activity but also stabilize the intermediate that remains sequestered in the local environment provided by the enzyme, which therefore thermodynamically contributes to the reaction as well. Without that stabilizing contribution from the enzyme, the equilibrium values of the concentration of free aa-AMP formed from ATP and amino acids would be negligible (Wells and Fersht, 1986), which raises the chicken-and-egg question of how this process could have emerged in the absence of enzymes. This observation strongly suggests that translation evolved in an environment providing strong activation processes in which adenylates or other mixed anhydrides could be available from alternative pathways (Pascal et al., 2005; Pascal and Boiteau, 2011 b). Several groups, including ours, have provided evidence that α -amino acids and peptides, strongly activated under the forms of *N*-carboxyanhydrides (NCAs) (Biron et al., 2004, 2005; Leman et al., 2006) or 5(4*H*)-oxazolones (Liu et al., 2014, 2016, 2017), respectively, react spontaneously with inorganic phosphate and phosphate esters to give mixed anhydrides including aaAMPs at equilibrium in diluted neutral aqueous solution (Scheme 2). One of the most logical consequences of these observations is that the possible formation of peptides

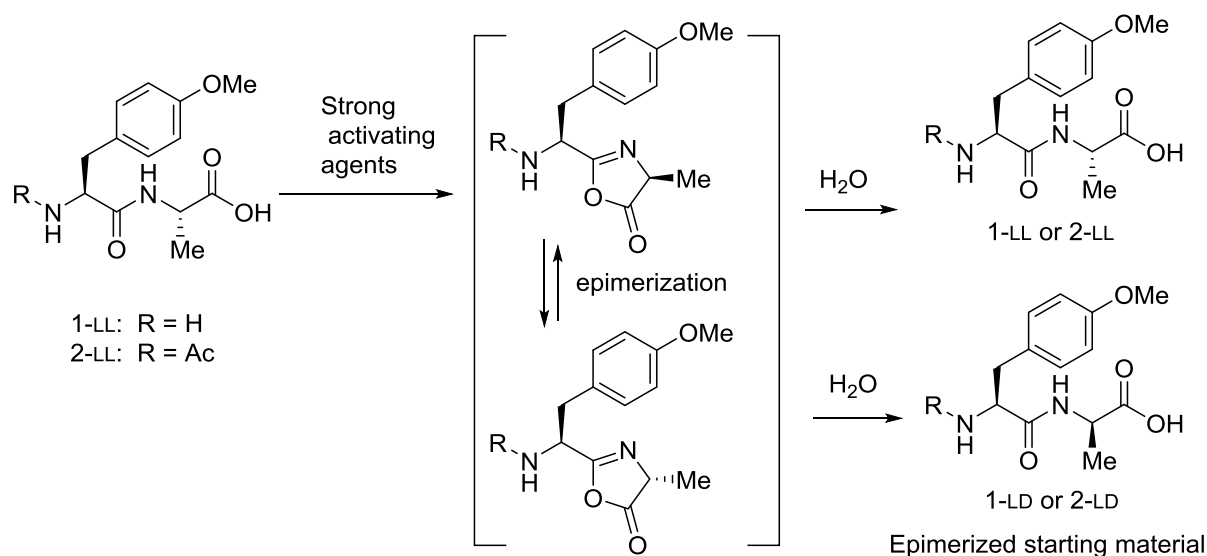
involving for instance mild wetting/drying cycles (Forsythe et al., 2015; Rodriguez-Garcia et al., 2015; Yu et al., 2017) at early stages of life does not prevent the need of processes involving strong reagents for life to emerge.



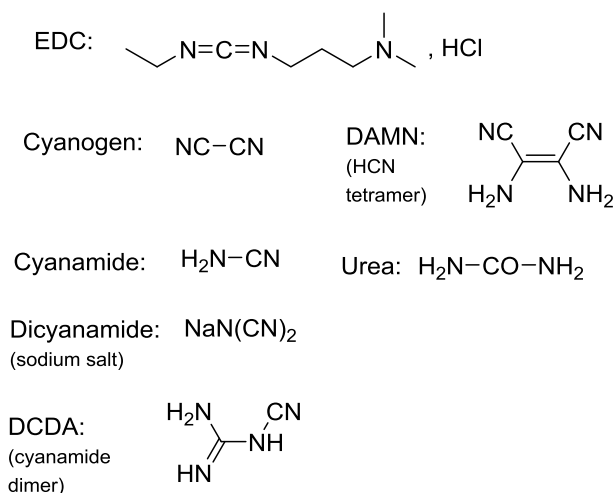
Scheme 2. Formation of mixed anhydrides from NCA and 5(4*H*)-oxazolones.

As far as strong activating agents are involved, *N*-carboxyanhydrides (NCAs) or 5(4*H*)-oxazolones are essential intermediates during the activation of α -amino acids or peptides, respectively (Danger et al., 2012). Some examples of prebiotically plausible processes for the formation of these activated intermediates have been reported in the literature. *N*-Carbamoyl amino acids yield NCAs by nitrosation in the dry state (Taillades et al., 1999) or, more directly, by heating neutral aqueous solution (Danger et al., 2006). Carbonyl sulfide (COS) is also efficient in producing NCAs from free amino acids in the presence of oxidizing or alkylating agents and transition metal ions (Leman et al., 2004). 5(4*H*)-Oxazolones are formed when acyl-amino acids or C-terminal residues in peptides are activated by strong activating agents like carbodiimides (Danger et al., 2013). The latter reaction significantly increases the rate of polymerization of amino acids promoted by carbodiimides (Danger et al., 2013). Though being considered as a drawback leading to a loss of chirality in the synthesis of peptides, 5(4*H*)-oxazolone formation has a very different outcome in prebiotic chemistry starting from racemic reagents since it may also be considered as a tool to induce a stereomer imbalance during polymerization (Danger et al., 2013). However, carbodiimides are not likely activating agents in an origin of life perspective. Cyanamide constitutes an alternative but its reactions turned out to be tedious (Danger et al., 2013) even at 80°C. We therefore considered the published work on amino acid

condensation agents (Brack, 1993, 2007). Many other reagents including cyanamide, dicyanamide, dicyandiamide (DCDA), diaminomaleonitrile (DAMN, HCN tetramer), and cyanogen leading to peptide bond formation can be found in the literature, although the yields were usually limited (Hulshof and Ponnampereuma, 1976). However, general conclusions are not easily drawn from these studies involving different conditions, different analytical methods, different starting materials, and in some cases using elevated temperatures or extreme values of pH. We realized that the formation of oxazolones from dipeptides constitutes a simple and practical way to characterize strong carboxyl activating agents (Scheme 3). The chiral instability of oxazolones rapidly results in a partial change of the configuration of the α -carbon of the amino acid residue, which can easily be monitored by analytical methods (e.g. HPLC) through the formation of diastereomers thus revealing the effect of powerful activating agents. The aim of the present work is to use this tool as a mean of investigating the reactivity of a series of prebiotically plausible condensing agents (Scheme 4) under conditions compatible with the environment of the primitive earth.



Scheme 3. The epimerization of free (1-LL) or acetylated (2-LL) Tyr(Me)-Ala dipeptides as a tool to monitor strong activation.



Scheme 4. Structures of the activating agents used in this study.

Experimental

Reagents and solvents were purchased from Sigma-Aldrich or Bachem, and were used without further purification. Dicyclohexyl ammonium methyl phosphate was prepared as described earlier (Dueymes et al., 2008). All aqueous solutions were prepared using pure water (18 M Ω) produced with a Milli-Q (Merck-Millipore) apparatus. NMR spectra in either DMSO-*d*₆ or D₂O solution were recorded on a Bruker DPX 300 spectrometer (300 MHz) or on a Bruker Avance 400 spectrometer (400 MHz); chemical shifts δ_{H} are reported in ppm; coupling constants *J* are reported in Hz. In all the experiments, the pH was monitored using a Thermo Orion 3-STAR pH-meter with a VWR electrode. UPLC analyses were carried out on a Thermo Scientific Dionex UltiMate 3000 Standard system including an autosampler unit, a thermostated column compartment and a photodiode array detector, using UV absorbance detection (acquisition in the 200–400 nm range) and the reactants and product were monitored at $\lambda = 273$ nm, which corresponds to the maximum of absorption of the aromatic ring of tyrosine. UPLC/ESI-MS analyses were carried out on a Waters UPLC Acquity H-Class system including a photodiode array detector (acquisition in the 200–400 nm range), coupled to a Waters Synapt G2-S mass spectrometer, with capillary and cone voltage of 30 kV and 30 V respectively, source and desolvation temperature of 140 °C and 450 °C respectively. ESI+ and ESI– refer to electrospray ionisation in positive and negative mode respectively. HRMS spectra were recorded on the same spectrometer, using the same source settings. HPLC conditions: Method A: Separations were carried out on a BDS-Hypersil C18 50×2.1 mm, 3 μm column, mobile phase made of solvent A: H₂O (0.1% TFA), B: CH₃CN (0.1% TFA); flow rate 0.2 mL/min; gradient: 0 min (5%

B), 15 min (15% B), 25 min (60% B), 26 min (100% B), 31 min (100% B); Method B: Separations were carried out on a BDS-Hypersil C18 50×2.1 mm, 3 μm column, mobile phase made of solvent A: H₂O (0.1% TFA), B: CH₃CN (0.1% TFA); flow rate 0.2 mL/min; gradient: 0 min (5% B), 25 min (25% B), 30 min (100% B), 32 min (100% B), and 33 min (5%). Method C (LC/ESI-MS): Kinetex C18 100A 100×2.1 mm, 2.6 μm column (Phenomenex), mobile phase made of solvent A: H₂O (0.1% formic acid, B: CH₃CN (0.1 % formic acid), flow rate 0.5 mL/min; ; linear gradient: 0 min (0% B) to 3 min (100% B).

Results

Activating agents were checked for strong activation using the dipeptide models **1-LL** (R¹ = H) and **2-LL** (R¹ = Ac) as starting materials (HPLC retentions times 9.6 min and 16.2 min respectively, method A). Any formation of 5(4*H*)-oxazolones from these substrates should result in the epimerization of the C-terminal alanine residue yielding the diastereomers **1-LD** (R¹ = H) and **2-LD** (R¹ = Ac) (Beaufils et al., 2014) (HPLC retentions times 16.5 min and 19.9 min respectively). Monitoring products is facilitated by the presence of the aromatic side-chain chromophore of tyrosine ($\lambda_{\text{max}} = 273 \text{ nm}$). Activation was carried out in a neutral to moderately acidic pH range considered as representative of aqueous environments of the primitive Earth with an atmosphere enriched in CO₂. The efficiency of the method was checked using the EDC activation reagent (Table 1 entries 1 and 2) known to induce 5(4*H*)-oxazolone formation (Beaufils et al., 2014) even in the case of unprotected dipeptide **1-LL**, which does not yield diketopiperazine owing to specific stereochemical properties (Beaufils et al., 2016) though in this case hydrophobic products having 23.3 to 24.9 min retention times were observed and correspond very probably to longer oligomers formed by reaction of the oxazolone with the free dipeptide. The results of the activation experiments using different reagents and conditions are listed in Table 1. None of the reagents checked during our investigations proved to reproduce an activity reaching that of EDC and, in most cases, no activity could be detected. Significant epimerization of the starting material was observed using cyanamide (Figure 1, Table 1, entry 7), confirming earlier preliminary results (Danger et al., 2013), and the related dicyanamide (Table 1, entries 17 and 18). However, the process required heating of the mixture to 80–90°C for long periods.

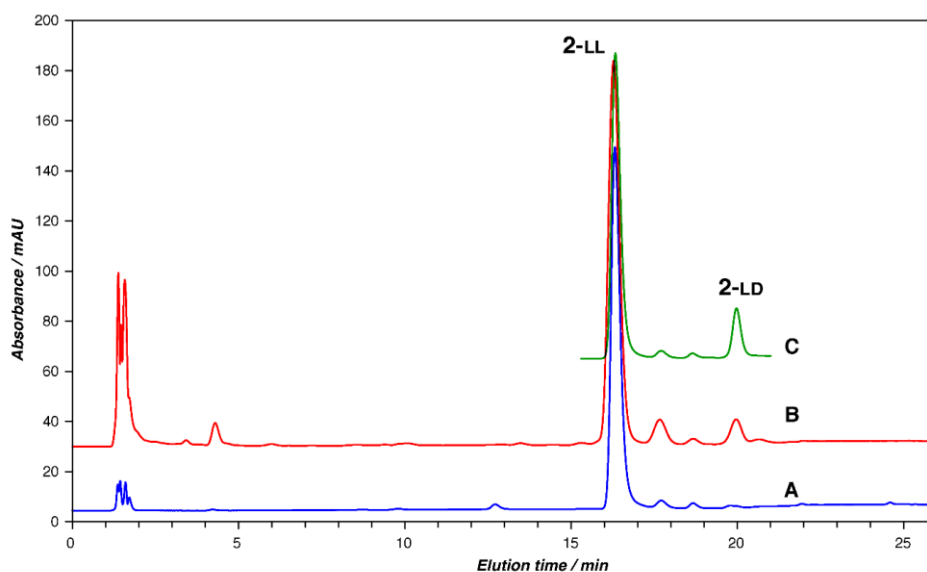


Figure 1. Reaction of 1 mM Ac-Tyr(Me)-Ala dipeptide, 2-LL, with 40 mM cyanamide at pH 5.5 (100 mM MES buffer) and 80°C. HPLC chromatograms (method A, UV detection 273 nm) A: starting material 2-LL in MES buffer; B: reaction of 2-LL with cyanamide after 20 days; C: comparison with the mixture of dipeptide epimers 2-LL and 2-LD obtained from the reaction of 1 mM 2-LL with 1 mM EDC in 100 mM MES buffer pH 6.5 at after 3 days.

Other reagents observed earlier to induce free amino acid polymerization in solution turned out to be inert with the acetylated dipeptide. This was indeed the case for cyanogen (Table 1, entries 3 and 21) formed in situ by the reaction of CuSO₄ and KCN (Brotherton and Lynn, 1959) and diaminomaleonitrile (DAMN, entries 5, 6 and 22). The acetylated dipeptide **2-LL** remained unchanged in these experiments revealing that the activity observed previously should result from a pathway different from direct carboxyl group activation. The replacement of the acetylated derivative by the dipeptide having a free amine gave an indication about the process of activation observed in the literature. A partial carbamoylation of the free amino group of dipeptide **1-LL** was observed through the observation of an additional HPLC peak with a retention time of 14.4 min (Method A) and signals at m/z 310.14 [M+H⁺] and 332.12 [M+Na⁺] in the HPLC-ESI-MS (positive mode) analysis of the mixture, which was particularly well established from the reaction of CuSO₄ and KCN. Similar signals were observed from DAMN. In fact, any hydrolysis of cyanogen formed during the reaction should lead to cyanate (Brotherton and Lynn, 1959) and then to the carbamoylation of free amines (Taillades et al. 2001). The activation observed in the literature should therefore result from the formation of carbamoyl-amino acids and their further conversion into NCAs as demonstrated by a re-examination of the activation of amino acids by cyanate

([Danger et al., 2006](#)). The result obtained from DAMN may be accounted for in a similar way since it was suggested in the literature that it can disproportionate into cyanogen and amino acetonitrile ([Mamajanova and Herzfeld, 2009](#)). No other combination of reagents proved to be effective. Interestingly, even the procedure reported recently ([Tsanakopoulou and Sutherland, 2017](#)) to improve the rate of formation of cyclic phosphodiester by a catalytic cycle based on the reactivity of glyoxylate or pyruvate with cyanamide at moderate temperature proved to be ineffective (Table 1, entry 11) demonstrating that, in this case, the increase in rate is brought about at the expense of the strength of the activating agent. In the same way, the use of a nucleophilic methyl phosphate buffer expected to react with cyanamide to give a reactive adduct proved to be ineffective (Table 1, entry 9). Further attempts to add metal ions to the reaction mixture, with the aim of binding both the reagent and the activating agent and bringing reactive centres in close proximity in the coordination sphere of the metal failed. A theoretical analysis of the conditions required for a steady state based on DKS led to the conclusion that energy has to be provided to the system with a potential equivalent or exceeding than that of visible light ([Pascal, 2012, 2013](#)). It would have been logical to investigate potential activation processes based on visible or UV irradiation of solutions containing our reagents and a series of potential activating agent precursors. However, preliminary experiments demonstrated that the dipeptide models used in the present study were not stable under irradiation at 254 nm. These investigations will require the development of a more sophisticated methodology avoiding a light-sensitive substrate.

Table 1 Screening of a variety of activating agents and reaction conditions to detect the epimerization of the two dipeptides H-Tyr(Me)-L-Ala-OH **1-LL** and Ac-Tyr(Me)-L-Ala-OH **2-LL** in buffered aqueous solution (100 mM MES unless otherwise mentioned). The solutions resulting from the activation experiments were analyzed by HPLC (method A, detection 273 nm unless otherwise mentioned) and potential products were characterized by mass spectrometry or NMR. Abbreviations: s.m. and n.d. stand for starting material and not detected, respectively.

Entry	Substrate	Activating agent and/or catalyst	Duration, temperature, pH	Result of activation (products, amounts)	Identification	Fig. ^a
1	1 mM 1-LL	1 mM EDC	4 d, <i>r.t.</i> , 6.5	Partial epimerization s.m. 1-LL + 3.7% 1-LD ^b + side products	HPLC retention time identical to pure 1-LD	S1
2	1 mM 2-LL	1 mM EDC	3 d, <i>r.t.</i> , 6.5	Partial epimerization s.m. 2-LL + 12.8% 2-LD ^b	HPLC retention time identical to pure 2-LD	S2
3	1 mM 2-LL	5 mM CuSO ₄ , 10 mM KCN	18 d, <i>r.t.</i> , 6.5	n.d. , unchgd. s.m. 2-LL		S3
4	1 mM 2-LL	5 mM CuSO ₄ , 10 mM KCN in 50 mM methyl phosphate buffer	4 d, <i>r.t.</i> 7.1	n.d. , unchgd s.m. 2-LL		S4
5	1 mM 2-LL	10 mM DAMN	11 d, <i>r.t.</i> 6.5	n.d. , unchgd s.m. 2-LL		S5
6	10 mM 2-LL	20 mM DAMN	25 d, 90 °C, 6.5	n.d. , unchgd s.m. 2-LL		S6
7	1 mM 2-LL	40 mM Cyanamide	20 d, 80 °C, 5.5	Partial epimerization s.m. 2-LL + epimer 2-LD 5.4%	LC-MS <i>m/z</i> 309.15 [M+H] ⁺ and 331.13 [M+Na] ⁺ (ESI ⁺)	1
8	1 mM 2-LL	10 mM Cyanamide in 5 mM imidazole buffer	7 d, <i>r.t.</i> , 6.68	n.d. , unchgd s.m.		S7
9	1 mM 2-LL	10 mM Cyanamide in 50 mM methyl phosphate buffer	13 d, <i>r.t.</i> , 7.1	n.d. , unchgd s.m.		S8
10	1 mM 2-LL	15 mM Cyanamide, 1 mM CuCl ₂	5 d, <i>r.t.</i> , 6.5	n.d. , unchgd s.m.		S9
11	1 mM 2-LL	5 mM Cyanamide/ 5 mM glyoxylic acid	5 d, 40 °C, 5.0	n.d. , unchgd s.m.	HPLC method B	S10
12	1 mM 2-LL	20 mM Urea	20 d, 80 °C, 5.5	n.d. , unchgd s.m.		S11
13	1 mM 2-LL	5 mM Dicyanamide	6 d, <i>r.t.</i> , 5.5	n.d. , unchgd s.m.		S12
14	1 mM 2-LL	5 mM Dicyanamide / 5 mM Cu(OAc) ₂	6 d, <i>r.t.</i> , 5.5	n.d. , unchgd s.m.		S12
15	1 mM 2-LL	5 mM Dicyanamide / 5 mM NiSO ₄	6 d, <i>r.t.</i> , 5.5	n.d. , unchgd s.m.		S12
16	1 mM 2-LL	5 mM Dicyanamide / 5 mM MnSO ₄	6 d, <i>r.t.</i> , 5.5	n.d. , unchgd s.m.		S12

(continues on the next page)

Table 1. (continued)

17	1 mM 2-LL	5 mM Dicyanamide	11 d, 85 °C, 5.5	Partial epimerization s.m. 2-LL + 2-LD 12.8%		S13
18	10 mM 2-LL	30 mM Dicyanamide	3 d, 85 °C, 5.5	Partial epimerization s.m. 2-LL + 2-LD 15.4%	LC-MS ESI ⁺ : <i>m/z</i> 309.15 [M+H] ⁺ , 331.13 [M+Na] ⁺	S14
19	1 mM 2-LL	10 mM DCDA	3 d, R.T., 5.5, dark	n.d. , unchgd s.m.		S15
20	1 mM 2-LL	10 mM DCDA	22 d, 80 °C, 5.5, dark	Limited epimerization s.m. 2-LL + 2-LD 2.1% ^a	HLPC retention time identical to 2-LD	S16
21	1 mM 1-LL	5 mM CuSO ₄ + 10 mM KCN	5 d, <i>r.t.</i> , 6.5	n.d. , unchgd s.m. 1-LL + <i>N</i> -carbamoyl dipeptide	LC-MS ESI ⁺ : <i>m/z</i> 310.14 [M+H] ⁺ , 332.12 [M+Na] ⁺ ; NMR [°]	S17
22	1 mM 1-LL	10 mM DAMN	7 d, <i>r.t.</i> , 6.5	n.d. , unchgd s.m. 1-LL + <i>N</i> -carbamoyl dipeptide + non-identified side product ^d	LC-MS ESI ⁺ : <i>m/z</i> 310.14 [M+H] ⁺ , 332.12 [M+Na] ⁺ ; ESI ⁻ : 308.20 [M-H] ⁻	S18

^a For figures S1 to S18, see Supporting Information; ^b Relative to all species absorbing at 273 nm; ^c NMR analysis of separated products; ^d LC-MS ESI⁺: *m/z* 418.18 for the unidentified side-product.

Conclusions

The presence of strong activating agents or of energy-rich carriers is a crucial parameter to induce self-organization as a consequence of a selection process among variants of entities capable of reproducing themselves (Eigen, 1971; Lifson, 1997; Szathmáry and Gladkih, 1989; Pross, 2016). In addition to the need of unlimited possibilities of variations that can be brought about by mutations in the sequence of a polymer, approaches based on the requirements for the settlement of DKS states emphasize on the need of sources of energy capable of feeding these systems and holding them in a state that is sufficiently far from equilibrium for a reproduction loop to work in an irreversible way (Pascal et al., 2013). The results of the present study indicate that a process able to activate the C-terminal end in a peptide in a way consistent with this approach is not available yet. Indeed, carbodiimides are not likely prebiotic reagents and cyanamide does not constitute an appropriate alternative because of its sluggish reaction even at high temperature. The only established processes leading to strong activation in prebiotically plausible environments are at present those based on the formation of *N*-carboxyanhydrides of amino acids (Taillades et al., 1999; Leman et al., 2004; Danger et al., 2006). The occurrence of a presently unknown catalytic process similar to that already established with oxo-acids (Tsanakopoulou and Sutherland, 2017) but that do not reduce the potential of

cyanamide reagent could be a possibility. In the absence of a catalytic pathway, the lack of reactivity of cyanamide and other nitriles is a drawback but it must be acknowledged that limited spontaneous breakdown rates are also necessary for the emergence of a metabolism in which reactants must be protected by kinetic barriers (Eschenmoser, 1994, 2007). Compounds containing triple bonds including nitriles and cyanamides are very likely candidate for this role (Pascal and Boiteau, 2011 a). The kinetic barriers protecting them from spontaneous hydrolysis actually represent a property allowing them to migrate from the location of their formation to that of a self-organizing system potentially requiring a different context. This hypothesis is however also dependent on the occurrence of efficient catalytic systems that could trigger the transfer of their energy potential into the metabolism of a self-reproducing entity. In a systems chemistry approach to the origin of life, a very attractive possibility for the conversion of the free energy of nitriles could involve an autocatalytic process able to contribute to a steady state behaving in conformity with a DKS drive and associated with possibilities of variations allowing the initiation of an open-ended evolutionary process. Finally, this observation means that our failure in detecting any possibility of strong activation of C-terminus in peptide must not be considered as a definitive conclusion and should encourage further investigations in this direction (Mariani et al., 2018). This potential of nitriles or other simple species bearing triple bonds in activation might be on the contrary be considered as in line with the role of hydrogen cyanide that has been recently uncovered in the formation of a wide range of biochemical building blocks through photocatalytic redox cycles (Ritson and Sutherland, 2012; Patel et al., 2015; Xu et al., 2018).

Acknowledgments

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Contributions

GA and JCR designed and performed the experiments. RP supervised the project. GA, JCR, LB and RP analyzed results and wrote the paper.

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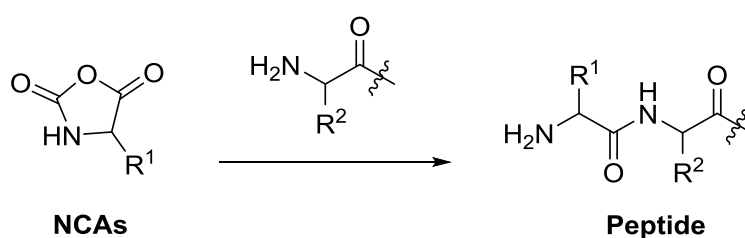
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Chapter 2. The prebiotic C-terminal elongation of peptides initiated by *N*-carbamoyl amino acid

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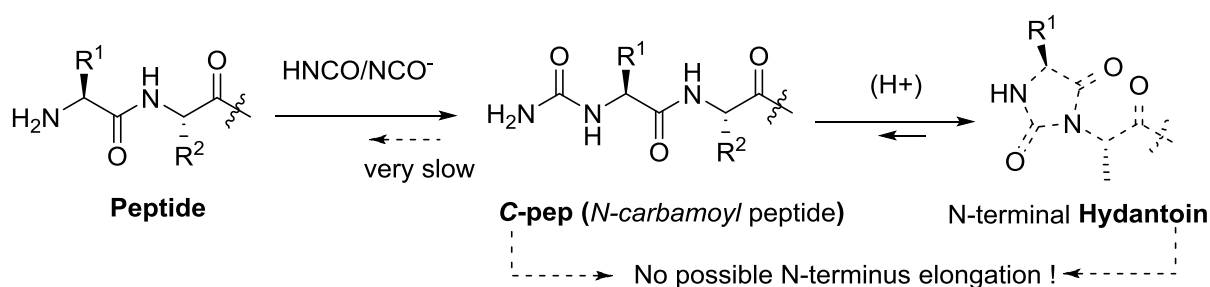
Overview:

Several plausible pathways have been proposed for the formation of oligopeptides from amino acids. As was discussed in the state of the art section, amino acids (or *N*-carbamoyl amino acids) can be activated as *N*-carboxyanhydrides (NCAs) through different pathways. It is worth emphasizing the specific role of *N*-carbamoyl amino acids since, as soon as CO₂ is present, these quite stable compounds are formed from aminonitriles through the Bucherer-Berg reaction easier than α-amino acids are produced by the Strecker pathway (**see State of the Art section**). Moreover, *N*-carbamoyl amino acids can also be formed under abiotic conditions from the reaction of the amino acid with cyanate (**Taillades et al. 2001**). Starting from amino acids, NCAs can be formed using carbonyl sulfide (COS) (**Leman et al. 2004, 2006**). Starting from *N*-carbamoyl amino acids, our group showed that NCAs could be formed in oxidative (NO+O₂) (**Taillades et al., 1999**) and neutral environments (80°C) (**Danger et al., 2006**). Peptides elongation (in the C- to N-terminal direction) can occur starting from the NCAs in the presence of a free amino group (amino acids or peptides) in aqueous solutions (**Scheme 1**).



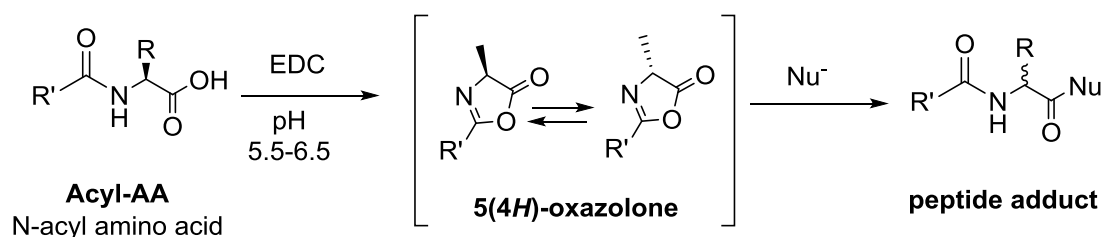
Scheme 1. Peptides elongation (C- to N-terminal direction) can occur starting from the NCAs in the presence of free amino group (amino acids or peptides) in aqueous solutions

However, the presence of cyanate (HNCO/NCO⁻) during the N-terminal elongation leads to the formation of the *N*-carbamoyl peptide and hydantoin (**Danger et al., 2006**), thereby blocking the elongation into a longer peptide (**Scheme 2**). The slow hydrolysis of the hydantoin could, however, recycle them into shorter peptide and amino acids (**Danger et al., 2006**).



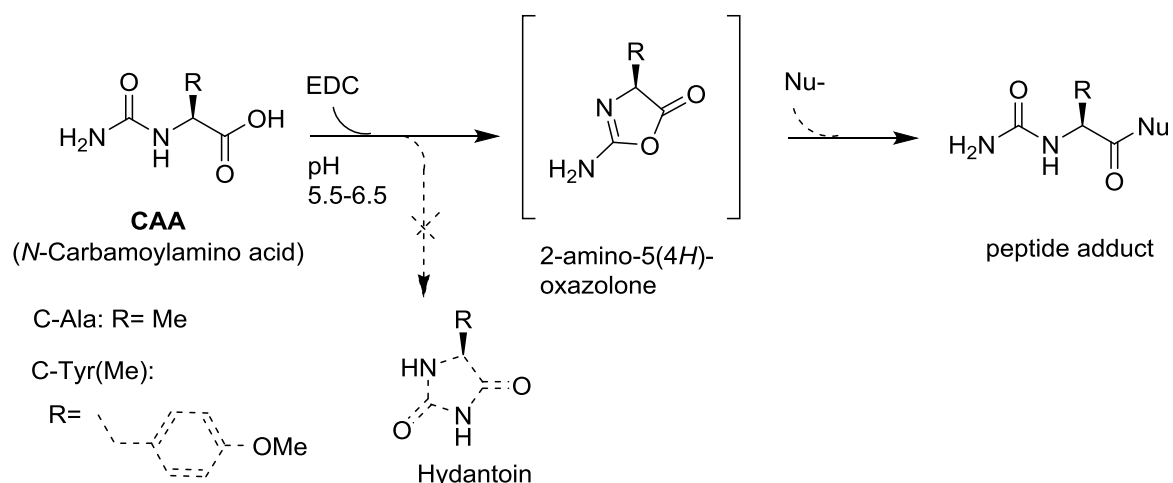
Scheme 2. Reactivity of a peptide N-terminus in the presence of cyanate with N-terminal hydantoin formation.

However, these drawbacks could in principle be circumvented through a C-terminal elongation of peptides for which there is no interference with the N-terminus. Cyanamide constitutes a potential C-terminal prebiotic activating agent of *N*-protected amino acid and peptides (**Danger et al., 2013**); its activity could be modeled by the carbodiimide (**Danger et al., 2013**), the reaction of which proceeds via the formation of a cyclic 5(4*H*)-oxazolone (**Scheme 3**). The later further readily react with a nucleophile, thus allowing the C-terminal elongation (**Scheme 3**).



Scheme 3. Activation of an *N*-acyl amino acid through cyclisation into a 5(4*H*)-oxazolone.

However, no experimental evidence indicated that the reaction *N*-carbamoyl amino acids (CAA) could proceed through 5(4*H*)-oxazolone intermediates. Therefore we undertook a study of the EDC promoted activation of the *N*-carbamoyl amino acids (CAA) derivative of Tyr (Me) or Ala in dilute aqueous medium in the presence of an excess of amino acid (nucleophile) at pH 5.5-6.5 (**Scheme 4**). EDC proved to be efficient in the formation of dipeptides with rates compatible with the presence of a 5(4*H*)-oxazolone intermediate. However, no epimerization could be detected in the product indicating that any putative 2-amino-5(4*H*)-oxazolone intermediate (**Scheme 4**) should be chirally stable compared to the analogs formed from usual acyl groups. The reactions identified in the following study clearly show a part of a chemical system involving the activation of the C-terminal group of the *N*-carbamoyl amino acids to form peptides that might have contributed to the chemical evolution of peptides.



Scheme 4. The EDC promoted activation of the *N*-carbamoyl amino acids (CAA) derivative of Tyr (Me) or Ala in dilute aqueous medium in the presence of an excess of a nucleophile (amino acid) at pH 5.5-6.5 yields peptide adducts without epimerization of the amino acid residue.

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Article 2.

The prebiotic C-terminal elongation of peptides can be initiated by *N*-carbamoyl amino acids

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Abstract

The formation of peptides upon EDC promoted activation of *N*-carbamoylamino acids (CAA), was considered in the scope of our recent works on carbodiimide promoted C-terminus elongation of peptides in a prebiotic context. Thus EDC promoted activation of CAA derivatives of Tyr(Me) or Ala in dilute aqueous medium pH 5.5-6.5 in the presence of excess of AA, resulted in peptide formation via C-terminus activation / elongation. Kinetic results similar to those of EDC-mediated activation of *N*-acyl-AA lead us to postulate the formation of a 2-amino-5(4*H*)-oxazolone intermediate by cyclization of the activated CAA, in spite of the absence of epimerization occurred at CAA residues. Thus, in a prebiotic context, CAA may have played a similar role as *N*-acyl-AA in the initiation of C-terminus peptide elongation.

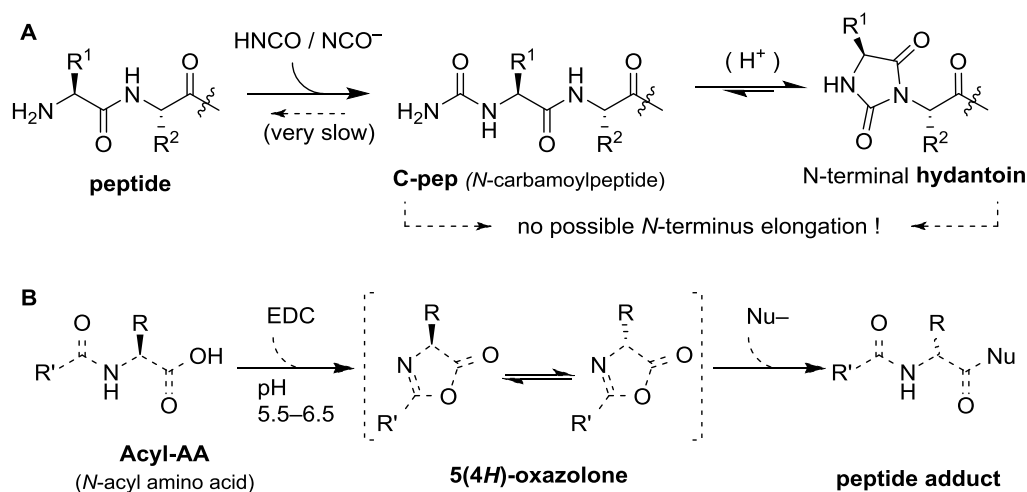
Keywords

amino acids • oxazolones • peptides • reactive intermediates • C-terminus activation

Introduction

The self-organization of organic matter on the primitive Earth is a long-lasting debate. Among molecules of interest, pathways toward oligopeptides and oligonucleotides in a prebiotic environment such as the primitive Earth have been particularly investigated. The presence of amino acid or nucleobase precursors is well documented from exogenous (Pizzarello et al., 2006) or endogenous sources (Patel et al., 2006). However, if we assume that prebiotic chemistry should proceed in water, the formation of such oligomers from building blocks is thermodynamically unfavorable (Danger et al., 2012 a). Amino acid monomers thus require activation in order to give rise to peptide formation. A plausible pathway has been proposed by Lemán et al., (Leman et al., 2004; Leman and Ghadiri, 2017) who demonstrated the possibility of using carbonyl sulfide (COS) to form oligopeptides from amino acids. An activated form of amino acid, the amino acid *N*-carboxyanhydride (NCA), is formed during this reaction and can react with another amino acid or the N-terminus of a peptide, which results in the formation or elongation of oligopeptides, respectively. Earlier, our group proposed other pathways of formation of NCAs from *N*-carbamoyl amino acids (CAAs) in oxidative (Commeyras et al., 2004) or neutral environments (Danger et al., 2006). Although, their contribution to the origins of life is not frequently considered, CAAs can easily be formed under abiotic conditions by reaction of cyanate and amino acids (Taillades et al., 2001). Moreover, an analysis of the kinetics of the conversion of α -aminonitriles (precursors of amino acids in the Strecker synthesis) demonstrated the prevalence of the CO₂-promoted pathway at moderate pH in aqueous solution, which leads to CAAs through the slow hydrolysis of hydantoins (Rousset et al., 1980; Taillades et al., 1998). The activation of CAAs under the form of NCAs induces an elongation of oligopeptides at the N-terminus. However, the presence of cyanate associated with the chemistry of CAAs would result in the *N*-carbamoylation at the free N-terminus, thus preventing any possibility of further elongation, since thus-formed urea groups lack any nucleophilic character and lead to the formation of N-terminal hydantoins (Danger et al., 2006) (Scheme 1, A). The sluggish hydrolysis of the latter could however recycle them into shorter oligopeptides and amino acids (Danger et al., 2006). Another option circumventing these drawbacks is the C-terminal elongation of amino acids or peptides. In addition to being a precursor of CAAs, cyanate has also been described as a potential prebiotic activating agent efficient on peptides presenting

a carboxyl group on the side chain of their C-terminal residue, such as aspartic acid (Danger et al., 2012 b). Peptide formation takes place through the cyclization of the activated carboxyl group into a cyclic anhydride. However, in the case of residue devoid of reactive functionalities on the side-chain, this process is inefficient (Danger et al., 2012 b), so that cyanate cannot play a role of universal prebiotic activating agent. Cyanamide constitutes a more general C-terminus activation agents as it was previously shown that its activity can be modeled by that carbodiimides (Danger et al., 2013). In this case, the *O*-acylurea intermediate is sufficiently reactive to induce the cyclization of the last two C-terminal residues into a 5(4*H*)-oxazolone in aqueous media, which further readily reacts with a nucleophile (Danger et al., 2013), thus allowing C-terminal elongation (Scheme 1). Noteworthy, due to the intermediacy of an oxazolone, the C-terminal residue undergoes epimerization during this process (Danger et al., 2013; Beaufils et al., 2014). We devised that the carbodiimide activation could constitute another way to circumvent the sluggish or non-suitable reactivity of CAAs since they could be able to initiate a C-terminus elongation of peptides. An investigation of the possible involvement of CAA in such pathways was therefore initiated.



Scheme 1. (A) Reactivity of a peptide N-terminus in the presence of cyanate with N-terminal hydantoin formation. (B) Activation of an *N*-acyl amino acid through cyclisation into an 5(4*H*)-oxazolone.

Results and Discussion

The formation of an oxazolone from an *N*-acyl amino acid (Acyl-AA) in presence of EDC was clearly demonstrated earlier: for instance, it resulted in D₂O in a rapid H/D exchange at the α position, easily monitored by ¹H NMR (Danger et al., 2013). We carried out a similar experiment to verify whether CAAs yield oxazolones upon EDC

activation, thus *N*-carbamoylalanine (C-Ala) was reacted with EDC in D₂O under ¹H NMR monitoring: conversely to Ac-Ala however, the C-Ala substrate remained unchanged while no H/D exchange was observed. Nevertheless, when the same reaction was performed in presence of 10 eq Gly-OBn in unbuffered H₂O, after 30 min reaction, the ¹H NMR spectrum in DMSO-*d*₆ (Fig. 1) showed the appearance of a singlet at 5.12 ppm, a quadruplet at 4.17 ppm and a doublet at 1.13 ppm with relative integration values of 2, 1 and 3, respectively. These resonances are likely to correspond to a product including both Ala and Gly-OBn moieties and can be attributed to the two benzyl hydrogens, the H_α and the methyl of Ala, respectively. Mass spectrometry analysis of the crude product in the range *m/z* = 270–320, gave ions at *m/z* = 280.2 and 302.1 Da, which can correspond to the pseudo-molecular ion [M + H⁺] and the sodium adduct [M + Na⁺] of the *N*-carbamoyl dipeptide C-Ala-Gly-OBn (279 Da). Without EDC, no such reaction was observed. Consequently, the CAA is sufficiently activated in the presence of EDC to induce peptide coupling even though it does not induce hydrogen exchange at the α position.

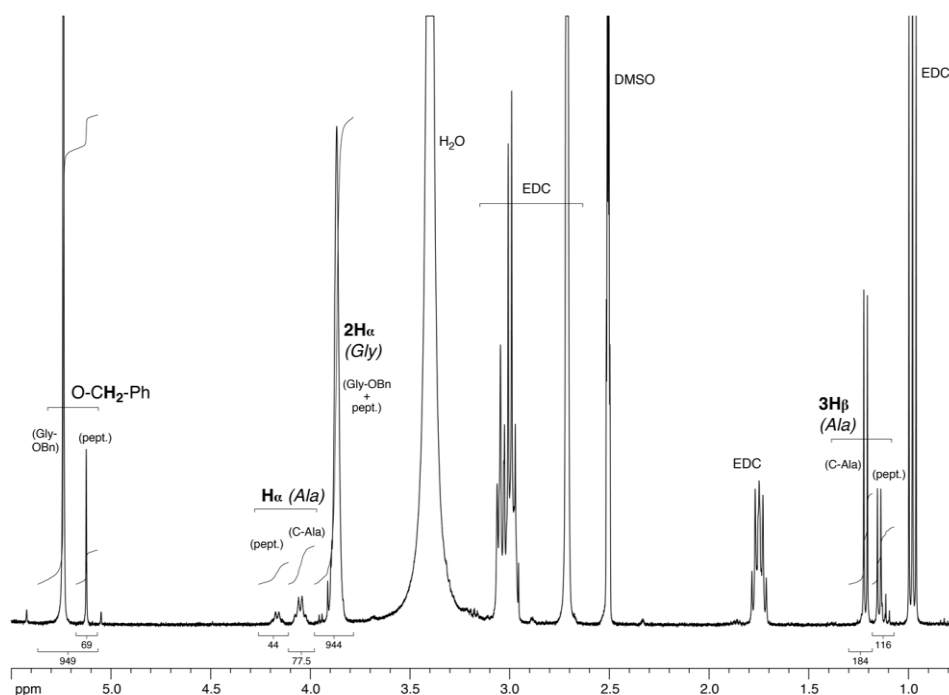


Figure 1. ¹H NMR spectrum (DMSO-*d*₆, 400 MHz) of the freeze-dried crude product of the reaction between 10 mM of C-Ala, 20 mM EDC and 50 mM Gly-OBn in unbuffered H₂O at 25°C.

To confirm these results, the same reaction was performed in MES buffer with 1 mM C-L-Tyr(Me)-OH and EDC in the presence of an excess of L-Ala-OMe at pH 5.5. HPLC

monitoring showed the disappearance of the starting C-L-Tyr(Me)-OH ($t_r = 14.6$ min, Fig. 2 trace A), together with the appearance of a peak at $t_r = 20.4$ min (Fig. 2 trace B) which was assigned to the *N*-carbamoyl dipeptide C-Tyr(Me)-Ala-OMe after HPLC-MS analysis exhibiting an $m/z = 322.14$ Da corresponding to the $[M - H^+]$ ion in ESI⁻ mode. The identity of the dipeptide was further confirmed by comparison with an authentic sample obtained at semi-preparative scale then characterized by ¹H NMR and HPLC-MS (cf. Supporting Information, Fig. S3). When the EDC activation was carried out with the opposite enantiomer C-D-Tyr(Me)-OH (in the presence of L-Ala-OMe), it showed the appearance of another HPLC peak at $t_r = 21.2$ min (Fig. 2 trace C) with an $m/z = 322.14$ Da, which was assigned to the *N*-carbamoyl dipeptide stereoisomer C-D-Tyr(Me)-Ala-OMe. In both cases, and conversely to similar EDC activation experiments involving *N*-acyl AAs (Danger et al., 2013), only one *N*-carbamoyl-dipeptide epimer was formed, thus meaning that no significant epimerization occurred upon EDC activation of C-Tyr(Me). Kinetically speaking, at pH 5.5, the C-Tyr(Me)-OH was totally consumed within 800 min, while at pH 6.5 30 % of the initial CAA was still remaining (Fig. S1). A faster reaction of EDC at low pH may explain these last observations.

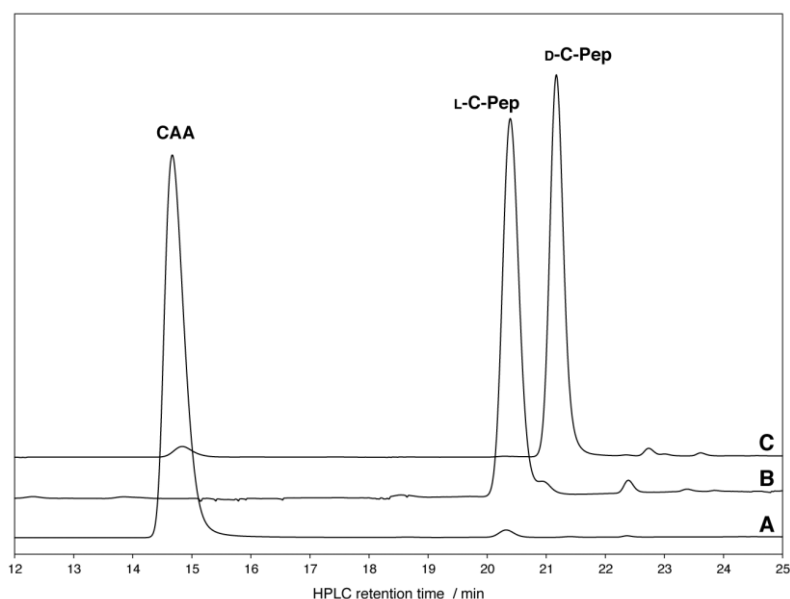
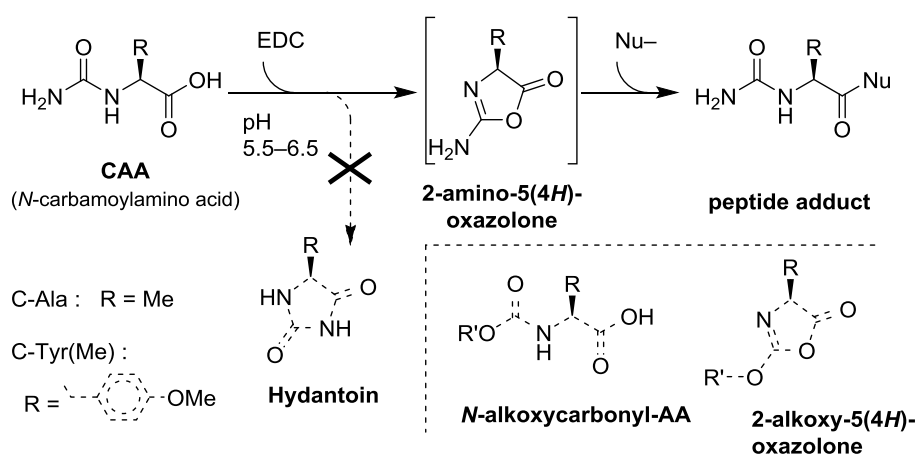


Figure 2. HPLC analysis (method B, cf. S.I.) of reactions of 1 mM C-Tyr(Me)-OH (either L or D) with 5 mM EDC and 10 mM Ala-OMe in 100 mM MES buffer pH 5.5, after 2 days at 25°C: (A) L-CAA substrate; (B) reaction mixture from L-CAA; (C) reaction mixture from D-CAA. Baselines shifted and chromatograms normalized for better legibility. Assignments of peaks were confirmed by HPLC-MS. Abbreviations: D/L-CAA and D/L-C-Pep, stand for C-D/L-Tyr(Me)-OH and C-D/L-Tyr(Me)-Ala-OMe respectively.

Since no CAA epimerization was observed, we cannot determine whether a 2-amino-5(4*H*)-oxazolone is transiently formed or if the coupling results from the direct reaction of the *O*-acylisourea intermediate. However, the kinetic profile of the reaction of C-Tyr(Me)-OH is very similar to that of a control reaction of Ac-Tyr(Me)-OH (Fig. S2). The similar values of estimated reaction half-times, 75 min and 145 min respectively, strongly suggest that the EDC activation of C-Tyr(Me)-OH also proceeds intramolecularly through an oxazolone (Danger *et al.*, 2013). Carboxylic acids unable to react intramolecularly have indeed been shown to exhibit reaction rates one to two orders of magnitude slower than an *N*-acyl-amino acid (Danger *et al.*, 2013). From these observations, the oxazolone can thus be considered as a plausible intermediate during the amino acid coupling. Nevertheless, the absence of racemization of C-Tyr(Me)-OH remains intriguing. It is noteworthy that Benoiton *et al.* have observed a similar behaviour upon activation of *N*-alkoxycarbonylamino acids with carbodiimides in organic solvents (Benoiton *et al.*, 1981). They clearly demonstrated that 2-alkoxy-5(4*H*)-oxazolones are formed during such reaction, though they hardly racemize conversely to oxazolones derived from peptides or acylated amino acids. In this case, the non racemization was explained by “the electron donating property of the 2-alkoxy substituent which lowers the lability of the 4-proton of the oxazolone” (Benoiton and Chen, 1981). Considering our kinetic results and the structural analogy between CAA and *N*-alkoxycarbonyl-AA (scheme 2), we suggest that during the reaction of CAA with a carbodiimide, an oxazolone is also formed, which does not racemize due to similar electron donating property of the 2-NH₂ substituent compared to the 2-alkoxy substituent.



Scheme 2. The observed reactivity of CAA in the presence of EDC in diluted aqueous medium pH 5.5–6.5, with the postulated 2-amino-5(4*H*)-oxazolone intermediate; no hydantoin formation was observed. 2-Alkoxy-carbonyl-AA and its oxazolone counterpart are shown for comparison.

In a last experiment aimed at demonstrating the possibility of oligopeptide formation from CAAs, 1 mM of C-L-Tyr(Me)-OH was reacted with 5 mM of EDC in the presence of 10 mM of free alanine (L-Ala-OH) at pH 5.5 and 25°C (where further C-terminus activation is possible conversely to the above Ala-OMe case). HPLC-MS analysis after ca. 7 h reaction showed the presence of new peaks (Fig. 3) which could be assigned to (oligo)Ala adducts of the CAA: *N*-carbamoyl-dipeptides with $m/z = 308.10$ Da (the 2 stereoisomers C-L-Tyr(Me)-L/D-Ala-OH, labels **2** on Fig. 3), -tripeptides with $m/z = 379.13$ Da (the 4 possible stereoisomers C-L-Tyr(Me)-[L/D-Ala]₂-OH, labels **3** on Fig. 3), and -tetrapeptides with $m/z = 450.17$ Da (two non-identified stereoisomers C-L-Tyr(Me)-[L/D-Ala]₃-OH, labels **4** on Fig. 3). While it is reasonable to hypothesize that the configuration of the C-Tyr(Me) moiety remained unchanged, beyond the dipeptide stage further EDC activation is very likely to induce 5(4*H*)-oxazolone formation hence rapid epimerisation of the peptide C-termini (Ala residues) as previously observed (Beaufils et al., 2014), thus accounting for the presence of almost all possible peptide stereoisomers in the reaction products. The ratio of dipeptide stereoisomers C-L-Tyr(Me)-[L/D-Ala]-OH (28:72 from HPLC peak integration, labels **2** on Fig. 3), is consistent with our previous observations on similar oxazolone mediated reactions (Beaufils et al., 2014), where the stereoselectivity of peptide coupling was mostly governed by the configuration of the nucleophile, rather than by epimerization equilibrium displacement (inequal epimerization rates L→D vs. D→L) upon further C-terminus activation by EDC. Additional effects accounting for dipeptide diastereisomeric excess may be inequal consumption rates of the L,L and L,D stereoisomers upon further reaction with L-Ala. The distribution of tripeptide isomers (ca. 1:6:2:3 ratio in ascending *t_r* order, labels **3** on Fig. 3), actually follows a similar trend. It is also noteworthy that upon EDC activation at mild pH, no significant hydantoin formation occurred from either CAA or *N*-carbamoyl-peptides.

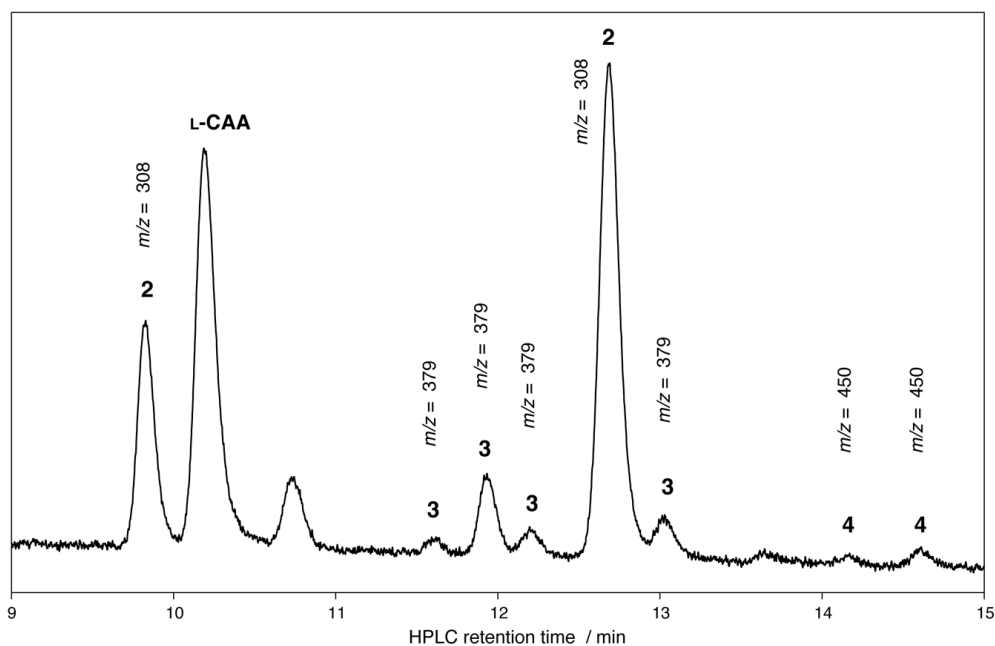


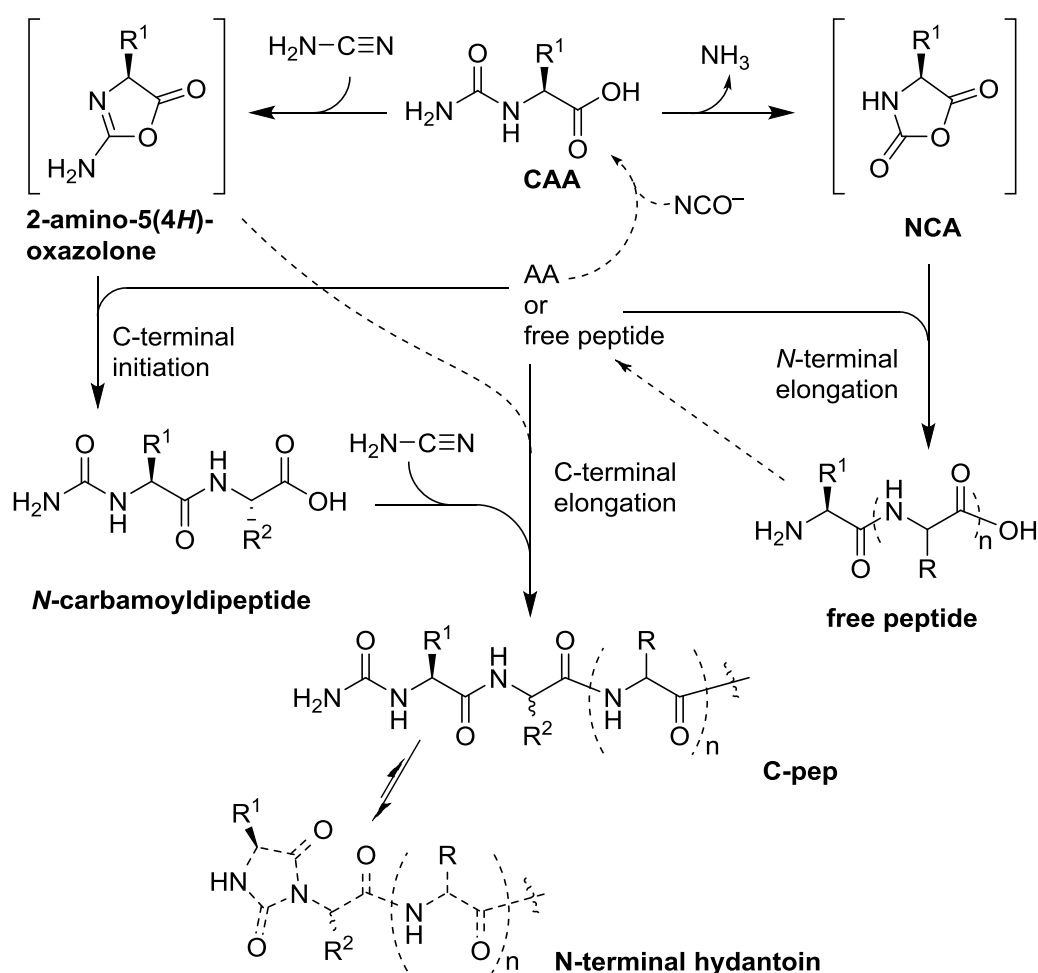
Figure 3. HPLC-MS analysis (method C, cf. S.I.) of the crude reaction mixture of 1 mM of C-L-Tyr(Me)-OH with 10 mM of L-Ala-OH and 5 mM of EDC in MES buffer pH 5.5 after 400 min at 25°C. Assignments of peaks were confirmed by HPLC-MS (see Supporting Information). Abbreviations: **L-CAA** stands for C-L-Tyr(Me)-OH; di-, tri- and tetrapeptides **2**, **3**, **4** (stereoisomers not identified), stand for C-L-Tyr(Me)-L/D-Ala-OH, C-L-Tyr(Me)-[L/D-Ala]₂-OH and C-L-Tyr(Me)-[L/D-Ala]₃-OH respectively.

Conclusion

These results demonstrate that CAAs constitute important intermediates in the formation of oligopeptides. The presence of cyanate on the primitive Earth is highly probable (Commeyras et al., 2004). Cyanate easily reacts with amino acids to form CAA. CAA can then constitute intermediates in the formation of oligopeptides through both N-terminal and C-terminal elongations. In an aqueous environment, they can indeed release ammonia leading to the formation of NCAs that then polymerize through N-terminal elongation (Danger et al., 2006). If carbodiimide derivatives or related activating agents are present, CAAs cyclize into oxazolones, which initiate elongation at the C-terminus. The resulting *N*-carbamoyl peptide (C-Pep) can undergo further elongation at the C-terminus, while the urea at the N-terminal position can be converted into a hydantoin. If a free N-terminal peptide is present a ligation can occur between *N*-carbamoyl peptide through its C-terminal activation (Scheme 3). Once formed, depending on their stability in the environment they can be degraded and recycled. The non-epimerization of CAA-oxazolones is assumed to have limited impact on previously discussed homochirality emergence scenarios (Beaufils et al., 2014), since it concerns only the first step of the peptide chain elongation — conversely noteworthy,

C-termini of peptide chains readily epimerize through oxazolone formation; in the latter case the stereoselectivity turn out to be mainly governed by the relative configuration of the nucleophile.

The reactions identified in the present study help in understanding how amino acids, and energy carriers – cyanate and carbodiimides – can help in building a chemical system involving the activation of amino acids to form oligopeptides at low concentration (millimolar) in water at pH 5.5–6.5. This chemical system is an example of a system that associates organic matter and energy and directs the self-organization of organic matter. The corresponding chemical processes are likely to have contributed to the chemical evolution of peptides (Pascal and Pross, 2015).



Scheme 3. Formation and reactivity of CAA for the prebiotic formation of oligopeptides from N- or C-terminal elongation in water at pH 5.5–6.5.

Acknowledgements

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ANR-14-CE33-0020. We are indebted to the CNES (Centre National d'Études Spatiales) through its Exobiology program for supports, and to the EU COST Action CM1304 "Emergence and Evolution of Complex Chemical Systems" for providing the possibility of fruitful scientific exchanges during the realization of this work.

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Part 2

Co-evolution of RNA and peptides – connecting the activation processes

Chapter 3. 5(4*H*)-Oxazolones as effective aminoacylation reagents for the 3'-terminus of RNA

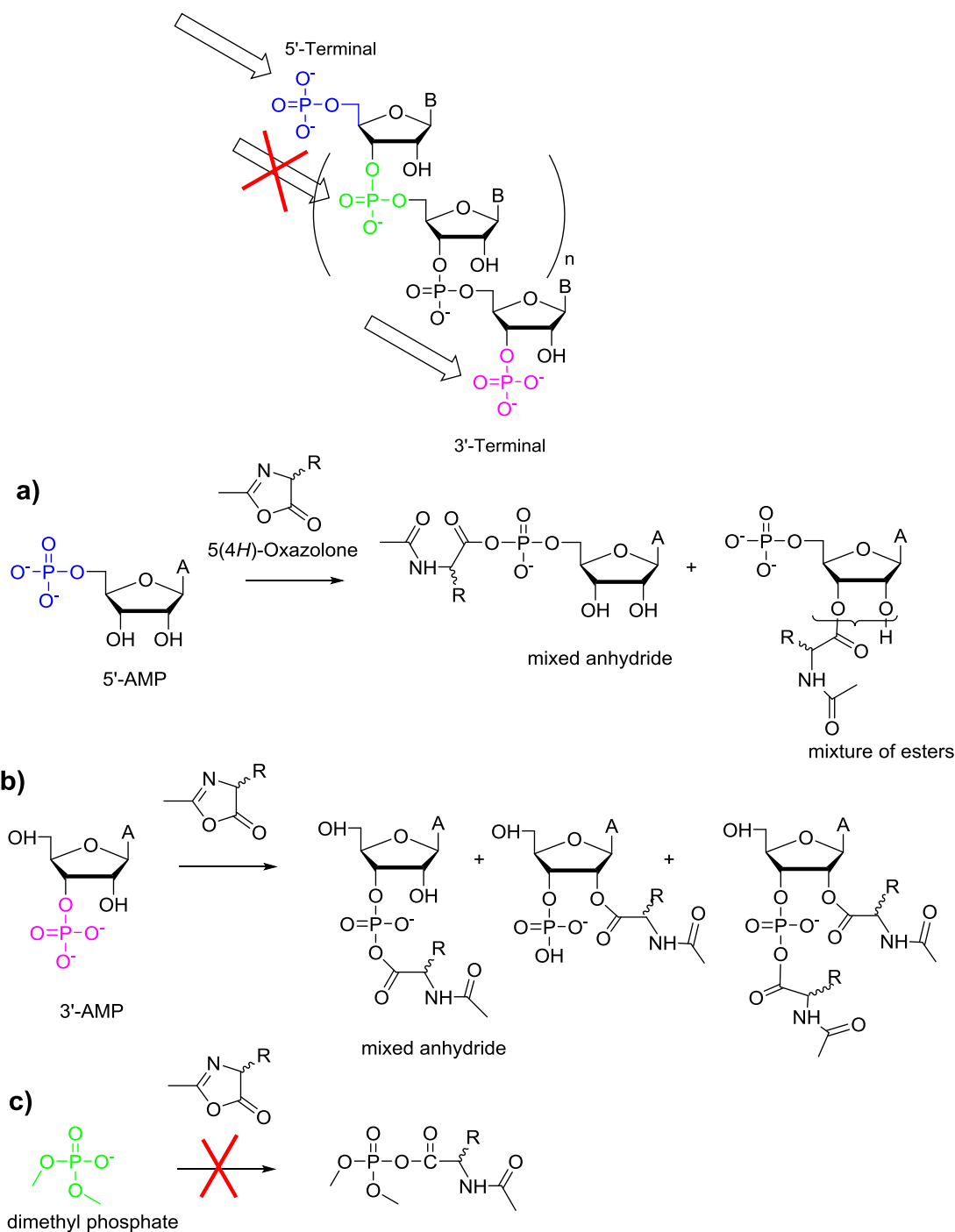
Published in *Synlett*, 2017, 28, 73–77 (special issue “prebiotic chemistry”)

Overview:

Regarding the emergence of translation, studying the interactions between α -amino acids and nucleotides constitutes one of the few chemical methods for understanding on which chemical grounds translation could have emerged and how the ribosomal machinery evolved. The preceding approach is in line with a systems chemistry views about the benefits from linking various systems to each other (**Kindermann et al., 2005; Ludlow and Otto, 2008**). However, our interest in the common chemistry of amino acids and nucleotides dates back to a time when systems chemistry idea was not yet proposed. Studies have been undertaken in our group or other groups as early as in 2004, 2005, 2006 (**Biron et al., 2004, 2005; Leman et al., 2006**). The work was in agreement with a linked origin of RNA and coded peptides (**Borsenberger et al., 2004**).

Aminoacyl adenylates (aa-AMPs) are the central intermediates in the biochemical synthesis of proteins. It is often claimed that aqueous solution polymerization of aa-AMPs is a potential route to abiotic peptides despite their very fast hydrolysis in the presence of CO₂. Liu et al., have investigated the efficiency and importance of this pathway by using models of amino acid phosphate mixed anhydrides, including aa-AMPs (**Liu et al., 2014**). They demonstrated, that at the CO₂ concentrations of the current oceans or physiological fluids, a direct polymerization pathway of the mixed anhydrides (aa-AMPs) could be ruled out. In contrast, a CO₂-promoted pathway was established where aa-AMPs reacted through the formation of NCAs, which accounts for the polymerization into peptides (**see the State of Art, scheme 9a**). Even taking into consideration that the CO₂ concentrations in the liquid environment of the primitive Earth were equivalent to current levels, the cyclisation process that takes place through NCAs was predominant. The possible involvement of NCAs as biochemical metabolites at some stage of evolution could be raised. Moreover, they showed that peptidyl-phosphate mixed anhydrides were formed from the reaction of inorganic phosphate with 5(4*H*)-oxazolones (the activated form of peptides), they also have

observed the reverse cyclisation process that takes place through the formation of oxazolone (**see the State of the art, scheme 9b**). These processes could form the core of a reactive network that can have evolved towards the emergence of translation. An extension of the preceding work was done by investigating more deeply into the formation of mixed anhydrides and the reactivity of the oxazolones with different sites of RNA (5' phosphoryl group, 3' phosphoryl group, internucleotidic phosphodiester linkage) (**Liu et al., 2016**). Different models were used (5'-AMP, 3'-AMP, dimethyl phosphate) (**Scheme1**). The authors showed that mixed anhydrides could be formed from relatively low concentrations of reactants with phosphates at two positions in RNA, namely the 5'- and the 3'-end (**Scheme 1a, 1b**). On the contrary, the use of dimethyl phosphate model provided evidence that mixed anhydrides could not be formed in the case of internucleotidic phosphodiester linkage (**Scheme 1c**). They concluded that mixed anhydrides could be formed from the interaction of oxazolones with phosphate monoesters and not phosphate diesters.



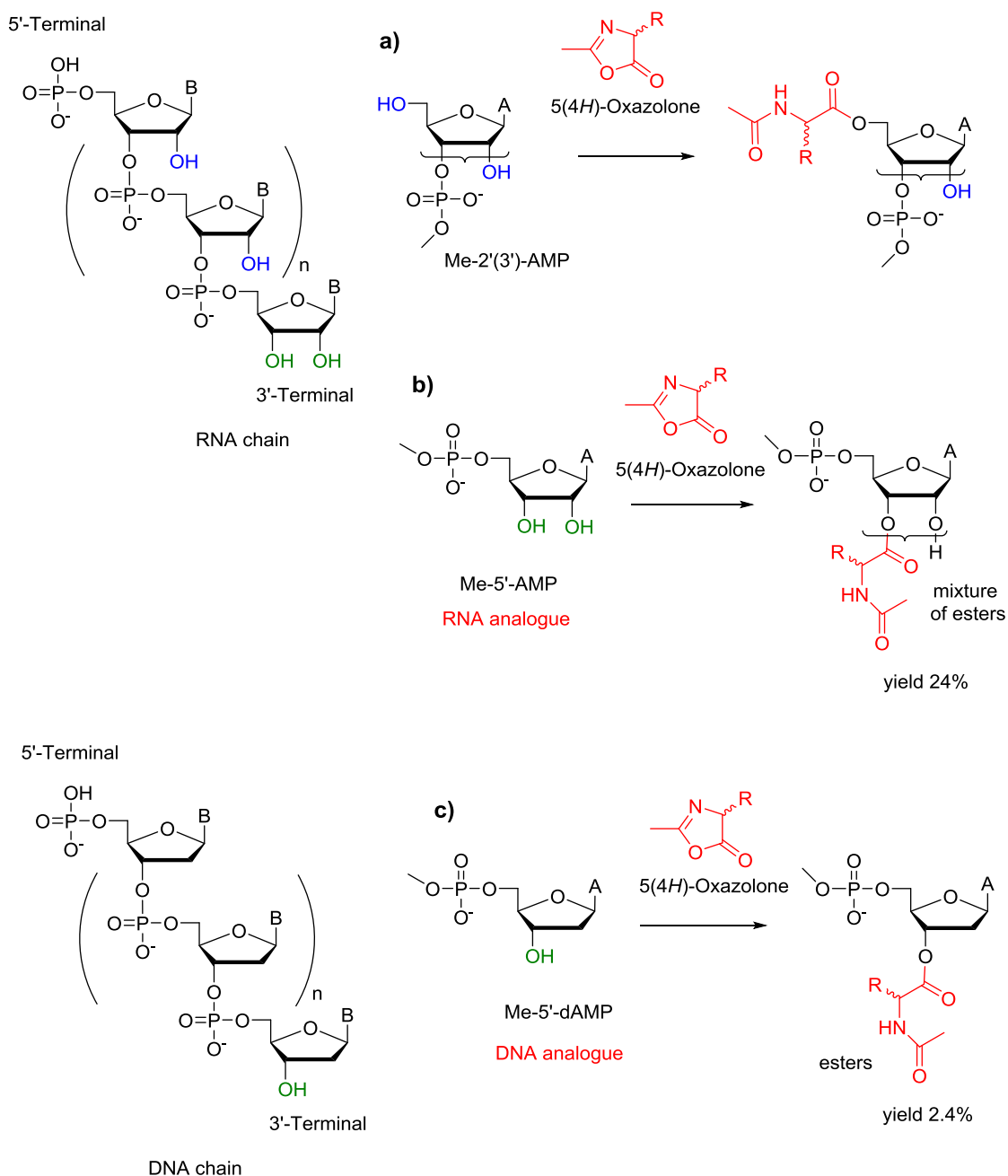
Scheme 1. Formation of covalent adducts from the interaction of the activated forms of protected amino acids (oxazolones) with different sites of RNA. **a)** Formation of mixed anhydrides at the 5'-end of 5'-AMP model and esters at the 2'(3') end of 5'-AMP. **b)** Formation of mixed anhydrides at the 3' end of 3'-AMP model. **c)** No mixed anhydrides were formed in case of using dimethyl phosphate model (internal phosphates).

The formation of the unexpectedly high yield of esters at the 2'- and 3'-positions of the ribose moiety at neutral pH (**Scheme 1a, Liu et al., 2016**), led our group to look further to non-enzymatic aminoacylation processes for the 3'-terminus of RNA. Therefore, the

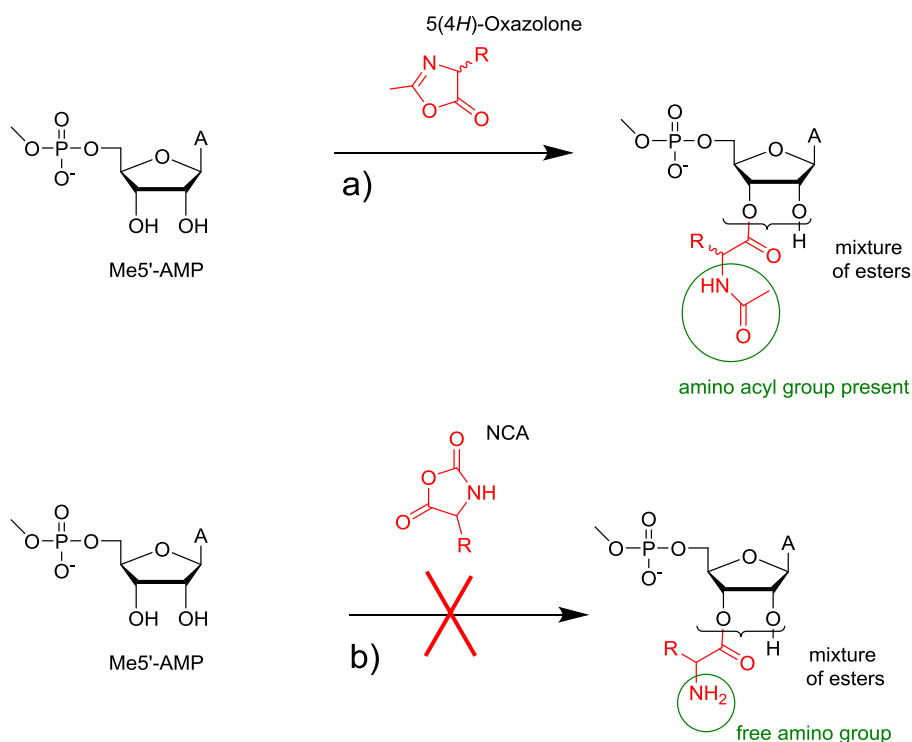
purpose of the work described in the following article was an examination of the reactivity of the oxazolones with the backbone and terminal hydroxyl groups of RNA and DNA, using different models (Me-5'-AMP, Me-2'(3')-AMP, 5'-AMP) (**Scheme 2**). The results of the following article are summarized below.

Me-2'(3')-AMP model was used for studying the reactivity of the hydroxyl group in the inner chain of RNA with oxazolones. No formation of esters was observed at the 2'-OH or 3'-OH of Me-2'(3')-AMP, but only esters at the free 5'-OH were formed in a moderate yield (7.5 %), which means that the inner hydroxyl group is not reactive (**Scheme 2a**). We used Me-5'-AMP bearing hydroxyl groups available at the 2'- and 3'- positions to study the reactivity of oxazolones with the terminal hydroxyl groups RNA chains. In this case, a substantial yield (24 %) of a mixture ester was obtained from the reaction of oxazolones with the RNA analogue Me-5'-AMP (**Scheme 2b**). On the other side, we have also studied the reactivity of the hydroxyl group at the terminus of DNA chain by using Me-5'-dAMP model. The reaction of oxazolone with DNA model (Me-5'-dAMP) led to the formation of esters at 3'-OH with a reduced yield (2.4 %) (**Scheme 2c**). From the preceding results, we concluded that RNA models (Me-5'-AMP) are about tenfold more efficient than DNA models (Me-5'-dAMP). Moreover, we deduced that the presence of two free hydroxyl groups (2'-OH, 3'-OH) at the 3-terminus of an RNA strand is a key factor in facilitating aminoacylation. The results confirms that the structure of RNA is better adapted to aminoacylation, and therefore accounts for the involvement of RNA in translation.

However, the aminoacylated nucleotide esters formed from the reaction of 5'-Me-AMP with oxazolone have an acyl group on the amino group (**Scheme 3a**). Therefore, we were interested in investigating the reactivity of the reaction of Me-5'-AMP with NCAs. It turned out that no esters could be observed in this case (**Scheme 3b**). A different mechanism of reaction should explain the difference in reactivity between NCAs and oxazolones. Therefore, NCAs are not able to aminoacylate directly the 3'-end of RNAs and give rise to aminoacyl-esters with a free amino group.



Scheme 2. Formation of nucleotide esters. **a)** No aminoacyl esters formed at the 2'(3')-OH of Me-2'(3')-AMP but only at the free 5'-OH were formed. **b)** Aminoacyl esters formed at the 2(3')-OH of the Me-5'-AMP model. **c)** Aminoacyl esters formed at the 3'-OH of Me-5'-dAMP model.



Scheme 3. **a)** aminoacylation of 2'(3')-OH of Me-5'-AMP by oxazolone. **b)** no aminoacylation of the 2'(3')-OH of Me-5'-AMP by NCA.

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Article 3.

5(4*H*)-oxazolones as effective aminoacylation reagents for the 3'-terminus of RNA

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Abstract:

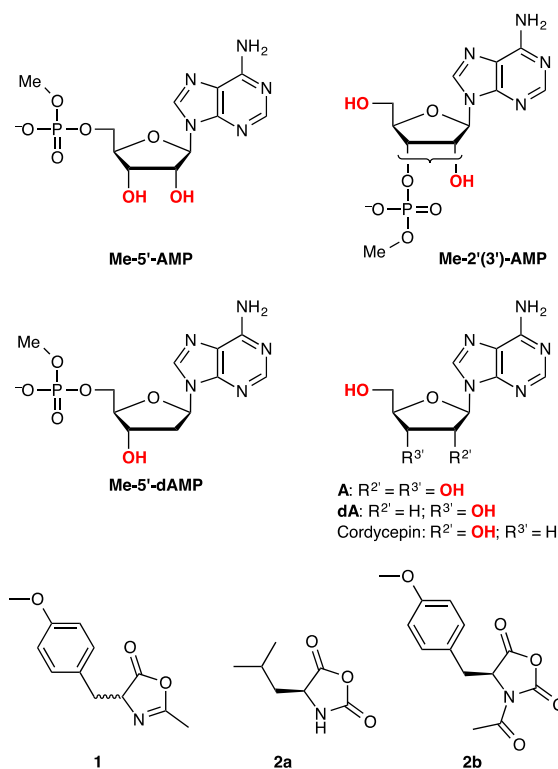
Nucleosides and methylated nucleotide models were used as substrates to identify pathways for the chemical aminoacylation of ribonucleic acids (RNAs) as a prerequisite for the evolution of translation. A selective and comparatively efficient reaction of a 5(4*H*)-oxazolone with the 2'- and 3'-OH of the ribonucleotide models was observed. Surprisingly, a similar reaction starting from an α -amino acid *N*-carboxyanhydride (NCA), selected as an acylating agent potentially leading to the unprotected ester required for translation, was not observed, which was confirmed using an acylated NCA equivalent. The reasons for this difference are analyzed.

Keywords:

Origin of translation, prebiotic chemistry, peptides, nucleotides, co-evolution.

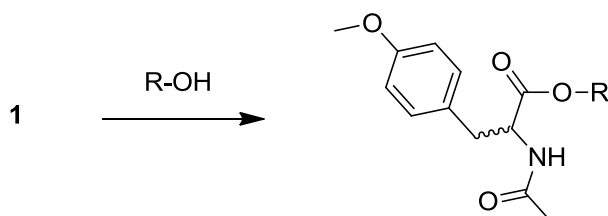
Introduction:

Elucidating how RNA could be chemically aminoacylated without the intervention of enzymes is one of the major issues in explaining how life could have emerged and how the translation process originated (Orgel, 1989; Wickramasinghe and Lacey, 1994). Imidazolides proved to be chemically competent in this process (Profy and Usher, 1984). Other pathways involving ribozyme catalysis have been proposed in the context of the RNA world hypothesis starting either from α -amino acid adenylates (Illangasekare, M. *et al.*, 1995) or cyanomethyl esters (Saito and Suga, 2001). However, the idea that life evolved from different families of biomolecules and biopolymers (Borsenberger *et al.*, 2004; Pascal *et al.*, 2005 ; Ruiz-Mirazo *et al.*, 2014) through a co-evolutionary process is gaining momentum because of the difficulties associated with RNA polymerization from hypothetical mixtures of ribonucleotides (Joyce and Orgel, 1999). Giving a satisfactory answer to the question of chemical aminoacylation of RNA is essential to understand how peptides and nucleotides may have interacted in an abiotic environment. In this context, we recently studied the formation of mixed anhydrides (Liu *et al.*, 2016) by reaction of nucleotides with 5(4*H*)-oxazolones, earlier demonstrated as prebiotically relevant activated peptides (Danger *et al.*, 2013). Esters at the 2'- and 3'-positions of the ribose moiety were additionally formed in unexpectedly high yield at neutral pH (Liu *et al.*, 2016). To account for this observation, we embarked on an investigation of the reaction of these activated forms of peptides with nucleoside models or nucleotides bearing no reactive phosphoryl group (*i.e.* without phosphoryl group present as a dianion, Scheme 1). The results of these investigations are presented in this report and show that significant aminoacylation yields can be observed at the 2'- or 3'-hydroxyl groups of the ribose moiety and that this efficiency in ester formation is not observed starting from α -amino acid *N*-carboxyanhydrides (NCAs) instead of 5(4*H*)-oxazolones though these species could be considered as closely related. The reasons of this discrepancy are analyzed and proposed to correspond to a different rate-determining step for aminoacylation, which, by consequence, suggests an explanation for the high reactivity of the ribose vicinal diols through proton transfer or hydrogen bonding when the breakdown the tetrahedral intermediate is rate-limiting.



Scheme 1. The different nucleosides and nucleotide models with potential acylation sites (bold red) as well as the activated aminoacylating species used in this work.

The reaction of the 5(4*H*)-oxazolone **1** (Scheme 2) derived from methylated *N*-acetyl-tyrosine with methylated AMP (**Me-5'-AMP**)^[1] was selected as a model of the aminoacylation of the 3'-terminus of RNA strands.^[2] We observed essentially the formation of four isomers (Figure 1) having the composition of a stoichiometric adduct (HPLC-ESI-HRMS negative mode, calcd. for C₂₃H₂₈N₆O₁₀P⁻: 579,1610; found 579.1605). The formation of these isomers can be accounted for by the well-known regio-isomerization of acyl groups between the 2'- and 3'-hydroxyl groups of the ribose moiety (Wolfenden et al, 1964) and the presence of the amino acid moiety as a mixture of the two chiral configurations resulting from the fast epimerization of 5(4*H*)-oxazolone **1** (Beaufils et al., 2014).



Scheme 2. The reaction of 2-methyl-4-(4-methoxybenzyl)-5(4*H*)-oxazolone **1** with the hydroxyl groups of nucleotide models (R–OH).

The isomers were identified by comparison with mixtures prepared from Ac-L-Tyr(Me)-OH or Ac-D-Tyr(Me)-OH and carbonyl diimidazole (CDI) using the procedure of Gottikh ([Gottikh et al., 1970](#)) (Figure 1). A COSY-NMR analysis of these isomeric mixtures (Supporting Information) allowed the identification of the four isomers as the D-2', L-2', D-3', and L-3'-configurations for the HPLC peaks with retention times in Figure 1 of 14.6, 15.0, 16.3, and 16.9 min, respectively. It is worth noting that the distribution of stereo/regioisomers is consistent with earlier observations that a stereochemical preference for the L-isomer is observed for esters formed at the 3'-position whereas the D-isomer is predominant at the 2'-position ([Lacey et al., 1988, 1993](#)). The reactions of several other nucleoside and nucleotide models with 5(4*H*)-oxazolone **1** were also studied under similar conditions (Table 1). It turned out that the yields of esters are in every case lower than that of the reaction of **Me-5'-AMP** considered as a model of a 3'-terminus of an RNA strand. Notably, deoxyribose derivatives **dA**, **dAMP** and **Me-5'-dAMP** were much less prone to undergo ester formation, which confirms the clear advantage of RNA over DNA with respect to aminoacylation ([Wickramasinghe and Lacey, 1994](#)) and may explain the unique role of RNA in translation by allowing the aminoacylation of tRNA. The presence of two free hydroxyl groups at the 3'-terminus of an RNA strand is therefore a key factor in facilitating aminoacylation, which is also supported by the probable absence of reactivity of isomers of methylated **Me-2'(3')-AMP** (Table 1) suggesting that the reaction of 5(4*H*)-oxazolone **1** is not possible at intra chain 2'-OH groups with the notable exception of the 3'-end. Other significant differences, for which no simple explanation can be found, are probably related to changes in the geometry of the ribose/deoxyribose ring.

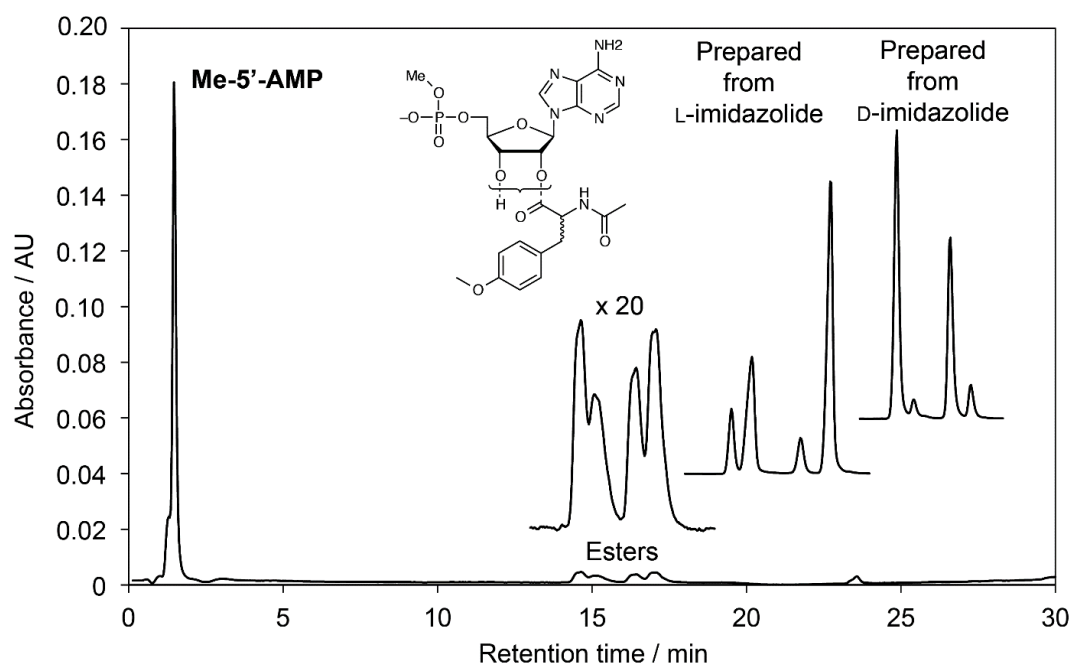


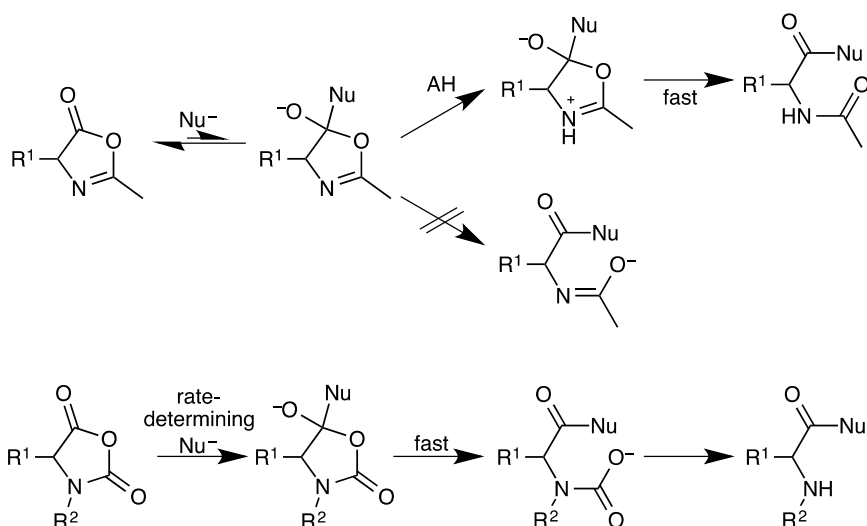
Figure 1. HPLC trace of adducts of amino acid with **Me-5'-AMP** after 24 h of reaction of 25 mM Ac-Tyr(Me)-OH-derived 5(4*H*)-oxazolone with 5 mM **Me-5'AMP** in 100 mM MES-buffered aqueous solution (pH 6.5). Before analysis, the reaction medium was acidified and extracted twice with ethyl acetate to remove Ac-Tyr(Me)-OH (retention time 15.1 min), then an aliquot (50 μ L) was withdrawn and diluted to 1 mL with water (see method A in Ref. 8 for HPLC analysis). The inset shows an amplification of the peaks corresponding to stereo/regioisomers of esters between 13 and 18 min resulting from the reaction of racemic 5(4*H*)-oxazolone **1**. Isomers can be identified by comparison with the products obtained by reactions of both L- and D- imidazolides.

Table 1. Reactions of different nucleosides/tides (5 mM) with Ac-Tyr(Me)-oxazolone (25 mM) in 100 mM MES buffer (pH 6.5 before the addition of reactants). Reaction time: 24 h unless otherwise mentioned. Areas at 248 nm correspond principally to nucleic base absorption and can thus be considered as a gross estimate of the yield of nucleoside/tide converted into adducts.

	Sum of 2' and 3' esters as % of the area measured at 248 nm.	Relative area of 2'-isomers	Relative area of 3'-isomers	5'-adduct as % of the area measured at 248 nm.	Bis-adduct as % of the area measured at 248 nm.	Reference
Me-5'-AMP	23.8	2'-L (13.0%), 2'-D (27.5%)	3'-L (34.8%), 3'-D (24.8%)			This work
A	30.2 ^b	not determined	not determined	30.2 ^{a,b}	10.8	This work
dA (2'-dA)	5.4 ^c	not applicable	100%	5.4 ^{a,c}	not detected	This work
Cordycepin (3'-dA)	16.2 ^d	100%	not applicable	16.2 ^{a,d}	not detected	This work
AMP ^e	8.4	not determined	not determined	25.6 ^f	4.2	(Liu et al., 2016)
dAMP ^g	1.9	not applicable	100%	27.7 ^f	not detected	(Liu et al., 2016)
Me-5'-dAMP	2.4	not applicable	100%			This work
Me-2'(3')-AMP	not detected			7.5 ^a	not detected	This work

^a Ester; ^b Mixture of 2'-, 3'- and 5'-adducts; ^c Mixture of 3'- and 5'-adducts; ^d Mixture of 2'- and 5'-adducts; ^e Reaction time: 4 h; ^f Mixed anhydride; ^g Reaction time: 7 h.

The reactions of 5(4*H*)-oxazolones with nucleotides yield aminoacylated derivatives in which the amino acid is not free but acylated. Regarding the importance of aminoacylated RNAs in translation, we studied the potential of NCAs as aminoacylating agents for which prebiotically relevant pathways of formation have been proposed (Taillades et al., 1999; Leman et al. 2004; Danger et al., 2006). In a first stage, the reaction of Leu-NCA **2a** (Daly and Poché, 1988) with **Me-5'-AMP** as a model of 3'-terminus of an RNA strand was studied. At the difference of 5(4*H*)-oxazolones, the NCA oligomerization into peptides was expected to render the analysis of results less straightforward. We therefore performed the reactions at pH values as low as possible at which the amino terminus of peptide chains would be less reactive owing to its non-nucleophilic protonated state. To this aim, acetate buffers (pH 4.8) and initial concentrations of NCA **2a** in the range of 2 to 20 mM were used. Alternatively, a bicarbonate buffer was prepared starting from a 5 mM NaHCO₃ solution in which a saturated concentration of CO₂ was constantly maintained by bubbling the gas. However, no amino acid-nucleotide adduct could be observed and the reaction produced only the free amino acid by hydrolysis and polypeptides. Since aminoacylation could be circumvented by the faster polymerization of NCA reagents, we selected the acetylated derivative **2b**^[3] as an alternative model unable to undergo polymerization but in principle capable of reproducing the behaviour of free NCAs with the diol of the ribose moiety. Under the conditions used previously for the reaction of the 5(4*H*)-oxazolone **1** (25 mM acetylated NCA **2b**, 5 mM **Me-5'-AMP** in a pH 6.5 MES buffer), no peaks corresponding to adducts at the 2' and 3'-positions could be observed though the acetylated NCA proved independently capable of reacting with L-alaninamide in a control experiment carried out under similar conditions giving the dipeptide Ac-L-Tyr(Me)-L-Ala-NH₂ as a single stereoisomer with a retention time of 10.7 min identical to that of an authentic sample (Beaufils et al., 2014). This series of experiments with an NCA and an acylated derivative demonstrates that the specific ability of the ribose vicinal diol is strongly dependent on the nature of the electrophile. A rather straightforward explanation can be proposed for this very specific behavior (Scheme 3).



Scheme 3. The different reactivity of tetrahedral addition intermediates proposed in order to account for the behaviour of 5(4*H*)-oxazolones and NCAs in the reactions with nucleophiles. The poor leaving group ability of the amide anion ($pK_a > 14$) makes the acid-catalyzed pathway mandatory from the former, whereas no catalysis is needed for the expulsion of the weakly basic carbamate anion from usual NCAs or from the N-acylated derivatives.

The two different pathways putatively proposed in Scheme 3 for the reactions of nucleophiles with the 5(4*H*)-oxazolones and NCAs suggest that the rate determining step could be different simply because of the poor leaving group ability of an amide anion involved in the former reaction compared to that of the carbamate formed in the latter. This difference in leaving group ability constitutes the basis of a rationale to account for the experimental data reported above. A breakdown of the tetrahedral intermediate generated from the 5(4*H*)-oxazolone therefore requires the prior protonation at the nitrogen atom to increase the leaving group ability into that of the neutral amide oxygen. The efficiency of the vicinal 2',3'-diol of the ribose moiety in the reaction with 5(4*H*)-oxazolones could therefore be related to a role of the second hydroxyl group in the proton transfer at the transition state. Whether this participation involves the hydroxyl group as a proton shuttle or more simply includes stabilization by hydrogen bonding at the transition state eventually facilitating proton transfer from a different acid remains to be determined. Additional support to this explanation can be found in the occurrence of acid catalysis already demonstrated in the 5(4*H*)-oxazolone reaction with amines (Murillo-Sánchez et al., 2016). Reagents generating tetrahedral intermediates with good leaving groups (as for instance the carbamate involved in the NCA reaction) would therefore react with nucleophiles without requirement for catalysis by a neighbouring group, which obviously favours the reaction of water. Those generating poor

leaving groups, would, on the contrary, require prior assistance from an acid, generating a specific advantage for vicinal diols, through an involvement in proton transfer or in hydrogen bonding. The observation of selective chemical aminoacylation of tRNA starting from cyanomethyl esters, aminoacyl adenylates (Saito and Suga, 2001) or imidazolides (Profy and Usher, 1984 a, 1984 b) could therefore be discussed on a similar basis. Interestingly, Profy and Usher observed that the reaction of an imidazolidine with the 2'(3')-sites of RNA was less sensitive to imidazole buffer concentration (Profy and Usher, 1984 b) than the reaction at the other available positions suggesting a role in acid-base catalysis for the vicinal diol. Hence, the 2'(3')-hydroxyl groups constitute the preferred acylation sites at low concentrations of imidazole buffer.

In conclusion, the present study of RNA model aminoacylation demonstrates the specific ability of 2'- and 3'-hydroxyl groups to react with 5(4*H*)-oxazolones. Surprisingly, this ability is not conserved for other aminoacylating agents such as *N*-carboxyanhydrides. Therefore, the possibility of chemically aminoacylating the 3'-end of RNA strands, required for the synthesis of aminoacylated tRNAs cannot be considered as a general property of activated α -amino acids. On the contrary, it could be specific of some activated species for which the breakdown of the tetrahedral intermediate becomes rate determining so that neighbouring group participation can give an advantage to the reaction of a vicinal diol compared to that of water, otherwise advantaged as the most abundant oxygen nucleophile in aqueous solution. Additionally, the selection of aminoacyl adenylates (biosynthesized through the ATP activation of amino acids) as substrates of aminoacyl-tRNA synthetases in protein biosynthesis has been considered as puzzling because of the short lifetime of these species in neutral aqueous solution (Pascal and Boiteau, 2011; Liu et al., 2014), which are converted into NCAs in the presence of low concentrations of carbon dioxide. The advantage of vicinal diols in the subsequent aminoacylation could therefore provide a rationale for that selection because NCAs proved to be incompetent whereas phosphate mixed anhydride, possibly requiring acid catalysis or other forms of assistance to phosphoryl transfer, might constitute efficient reagents. Another observation (Wickramasinghe and Lacey, 1994) confirmed by this study is the specific ability of RNA models to undergo aminoacylation, which exceeds that of DNA models by a factor of *c.a.* one order of magnitude and therefore accounts for the wide use of RNA in translation. The slight but significant preference for L-amino acids at the 3'-position of the D-ribose moiety, observed earlier by Lacey *et al.*, (Lacey et al., 1988, 1993) whereas D-amino acids are more easily bound to the 2'-OH, was confirmed in the present work. It is consistent with the involvement of 3'-aminoacylated-tRNAs as activated species in

the ribosomal biosynthesis of proteins, which is an indication that the system made of L-amino acids esterified at the 3'-position of D-ribonucleotides could be based on chemical grounds rather than the result of a random selection. The specific behaviour of vicinal diols in the reaction with 5(4*H*)-oxazolones can be analysed in relation with a similar role in ribozyme activity (Müller and Bartel, 2003) and ester aminolysis (Rangelov et al., 2010; Weinger et al., 2004; Weinger and Strobel, 2006; Bernhardt, 2015) including the peptidyl transfer of the ribosomal reaction. The study of the activity of potential prebiotic reagents for the non-enzymatic aminoacylation reactions of RNA models therefore provides valuable information on the chemistry that could have supported the first steps of life.

Notes

[1] 5'-AMP was methylated by reaction with dicyclohexylcarbodiimide in methanol as described in Ref. (Liu et al., 2016)

[2] Reactions were carried out in aqueous solution using non-nucleophilic MES (MES = morpholinoethane sulfonic acid) buffers at pH 6.5. *O*-methylated tyrosine (Tyr(Me)) was used as a model of usual amino acid derivatives. *N*-Ac-Tyr(Me)-5(4*H*)-oxazolone is stable at -20°C as a solid or in CH₃CN solution for several weeks (see ref. Liu et al., 2016).

[3] The acetylated NCA **2b** was prepared by acylation of the non-substituted derivative (prepared as described in Refs. Daly and Poche, 1988; Liu et al., 2014) with acetyl chloride in the presence of *N*-methylmorpholine according to the general procedure described in Refs. Fuller et al., 1990, 1996) for urethane-*N*-carboxyanhydrides. Tyr(Me)-NCA (0.3 g, 1.36 mmol) and acetyl chloride (0.101 mL, 1.43 mmol) were dissolved in dry THF (5 mL) under a N₂ stream. The system was kept at -10°C using a methanol/ice (50/50) bath. Dry *N*-methylmorpholine (NMM, 0.22 mL, 2 mmol) was added dropwise into flask under N₂. The slurry was stirred at -10°C for 1.5 h. Excess NMM was neutralized by adjusting the pH to 3-5 by the addition of HCl (0.15 ml of a 4 M solution in dioxane). The precipitate was removed by filtration then washed by THF twice. After concentration of the filtrate under reduced pressure, the resulting oil was dissolved in THF (minimum) and hexane was added until crystallization started. The solution was placed at -15°C overnight. The white product was collected by filtration and dried under vacuum to give Ac-Tyr(Me) NCA as a solid (138 mg, 38.7%). ¹H NMR (300 MHz, CDCl₃) δ_H = 7.01 – 6.91 (m, 2H), 6.90 – 6.80 (m, 2H), 5.01 (dd, *J* = 5.7, 2.4 Hz, 1H), 3.79 (s, 3H), 3.60 – 3.20 (m, 2H), 2.54 (s, 3H). ¹³C NMR (75 MHz, CDCl₃) δ_C = 169.06, 165.77, 159.48, 148.19, 130.42, 124.09, 114.55, 60.49, 55.21, 33.79, 24.69.

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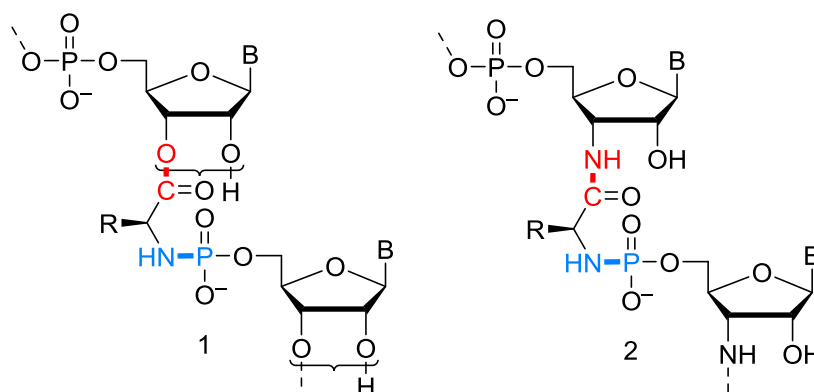
Chapter 4. The potential role of co-oligomers of ribonucleotides and amino acids in chemical evolution

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(after text revisions, with updated title: “*The chemical likelihood of ribonucleotide – α -amino acid copolymers as players for early stages of evolution*”)

Overview:

Building on an early background (**Biron et al., 2004, 2005; Leman et al., 2006**) as well as on more recent studies (**Liu et al., 2014, 2016, 2017**), we chose to investigate the potential role of copolymers of α -amino acids and nucleotides **1** linked by phosphoramidate and ester linkages (**Scheme 1**) as another option to the restrictions of both peptide and RNA worlds. Since RNA polymerization is limited by the low efficiency of the nucleophilic attack of the ribose 3'-OH, many investigations have used a 3'-NH₂ modified ribose moiety to facilitate polymerization and study template-directed polymerization (**Zielinski and Orgel, 1985; Zielinski and Orgel, 1987 a; Zielinski and Orgel, 1987 b; von Kiedrowski et al., 1991; Sievers and von Kiedrowski, 1994; Kaiser et al., 2012; Zhang et al. 2013**). Moreover, an interesting structure mentioned in the literature is the corresponding – amide-linked – aminoacylated nucleotide modified by an amino group **2** (**Scheme 1**) that also yielded efficiently phosphoramidates upon reaction with nucleotides (**Zielinski and Orgel, 1989**).



Scheme 1. The structure of co-oligomers of α -amino acids and nucleotides **1** involving carboxylic acid esters (red) and phosphoramidates (blue) as key linkages and the stable amide structural analogues of 3'-amino-3'-deoxy-nucleotides **2** (X = NH) studied by Zielinski and Orgel (**Zielinski and Orgel, 1989**).

In the following article, we have investigated the possibility of finding plausible chemical processes allowing the formation of the copolymers **1** and performed experimental kinetic studies to check their hydrolytic stability. The results of the following article are summarized below, and the structures used in the present study are presented in figure 1. We have used a simple trimer models **3a** (with a Tyr(Me) amino acid residue) containing the phosphoramidate and ester linkages and a 5'-terminus blocked by a methyl group (**Figure 1**).

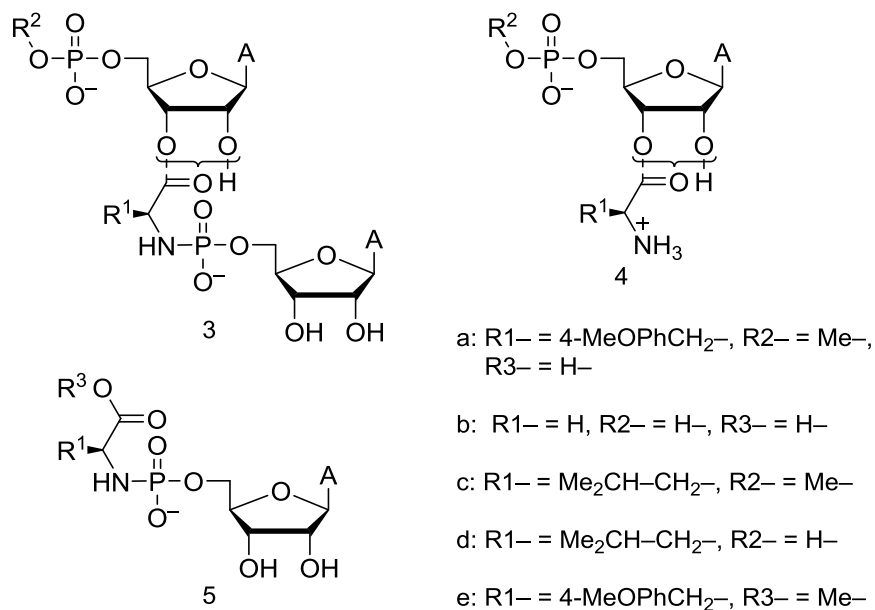


Figure 1. Structures used in the present study.

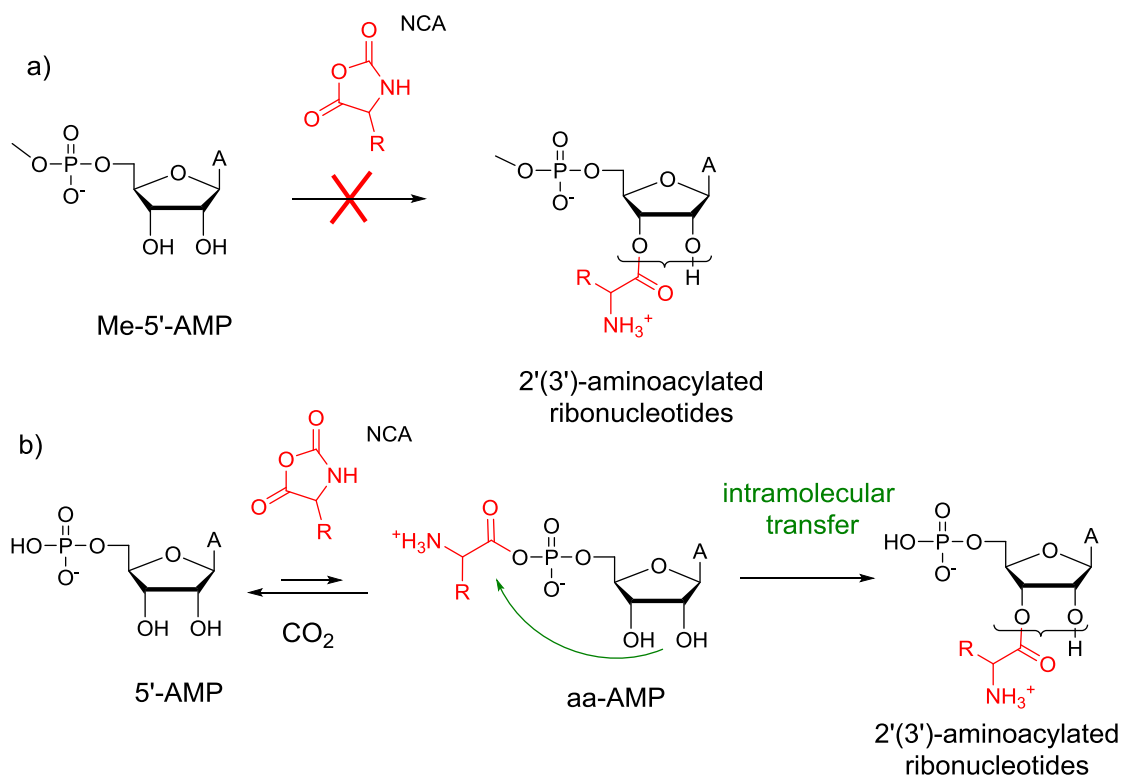
Formation of the trimer model **3a**:

Firstly, we started by searching for a chemical processes to synthesize the phosphoramidate linkage and then to find a prebiotic way for the aminoacylation of nucleotides (formation of ester linkage).

- ✓ **Formation of phosphoramidate linkage:** To the best of our knowledge, in the literature, the work of Shim et al. (**Shim et al., 1974**) is the only report of a pathway for the formation of a similar phosphoramidate adduct **3b**. Using ¹⁴C-labeled substrates, they reported that up to 80 % yield was formed from a reaction of 12.5 mM 2'(3')-Gly-AMP ester **4b** with the 12.5 mM AMP-imidazolide in the presence of 200 mM NaCl and 75 mM MgCl₂ and 50 mM poly-U as a template for oligomerization at 0°C. The authors also investigated the reaction of the glycine phosphoramidate of AMP **5b** with AMP in the presence of EDC as an activating agent, which also indicated the formation of

a similar product in up to 43.5% in the presence of poly-U and imidazole. They have studied the effect of poly-U as a template of oligomerization and therefore of alternative structures capable of template-directed oligomerization. In our hands, we have synthesized the copolymer **3a** from aminoacylated methylated AMP model **4a** and activated 5'-AMP in MES buffer (6.5) at room temperature. Moreover, we have made the same reaction at a low temperatures (5 °C) and the yield strongly increased. Therefore, the reaction yield is highly dependent of temperature and strongly increased at temperatures close to 0 °C. We have purified the copolymer model **3a** by preparative LC due to its stability (explained later). It has been identified by NMR and MS. The targeted polymer could, therefore, be obtained through an abiotically plausible process provided that aminoacylated derivatives of ribonucleotides analogous to **4a** are available.

- ✓ **Ester linkage (prebiotic aminoacylation process):** Identifying a plausible mechanism for the formation of 2'(3')-aminoacylated mononucleotides analogous to **4a** was one of our priorities. NCAs proved to be inefficient for the aminoacylation of methylated AMP (Me-5'-AMP), a simple model of the reactivity of RNA 3'-end (**Scheme 2a**, Liu et al., 2017). We have therefore considered the possibility of an intramolecular aminoacyl transfer published earlier by the group of Lacey (**Wickramasinghe et al., 1991**). In this pathway, the occurrence of an aminoacyl transfer from the 5'-OH position of the adenylate mixed anhydride (aa-AMP) to the 2'(3')-OH of the ribose moiety allows the formation of esters. In addition to Lacey's observation, we knewed from an earlier study, that the mixed anhydride could be formed from a reaction of 5'-AMP and NCAs and that the reaction is reversible in the presence of CO₂ (even in the low amount of CO₂). We, therefore, investigated the possibility that the NCA could react with AMP to yield a mixed anhydride at equilibrium, which could be subsequently trapped by intramolecular transfer. We were delighted to observe that this procedure worked efficiently (**Scheme 2b**), albeit in low yield.



Scheme 2. a) Impossibility of the direct aminoacylation of 2'(3')-OH of Me-5'-AMP by NCAs. b) Possibility of the indirect aminoacylation of 2'(3')-OH of 5'-AMP by NCAs through the intramolecular aminoacyl transfer from aa-AMP, which can compete with the breakdown of the mixed anhydride promoted by CO₂.

In addition to a route for their synthesis, a potential role of the co-oligomers would require a sufficient time stability. Therefore, we studied the hydrolytic stability of the copolymer structure **1** (Scheme 1).

Kinetical study of the stability of the phosphoramidate linkage and 2'(3')-aminoacylated nucleotides:

We have examined the hydrolysis of the selected model **3a** in aqueous buffers at different pH values. The copolymer has two hydrolysis sites: the ester linkage and the phosphoramidate linkage. In a way consistent with the presence of two hydrolysis site, we have shown that the pH-rate profile of the copolymer is mainly characterized by two linear domains corresponding to the acid-catalyzed phosphoramidate hydrolysis at pH values below 5 and the base-catalyzed cleavage of the ester above pH 6.5. This pH-rate profile was compared with that of analogues having a simpler structure as the aminoacylated RNA model **4c** which was also subjected to hydrolysis under similar conditions. We have observed that the negative charge of the phosphoramidate group has a substantial influence on the stability of the ester bond

in the co-trimer **3a** since the ester proved to be stabilized by a factor exceeding 3 orders of magnitude compared to esters with a protonated free amino group **4c** at pH 6.5. The above observation means that the ester bond of a 2'(3')-aminoacylated nucleotide, considered as an aminoacylating agent for protein biosynthesis, is stabilized upon phosphorylation, having lifetimes reaching several weeks or months at moderate temperatures and within a 5–7 pH range anticipated for most early Earth aqueous environments. So that it could constitute the potential building block of a reactive biopolymer having a lifetime sufficient to play a role in the early developments of life.

Preliminary kinetic hydrolysis studies of the ester (4c**) at 0 °C.**

After observing that the polymer was formed in a high yield at 0 °C from the ester **4c** and activated AMP at pH 6.5. We have investigated the stability of ester **4c** at 0 °C. We have observed that the half-life of ester **4c** hydrolysis increases from 3 h to 85 h by lowering the temperature from 25°C to 0°C. This result can explain the high yield of the copolymer formation at low temperature since the formation of the phosphoramidate linkage of the targeted copolymer competes favorably with the hydrolysis of the ester bond of the starting material **4c** at 0°C.

The main results in the following article are that: We have observed unexpected properties of the amino acid-nucleotides copolymers that were linked by phosphoramidate and ester linkages. Firstly, we have shown that upon phosphorylation of the amino group of aminoacyl nucleotides, the stability is strongly increased. Secondly, we have demonstrated a prebiotically plausible process for the formation of aminoacyl nucleotides via the intervention of NCAs by an indirect pathway. Thirdly, we have confirmed the formation of phosphoramidates are more efficient than the formation of the phosphodiester, because amino group is more nucleophilic than alcohols in phosphoryl exchange. All of the above support the idea of a potential role of these copolymers at the chemical stage of evolution, and we should not underestimate their role. As another important point raised in the course of this work, we have also observed the strong temperature dependence of the hydrolysis of 2'(3')-aminoacylated nucleotide. Though this observation does not rule out an origin of life at high temperatures, it makes the emergence of translation more

probable at a temperature close or below the melting temperature of water by a factor that could reach several orders of magnitude by comparison with a value of 70°C suggested for the Archean ocean. If the evidence that low temperatures are favorable to the emergence of translation is considered as valuable, it would mean that observation of an ancestor living at higher temperatures is the result of a later adaptation of life to higher temperatures. Therefore, it removes any relevance of phylogenetic determinations to the temperatures favorable for the origin of life. We can see that chemical investigation on the origin of life can also lead to conclusions on the more favorable conditions for its origin (including temperatures) and that these conclusions are valuable for astrobiology as a science.

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Article 4.

The potential role of co-oligomers of ribonucleotides and amino acids in chemical evolution

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(after text revisions, with updated title: “*The chemical likelihood of ribonucleotide – α -amino acid copolymers as players for early stages of evolution*”)

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Supporting information for this article will be found online on the journal website.

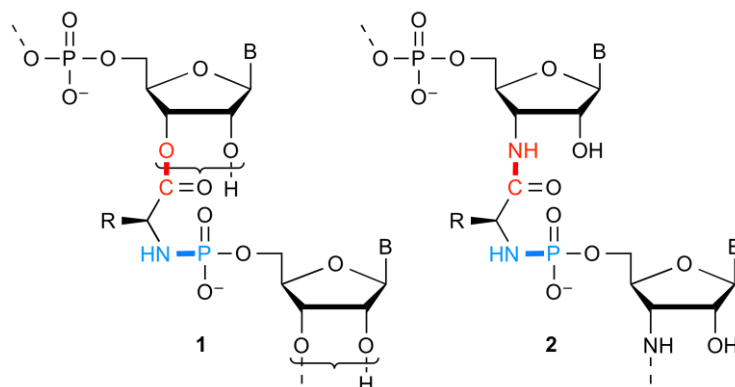
Abstract

How ribosomal translation could have evolved remains an open question in most available scenarios for the early developments of life. Here we show that copolymers of α -amino acids and nucleotides could have lifetimes of several weeks to months at neutral pH and 20°C owing to the *mutual protecting effect* of both neighbouring phosphoramidate and ester functional groups increasing their stability by factors of about 1 and 3 orders of magnitude, respectively. This protecting effect is reversible upon hydrolysis allowing the possibility of subsequent reactions. These copolymers, for which an abiotic synthesis pathway is supported by experiments, form a basis from which both polymerization and translation could have logically evolved. Low temperatures were identified as a critical parameter facilitating the formation of the polymers. This observation independently supports the views that any process involving RNA aminoacyl esters, outstandingly including the emergence of translation, was more probable at 0°C or below.

Introduction

The idea that the origin of life could result from a replication of the sequence of a biopolymer was a logical consequence of the discovery of the double helix structure of DNA in 1953 ([Watson and Crick, 1953](#)). This discovery emphasized the role of nucleic acids in the storage of information and as a main driver of life and its developments. By contrast, the results of Miller's experiment ([Miller, 1953](#)), published three weeks later, pointed towards the formation of α -amino acids under prebiotic conditions and therefore towards an easier synthesis of peptides, the other main class of biopolymers. However, the chemical replication and inheritability of peptide sequences without translation is unsolved yet and is likely to remain so. On the other hand, the possibilities of template replication of nucleotides as well as the role of RNA in biochemistry pointed towards a major contribution of RNA in early life ([Crick, 1968](#); [Orgel, 1968](#); [White, 1976](#)). Unfortunately, the abiotic synthesis of long strands of RNA capable of playing the role of polymerase has not been successful to date even though chemical pathways for the abiotic synthesis of nucleotides have been discovered ([Anastasi et al., 2006](#); [Powner et al., 2009](#)). Following the hypothesis of an RNA world, the transition of the RNA world to an RNA-protein world, a subsequent stage of evolution, requires a common chemistry of RNA and amino acids or peptides ([Szathmáry, 1999](#)). It would then be surprising that the corresponding chemistry could not have played a role at earlier stages since α -amino acids were likely present on the primitive Earth eventually leading to both RNA and coded peptides ([Borsenberger et al., 2004](#)). We decided to investigate the potential role of copolymers of α -amino acids and nucleotides **1** containing phosphoramidate and ester linkages (Scheme 1) as an alternative to the limitations of both peptide- and RNA-worlds. One of the advantages of these structures is the foreseen effectiveness of formation of a phosphoramidate linkage compared to that of phosphodiester demonstrated by the group of Orgel ([Lohrmann and Orgel, 1976](#); [Zielinski and Orgel, 1985](#)). The reaction of nucleotides having a 3'-hydroxyl substituted for an amino group easily produces phosphoramidate linkages upon activation by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and served as a tool to investigate template replication ([Zielinski and Orgel, 1985](#); [Zielinski and Orgel, 1987 a](#); [Zielinski and Orgel, 1987 b](#); [von Kiedrowski et al., 1991](#); [Sievers and von Kiedrowski, 1994](#); [Kaiser et al., 2012](#); [Zhang et al., 2013](#)). Interestingly, the

corresponding – amide-linked – aminoacylated nucleotide modified by an amino group **2** (Scheme 1) also yielded efficiently phosphoramidates upon reaction with nucleotides (Zielinski and Orgel, 1989).



Scheme 1. The structure of co-oligomers of α -amino acids and nucleotides **1** involving carboxylic acid esters (red) and phosphoramidates (blue) as key linkages and the stable amide structural analogues of 3'-amino-3'-deoxy-nucleotides **2** (X = NH) studied by Zielinski and Orgel (Zielinski and Orgel, 1989).

Orgel et coll. (Shim et al., 1974) even observed the formation of sequences corresponding to **1** from an – ester linked – 2'(3')-glycyl-5'-AMP. It is worth noting that the presence of a template was reported to greatly facilitate the reaction and the authors concluded in wondering about the potential role of oligomers of that kind in the context of prebiotic chemistry. The ester bond in the copolymer is analogous to that of aminoacyl-tRNAs (aatRNAs) known as the unstable activated species of peptide biosynthesis and that are present as an equilibrium mixture of 2'- and 3'-isomers (Wolfenden et al., 1964). Taking into account the fact that removing the positive charge of the aminoacyl moiety increases the kinetic stability of the ester bond in aa-tRNAs by two orders of magnitude (Wolfenden, 1963; Schuber and Pinck, 1974) and that this effect could be enhanced by the negative charge of the phosphoryl substituent, we deduced that the corresponding phosphoramidates could present a time stability suitable for allowing them to play a significant role in chemical evolution. To the best of our knowledge the pioneering work of Shim et al. (Shim et al., 1974), mainly motivated by the identification of structures capable of template replication, an ability that was not confirmed later using closely related amide-based derivatives **2** (Zielinski and Orgel, 1989), has not been investigated further. On the basis of our earlier experience in aminoacylation using α -amino acids strongly activated under the form of 5(4*H*)-oxazolones and *N*-carboxyanhydrides (NCAs) (Liu

et al., 2014 ; Liu et al., 2016 ; Liu et al., 2017), we embarked on a study of potential prebiotic pathways of formation of phosphoramidate-linked adducts as well as of their hydrolytic stability. We now report the first results demonstrating that abiotic pathways for the formation of both ester and phosphoramidate key linkages of structure **1** are indeed available and that their kinetic stability is compatible with a role of chemical intermediates in a system based on amino acids and nucleotides predating the better established RNA-protein world. Hypothetical pathways through which chemical evolution could have ensued are additionally proposed to illustrate the possible contribution of these polymers to the early stages of life from which translation and replication could have evolved at the same time without requiring a stage based on RNA only.

Results and discussion

The study of the co-oligomers was undertaken using the simple trimer models **3a** (with a Tyr(Me) amino acid residue) containing the key phosphoramidate and ester linkages and a 5'-terminus blocked by a methyl group (Figure 1). Using ¹⁴C-labeled substrates, Shim et al. (Shim et al., 1974) were able to detect the formation of a similar phosphoramidate adduct **3b** in up to 80% yield from a reaction of 12.5 mM 2'(3')-Gly-5'-AMP ester **4b** with the 12.5 mM 5'-AMP-imidazolide in the presence of 200 mM NaCl and 75 mM MgCl₂ and 50 mM poly-U as a template for oligomerization at 0°C. The authors also investigated the reaction of the glycine phosphoramidate of 5'-AMP **5b** with 5'-AMP in the presence of EDC as an activating agent, which also indicated the formation of a similar product in up to 43.5% in the presence of poly-U and imidazole.

Formation of the phosphoramidate linkage. We investigated phosphoramidate formation from the free 5'-AMP nucleotide that was activated and reacted with the aminoacylated methylated 5'-AMP model **4a** (Figure 1) in the presence of the EDC reagent well known to be efficient for the phosphoramidate ligation of nucleotides. (Lohrmann and Orgel, 1976; Zielinski and Orgel, 1985; Zielinski and Orgel, 1987 a; Zielinski and Orgel, 1987 b; von Kiedrowski et al., 1991; Sievers and von Kiedrowski, 1994; Kaiser et al., 2012; Zhang et al., 2013; Zielinski and Orgel, 1989). In our hands, this procedure proved to be convenient and yielded an adduct in moderate yield indicating that phosphoramidate formation is able to compete with the hydrolysis of the ester at reasonably low concentrations (Figure 1). For instance, a solution

containing 5 mM 5'-AMP, 5 mM ester **4a** and 500 mM EDC in a 100mM MES buffer (pH 6.5) was allowed to react at room temperature and monitored by HPLC (Figure 1). The selected HPLC method (Method A, Supporting Information) showed the presence of two regioisomers (retention times 11.2 and 13.6 min in a 3:7 ratio) of the starting material **4a** and the addition of EDC induced the formation of two new HPLC peaks (retention time 12.6 and 14.5 min in a 4:6 ratio). After 18.5 h, the starting ester was totally consumed and the mixture was analysed by HPLC-MS indicating the presence of two isomers with a mass corresponding to that of co-trimer **3a** (HPLC-MS, retention time 1.55 and 1.58 min, Method D, ESI negative mode, m/z calculated for $C_{31}H_{38}N_{11}O_{15}P_2^-$: 866.2030, found 866.20). The reaction was continued for 300 h without major change and the final yield was estimated to approximately 16% making the hypothesis that the uv absorption of the adenine moiety in the chromatogram is not modified by the reaction, which is reasonable with respect to the accuracy of the assessment. At that time the medium was acidified to pH 2 and the HPLC analysis was repeated after 3 days indicating that the hydrolysis of the adduct reverted the starting ester **4a** as two regioisomers (HPLC-MS, retention time 1.54 and 1.56 min, Method D, ESI negative mode, m/z calculated for $C_{21}H_{26}N_6O_9P^-$: 537.1504, found 537.10). The product's reversion in acid to the starting material is consistent with the presence of an acid-sensitive phosphoramidate linkage and with the attribution of the structure of co-trimer **3a** to the adduct, which is in agreement with the presence of two regioisomers as well. A similar reaction was carried out in D_2O and analysed by ^{31}P -NMR (Supporting Information Figure S1) indicating the presence of two signals at 5.99 and 6.27 ppm supporting the presence of isomers bearing a phosphoramidate moiety. The yield of the reaction could be substantially increased by performing the reaction at 4°C over longer periods with yields approaching the one published by Shim et al. (Shim et al., 1974). A rationale for this observation is that the hydrolysis of ester **4a** occurring at the same time as phosphoramidate formation is responsible for the low yield of co-trimer **3a** at 25°C and can be overcome owing to the unusually high influence of temperature on the rates of hydrolysis of ester **4a** (see below) by reducing the temperature to a value close to 0°C. Owing to its time stability, increased compared to aminoacylated nucleotide **4a**, the adduct **3a** could be separated by preparative chromatography in triethylammonium acetate buffers with an overall yield exceeding 50% and characterized by NMR and MS (Supporting Information). The targeted polymer could

therefore be obtained through an abiotically plausible process provided that aminoacylated derivatives of ribonucleotides analogous to **4a** are available.

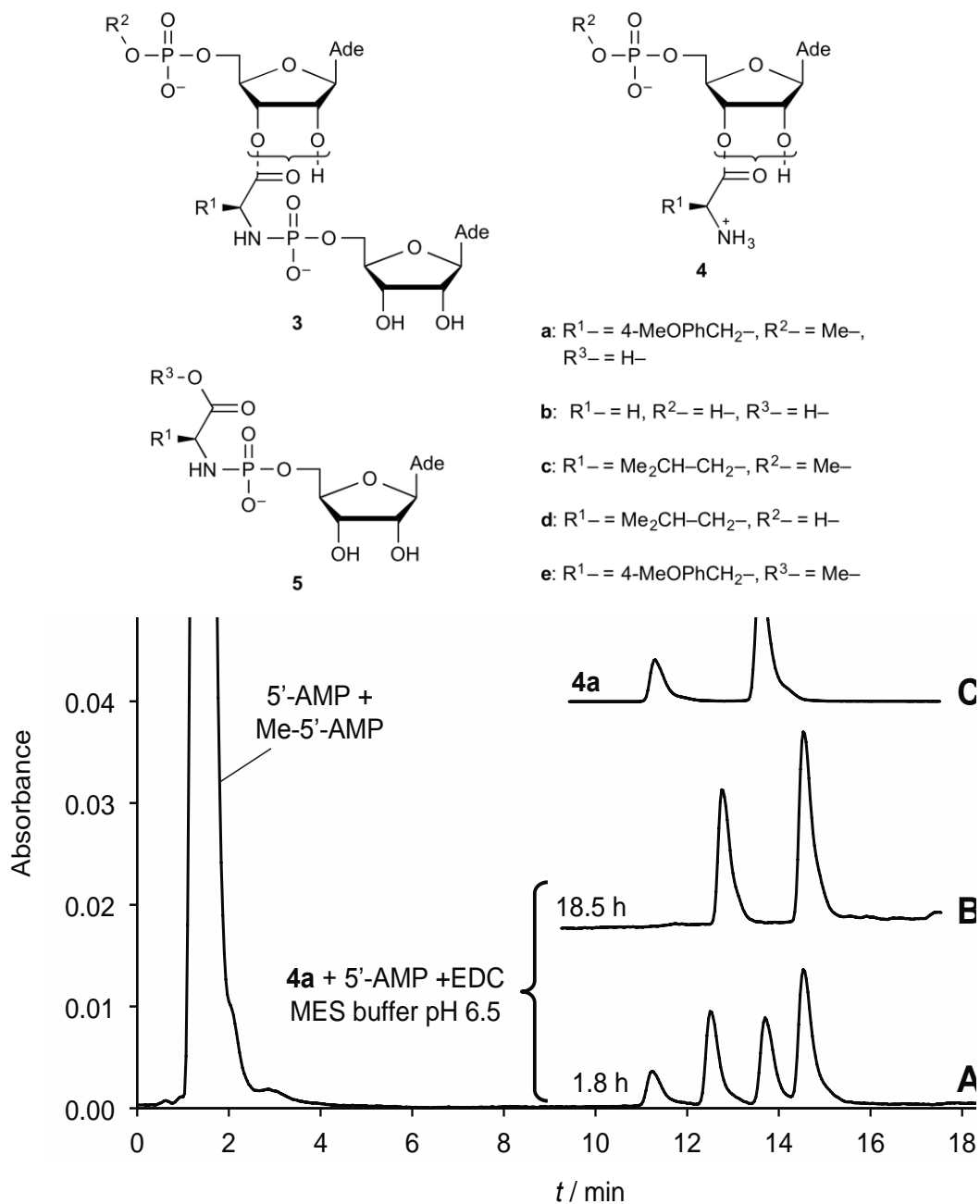
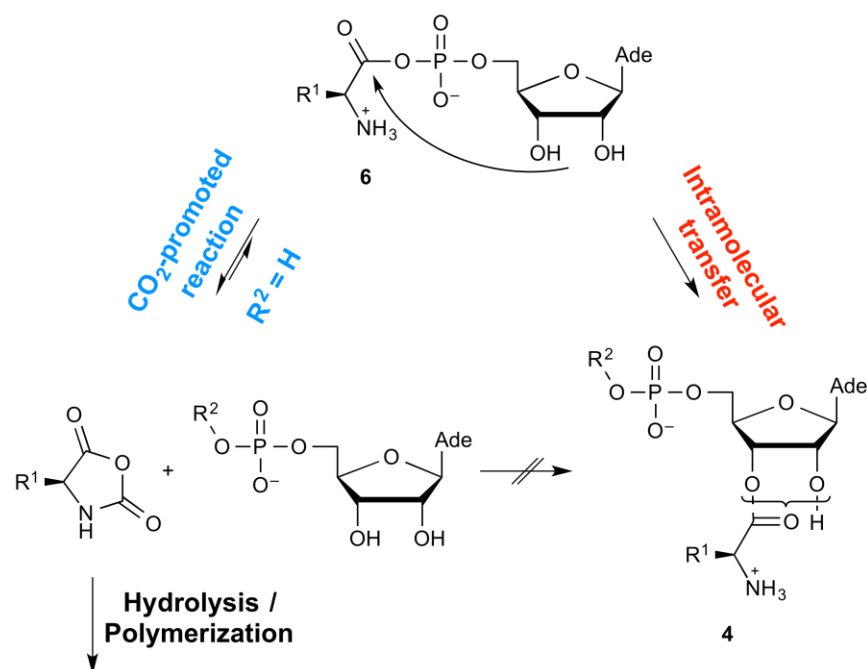


Figure 1. Upper box: structures of the chemical derivatives used in this work. Lower box: reaction of 5 mM ester **4a**, 5 mM 5'-AMP and 500 mM EDC in a 100 mM MES buffer pH 6.5; HPLC chromatograms of samples of the reaction medium (20 μ L diluted to 1 mL with water, method A, detection 248 nm at the minimum of the uv spectrum of Tyr(Me)); **A:** Reaction medium after 108 min of reaction; **B:** after 18.5h; **C:** Unreacted ester **4a** in water.

Aminoacylation. Although procedures for chemically aminoacylating ribonucleotides have been proposed, we considered the possibility of a reaction involving α -amino acid *N*-carboxyanhydrides (NCAs) as activated reagents and an intramolecular aminoacyl transfer (Scheme 2) for reasons that are listed below. Firstly, and from a synthetic perspective, imidazolides of *N*-protected amino acids turned out to be efficient for aminoacylation (Profy and Usher, 1984 a, 1984 b) though their occurrence in an abiotic context is questionable and the presence of a protecting group removes to the process most of its relevance to our present goal. Ribozymes active in aminoacylation and using adenylates, active esters or thioesters have been selected (Illangasekare et al., 1995, 1997; Lee et al., 2000) In an origin of life context, it is worthy to note that very short sequences proved to be active (Turk et al., 2010, 2011). However, strongly activated α -amino acid derivatives such thioesters (Brack, 1987) as well as aminoacyl adenylates (Wickramasinghe et al., 1991; Liu et al., 2014) having a free amino group are not stable in aqueous environments in the presence of CO₂ and the lifetime of adenylates at the present day concentration of bicarbonate in the ocean or in biological media has been assessed to a few seconds only (Liu et al., 2014), removing any utility of amino acid phosphate anhydride as activated precursors for the formation of peptides in a prebiotic environment richer in CO₂. The CO₂-promoted reaction, which strongly increases peptide formation (Liu et al., 2014), takes place through an NCA intermediate (Scheme 2), which can then be hydrolysed or polymerized. No advantage can therefore be found for adenylates as peptide precursors, which raises the question of their selection as essential intermediates of the biosynthesis of peptides. The important role of adenylates and other mixed anhydrides could however lie in the fact that NCAs proved to be inefficient for the aminoacylation of methylated 5'-AMP (Me-5'-AMP), a simple model of the reactivity of RNA 3'-end (Liu et al., 2017). This behaviour, contrasting with that of other reagents like 5(4*H*)-oxazolones as activated peptide segments was considered as resulting from a different reaction path avoiding any assistance from the vicinal diol of the ribose moiety (Liu et al., 2017). We therefore considered the Lacey's group invaluable observation (Wickramasinghe et al., 1991)

of the occurrence of an intramolecular aminoacyl transfer in aminoacyl adenylates allowing the formation of esters at the 2'(3')-ribose moiety **4** from the mixed anhydride **6** (Scheme 2). The reaction was reported to be highly effective at pH

values below 6 provided that CO₂ is rigorously excluded from the reaction medium (Wickramasinghe et al., 1991). Moreover, the authors observed a high degree of stereoselectivity in favour of the natural configuration – a D-ribose moiety induced a preference for aminoacylation by L-amino acids – supporting an important role for this process in the emergence of the chiral coupling between the two classes of biomolecules (Wickramasinghe et al., 1991). Though this purely chemical process presents the drawback of being largely surpassed by the fast CO₂-promoted hydrolysis of adenylates, even at low contents of this gas in the atmosphere (Wickramasinghe et al., 1991), we realized that the reverse path, the formation of adenylates from NCAs, would also take place (Biron et al., 2005 ; Leman et al., 2006 ; Liu et al., 2014) provided that other synthetic processes could deliver α -amino acid *N*-carboxyanhydrides in the medium. Therefore, the possibility of aminoacylation of nucleotides could involve an indirect rather than direct reaction of NCAs, reagents for which prebiotically plausible pathways of formation have been identified (Leman et al., 2004; Pascal et al., 2005; Danger et al., 2006).



Scheme 2. The inefficiency of the direct aminoacylation of nucleotides 5'-AMP ($R^2 = H$) and Me-5'-AMP ($R^2 = Me$) by α -amino acid *N*-carboxyanhydrides (Liu et al., 2017) can be overcome through the intramolecular aminoacyl transfer from adenylates like **6d** ($R^1 = Me_2CHCH_2$) (Wickramasinghe et al., 1991), which can compete with the breakdown of the mixed anhydride promoted by CO₂ (the reverse of the aminoacylation pathway based on the reaction of NCAs that is fast in the presence of CO₂).

Taking into account our previous observation that the decomposition of aminoacyl adenylates into NCAs promoted by CO₂ is reversible (Liu et al., 2017), we therefore investigated the possibility that the NCA could react with 5'-AMP to yield a mixed anhydride at equilibrium, which could be subsequently trapped by intramolecular transfer (Scheme 2). We were delighted to observe that this procedure worked efficiently, albeit in low yield (Figure 2). Two HPLC peaks (retention time 4.1 and 6.4 min, Method C) corresponding to the 2'(3')-regioisomers of the aminoacylated species **4d** were indeed observed by reaction of Leu-NCA (5 additions of a total of 25 mM over 4 h to reduce the occurrence of NCA polymerization) with 5'-AMP at pH 5.5 (100 mM MES buffer). A ca. 2-fold increase in yield could be observed in the presence of 50 mM Mg²⁺ and the intramolecular nature of the reaction could be confirmed by the absence of reaction of the methylated ester **4c** (Supporting information) (Liu et al., 2017).

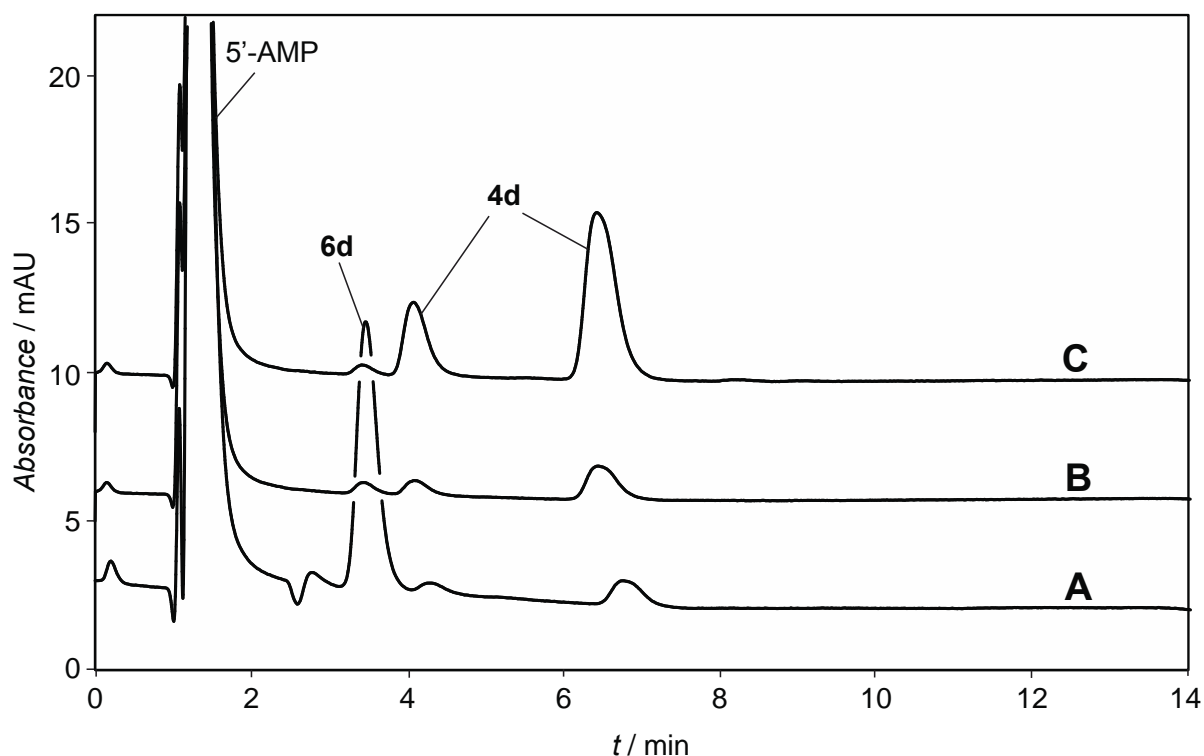


Figure 2. Reaction of Leu-NCA (25 mM overall added in 5 portions over 4h) with 1 mM 5'-AMP in a 100 mM MES buffer (pH 5.5) containing 50 mM MgCl₂ at room temperature. HPLC monitoring of the reaction progress (Method C, detection 248 nm): **A**: reaction medium after the addition of the last portion of Leu-NCA at 4h; **B**: reaction medium at 24 h; **C**: reaction medium at 24 h after addition of ester **4d**. Identification of HPLC peaks: 1.3 min, 5'-AMP, 3.5 min leucyl adenylate **6d**, and 4.1 and 6.4 min, 2'- and 3'-regioisomers of ester **4d**, respectively.

The intramolecular nature of the aminoacylation process, already supported by experiments carried out at very low concentration (Wickramasinghe et al., 1991), is confirmed here by the reaction of the NCA with 5'-AMP, which contrasts sharply with the absence of reaction observed earlier for Me-5'-AMP in which the phosphodiester moiety is unable to give a di-anion and therefore to yield a mixed anhydride (Supporting information) (Liu et al., 2017). We previously noticed that the fast decomposition of amino acid-nucleotide mixed anhydrides observed even at low concentration of CO₂ removes any utility as peptide precursors to these reagents since the NCA intermediates behave as active monomers in a much better way (Liu et al., 2014). Their early role in chemical evolution could therefore have been to act as aminoacylating agents rather than as activated precursors of peptides. The possibility of an intramolecular transfer of the aminoacyl moiety from adenylate anhydrides to the 3'-hydroxyl group is an additional example of the importance of intramolecular reactions in a prebiotic context and of *induced intramolecularity* for the emergence of catalysis (Pascal, 2003, 2015).

Kinetical study of the stability of the phosphoramidate linkage and of 2'(3')-aminoacylated nucleotides. The kinetic stability of copolymer structure **1** is an essential parameter of its possible contribution in early evolution. In aqueous solution, at the pH values used in the experiments, the hydrolysis of the selected model **3a** was monitored by HPLC (retention time of 2'- and 3'-regioisomers 12.4 min and 14.4 min, Method A) in aqueous buffers at different pH values (Figure 3). By contrast with the cleavage of the acid sensitive phosphoramidate moiety at pH 2 described in the above section on phosphoramidate formation, ester hydrolysis was observed at high pH values as for instance in a 50 mM borax buffer (pH 9.2) where two products were identified by HPLC-MS after 9 days of reaction as Me-5'-AMP (retention time 1.27 min, ESI negative mode, *m/z* calculated for C₁₁H₁₅N₅O₇P⁻: 360.0715, found 360.07) and the phosphoramidate **5a** (retention time 1.73 min, ESI negative mode, *m/z* calculated for C₂₀H₂₄N₆O₉P⁻: 523.1348, found 523.13). In a way consistent with the presence of two hydrolysis sites, the pH-rate profile of Figure 2 is mainly characterized by two linear domains corresponding to the acid-catalyzed phosphoramidate hydrolysis at pH values below 5 and to a the base-catalyzed cleavage of the ester above pH 6.5. A deviation from calculated curves may indicate the occurrence of a pH-independent ester hydrolysis at intermediate pH values. This pH-rate profile was compared with that of analogues having a simpler structure,

namely, the aminoacylated RNA model **4c**, the phosphoramidate **5a** and the corresponding methyl ester **5e** (Figure 3), which were also subjected to hydrolysis under similar conditions. The pH-rate profile for the aminoacylated nucleotide **4c** was similar to the behaviour published in the literature for aminoacylated-tRNAs (Wolfenden, 1963; Schuber and Pinck, 1974) that is characterized by a slower hydrolysis of the neutral aminoacyl moiety predominant at high pH compared to the protonated one (by a factor of ca. 2 orders of magnitude). A similar but stronger influence of the negative charge of the phosphoramidate group was observed for the co-trimer **4c** since the ester proved to be stabilized by a factor exceeding 3 orders of magnitude compared to esters with a protonated free amino group at pH 6.5. This observation means that the ester bond of a 2'(3')-aminoacylated nucleotide, considered as an aminoacylating agent for protein biosynthesis, is stabilized upon phosphorylation, having lifetimes reaching several weeks or months at moderate temperatures and within a 5–7 pH range anticipated for most early Earth aqueous environments, so that it could constitute the potential building block of a reactive biopolymer having a lifetime sufficient to play a role in the early developments of life. It is worth mentioning that the lifetime of these species could be increased to values measured in years at temperature close to the melting of ice, which were above noticed to increase substantially the yield of phosphoramidate formation.

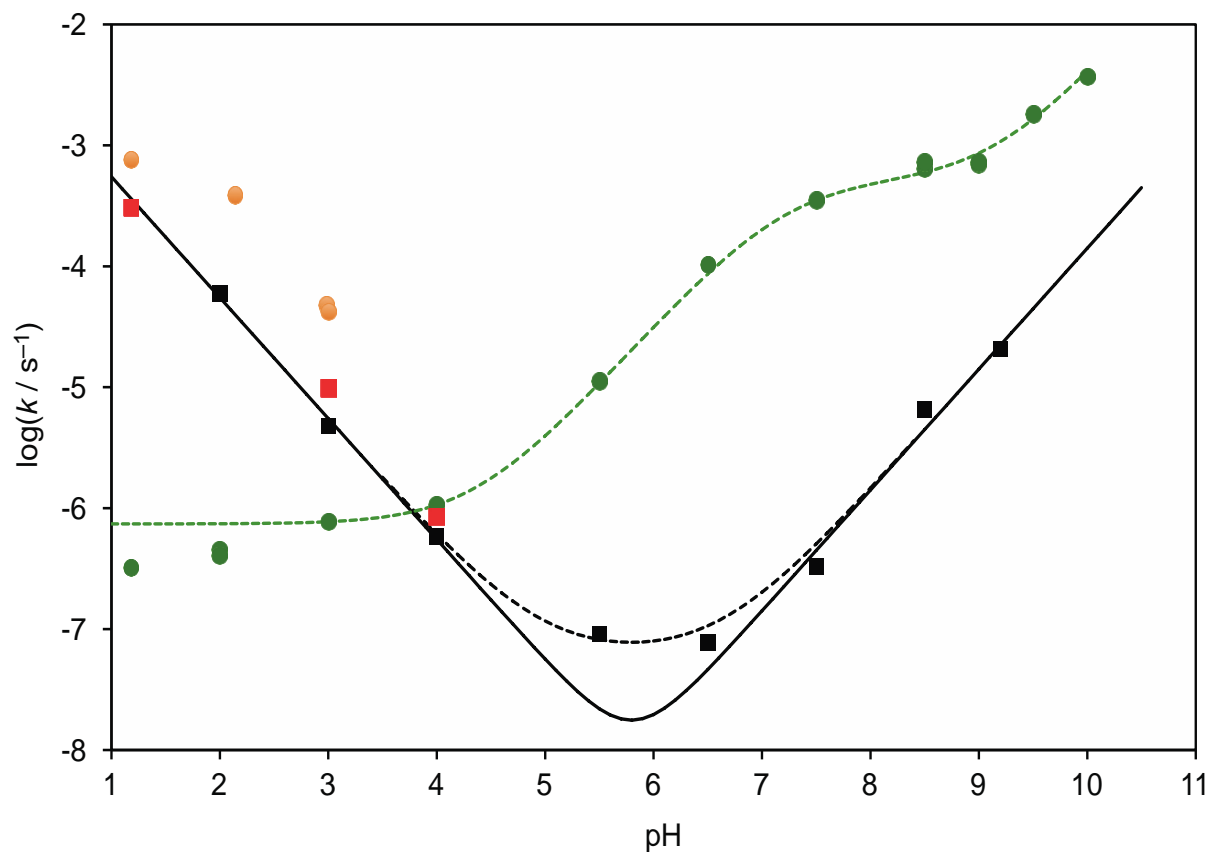


Figure 3. pH-rate profiles for the hydrolytic reactions of the copolymer model **3a** (black squares); the leucine ester **4c** (green circles); the phosphoramidate adduct of methylated tyrosine **5a** (orange circles) and its methyl ester **5e** (red squares); curves for the hydrolysis of co-trimer **3a** fitted to specific acid and base catalysed pathways (black line) and adding a pH-independent term (black dotted line); curve fitting for aminoacyl ester **4c** (green dotted line). The rate constant values used in the calculation are provided in the Supporting Information.

In the acid range ($1 \leq \text{pH} \leq 3$), the comparison with the methyl ester derivative **5e** indicates that the adenosine moiety has a limited influence on phosphoramidate hydrolysis. However, a higher sensitivity towards hydrolysis was observed for the compound **5a** having a free carboxyl group and that is produced by hydrolysis of the whole copolymer model **3a**. This faster hydrolysis of the acid by a factor of c.a. one order of magnitude can be accounted for by an intramolecular pathway involving the free carboxy group that is already well documented for this kind of derivatives (Benkovic and Benkovic, 1967; Sampson et al., 1973; Ora et al., 2007).

Finally a special mention must be made on the influence of temperature on the rates of hydrolysis of esters **4a** and **4c**, which is responsible for the increase in yield observed above for the formation of phosphoramidate **3a** at low temperature. The half-life of ester **4c** hydrolysis measured at pH 6.5 turned out to be increased from

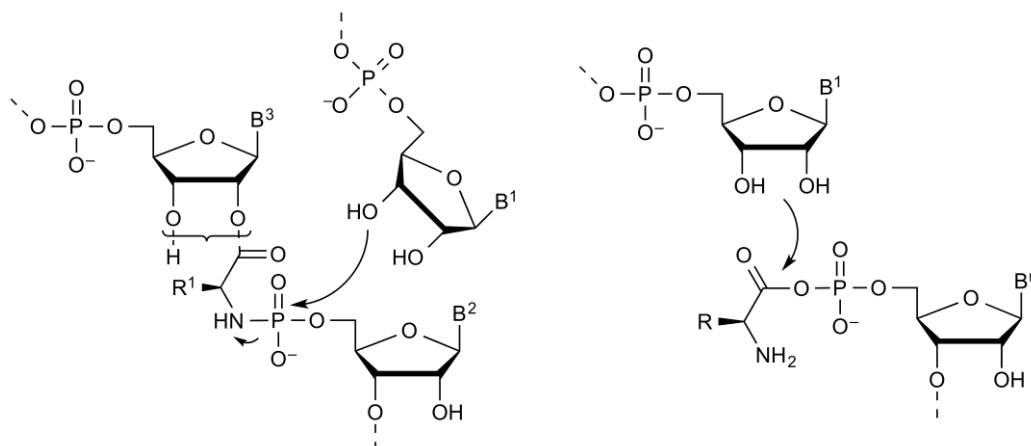
3 h to 85 h by lowering the temperature from 25°C to 0°C. This influence of temperature on the hydrolysis rates (a 29-fold increase much higher than the 5.7-fold deduced from the empiric rule that rates increase by a factor of 2-fold every 10°C, [Wolfenden et al., 1999](#)) is sufficient to explain why low temperatures favour the formation of phosphoramidate **3a**. ¹H and ¹³C NMR experiments carried out at 2°C using **4c** and its analogue labelled at leucyl-carbonyl carbon with ¹³C (Supporting information Figure S2 and S3) indicated no major change in the NMR spectra with temperature ruling out any shift of the equilibrium ratio of 2'- and 3'-isomers or even the occurrence of a stabilized tetrahedral hemioorthoester structure unable of undergoing hydrolysis ([Förster et al., 1994](#)). Anyway, the role of temperature in the efficiency of this reaction must be emphasized. It is a consequence of the kinetic stability of RNA aminoacyl esters that was identified here to be increased substantially at low temperatures, which independently support the hypothesis that the development of the common chemistry of α -amino acids and RNA, from which translation eventually emerged, should have occurred at temperature close to 0°C (or even below in eutectic phases in ice matrices, [Monnard et al., 2003](#)) and subsequently adapted to higher temperatures. It is an additional indication that low temperatures would not have been detrimental to life's early developments and should be considered in the light of the weak young Sun's output ([Kasting, 2010](#)) and the possible occurrence of frozen environments.

Conclusions and perspectives

Our results on the copolymer model **3a** suggest unexpected properties of amino acid-nucleotide copolymers built on ester and phosphoramidate linkages. Firstly, in the covalent assembly, the stability of the ester bond is strongly increased. Secondly, we identified a plausible mechanism for the formation of aminoacylated mononucleotides from NCAs through mixed anhydrides able to undergo intramolecular transfer. Lastly, the formation of phosphoramidates was confirmed to be effective owing to the increased nucleophilicity of amines compared to alcohols in phosphoryl transfer. Having demonstrated that oligomers based on phosphoramidate linkages between α -amino acid residues and nucleotides have strongly increased stabilities compared to esters at the 3'-end of RNAs and that their abiotic formation could not be considered as much less probable as that of "pure" RNA strands at the

chemical stage of evolution suggests a role for these structures in the emergence of life that should not be underestimated. This statement is worth to be analysed in the light of recent studies pointing towards a role for phosphoramidate intermediates in the formation of both peptide (Jauker et al., 2015 ; Griesser et al., 2017 a, 2017 b) and internucleotidic linkages (Ni et al., 2009). It should also be related to the observation that a single chemical network can lead to the formation of both amino acid and nucleotides building blocks from a photocatalytic redox cycle (Patel et al., 2015; Sutherland, 2017). The presence of both kinds of monomers at the same location is consistent with the formation of the copolymers considered here. It also accounts for the selection of adenylates and aminoacyl esters of nucleotides before the emergence of translation, which would otherwise be puzzling. Considering this fact in the light of the later developments of translation suggests that the contribution of copolymers to the process should be considered more thoroughly since it may point towards the nature of the evolutionary driving force that led to the development of ribosomal synthesis. The advantage of this approach lies in the chemical consistency of the presence of nucleotide and α -amino acid copolymers with the later evolution of replication of nucleic acids and translation. Though the behaviour of these copolymers will need deeper investigation, we can conceive that the folding behaviour of α -amino acid residues as well as the introduction of functional diversity from their side-chains is likely to bring about a repertoire of catalytic activities unattainable by ribozymes. They may additionally have played a dynamic role at least as intermediates with lifetimes reaching several weeks at 20°C and more at lower temperature values that could match the generation time of early entities. Several hypotheses can be considered with respect to that dynamic participation to a nucleotide metabolism owing to the electrophilic character of both the ester and the phosphoramidate linkages. The first one, resulting from the reaction of the ester moiety obviously predates ribosomal synthesis through the formation of non-coded peptides initiated by the free amino group of the aminoacylated 3'-end. The second one, eventually leading to phosphodiester (Scheme 3), is deduced from the observation that phosphoramidates correspond to reactive species in phosphoryl transfer (Ni et al., 2009) undergoing hydrolysis but that have also been identified as potential precursors of pyrophosphates by reaction with other phosphoryl groups and of phosphodiester by reaction with alcohols. Interestingly certain aminoacid

phosphoramidates have also been used as substrates for polymerases (Adelfinskaya et al., 2007).



Scheme 3. Hypothetic mechanisms through which amino acid – nucleotide phosphoramidates could serve as phosphoryl transfer agents (left) and of aminoacyl moieties could be transferred from mixed anhydrides (right) thanks to a precise positioning of reacting groups.

An additional hypothesis, not incompatible with the preceding ones can be put forward. The aminoacylation process occurring through intramolecular transfer in aminoacyl adenylates, which is stereoselective and highly efficient at low pH (below pH 6) provided that carbon dioxide remains at low concentrations (Wickramasinghe et al., 1991), can produce limited overall yields only. We additionally demonstrated that NCAs can serve as aminoacylating agents efficient at low concentrations where their polymerization could be controlled provided that the target nucleotide bears a phosphoryl group present as a di-anion. The efficient intramolecular transfer observed from the 5'-AMP adduct with NCAs as well as the earlier observation of similar processes from 3'-phosphorylated nucleotides (Biron et al., 2005) open the possibility of more sophisticated pathways with improved yields (Scheme 3). Certain strands of amino acid-ribonucleotide copolymers in which the carbonyl group of the mixed anhydride formed at the 5'-phosphorylated end is located in close proximity to a 3'-end could greatly facilitate the intramolecular transfer owing to a favourable folded structure or supramolecular assembly constituted by complementary strands as already observed from RNA mini-helices (Tamura and Schimmel, 2004). Combined with the earlier observation of an aminoacylation process of 3'-phosphorylated nucleosides by NCAs involving the intramolecular transfer from a mixed anhydride intermediate, this observation suggests a wider role of NCAs in aminoacylation from mixed anhydrides intermediates from pending phosphate

groups of RNA. The role of adenylates in biological aminoacylation could therefore have originated in the activity of an oligonucleotide or a copolymer having a phosphate pending group, for instance at the 5'-end, serving as a handle capable of transferring aminoacyl groups formed by reaction with NCA to the 3'-end of the folded structure or of that of another strand bound by Watson-Crick base-pairing. From an evolutionary perspective, it could be considered that the advent of protein aminoacyl-tRNA synthetases has removed any catalytic utility to the RNA chain and that the AMP moiety of adenylate mixed anhydrides is just the remnant of that early ribozyme from which all the bases that had been made useless by the protein catalytic activity were discarded by natural selection. It is worth noting that at least some free 5'-phosphoryl groups are present in the sequence of the short aminoacylation ribozymes identified by the group of Yarus ([Turk et al., 2010](#); [Turk et al., 2011](#)), which suggests that short oligonucleotides could play a role in aminoacylation by NCA as well. As short oligonucleotides, copolymers having increased folding abilities could also act as catalysts for the aminoacylation of the copolymers devised here. This latter possibility opens a new systems chemistry perspective since the acid lability of phosphoramidates may then turn into an advantage since the cleavage of one strand into two shorter ones would lead to a two-fold increase of the available aminoacylated 3'-ends and therefore to a possibility of autocatalytic growth of these short oligomers.

The results described here support the view that the biochemistry of translation evolved from earlier stages in which the reproduction of chemical entities could be based on the interaction of α -amino acids and nucleotides rather than from a stage in which life was based on one of these families of copolymers only ([Borsenberger et al., 2004](#)). Although the historical path will certainly not be reconstituted, our results obtained from an approach inspired by *systems chemistry* ([Kindermann et al., 2005](#); [Ludlow and Otto, 2008](#)) give an indication of how rudimentary chemical processes can be associated to this aim. They suggest that low temperatures would be favourable to the development of this path. At least, these results can legitimate a future search for networks of reactions derived from the chemistry presented here and that could be capable of potentially overcoming the improbability of complex structure formation owing to an ability to self-reproduce and therefore to grow

exponentially and become persistent (Pascal et al., 2013; Pascal and Pross, 2015; Pross, 2016).

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Author contributions

R.P. conceived the project, Z.L. carried out the cotrimer synthesis and the study of its hydrolysis, G.A. studied the hydrolysis of esters, R.P. wrote the paper with input from Z.L., G.A. and J.C.R. All authors discussed the results and commented on the manuscript.

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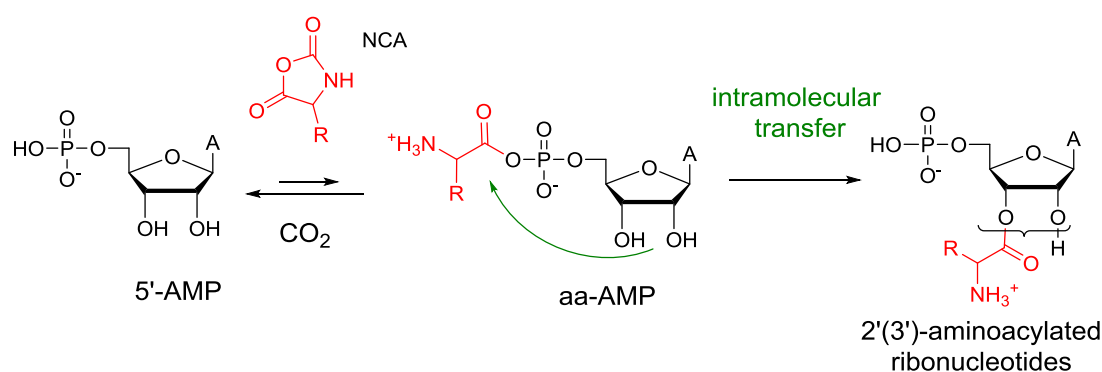
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Chapter 5. Investigation on possibilities of intramolecular aminoacyl transfer from a phosphorylated 5'-end to hydroxyl groups at the 3'-end

Introduction

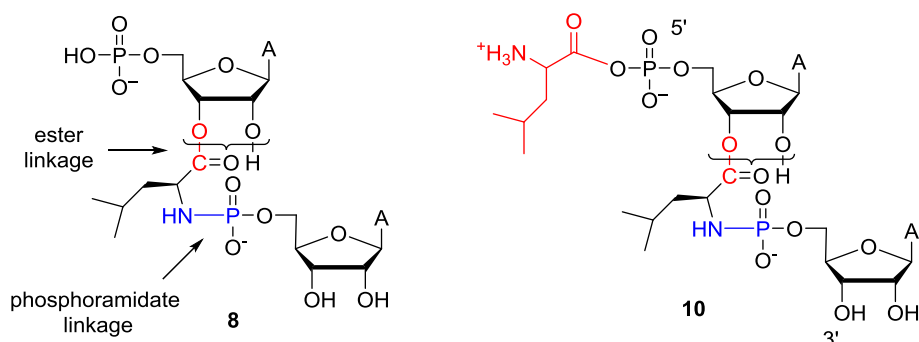
Finding a prebiotic chemical aminoacylation process for RNA is fundamental because it would give us answers about the emergence of translation and the evolution of the ribosomal machinery. We have shown that NCAs are not able to directly aminoacylate the 2'(3')-OH of RNA (**chapter 3**). However, more recently (**chapter 4**) we have shown that, provided that the CO₂ concentration is maintained as low as possible, a NCA could react with 5'-AMP to yield a mixed anhydride (aa-AMP) at equilibrium, that can be trapped by the intramolecular transfer (**Scheme 1**).



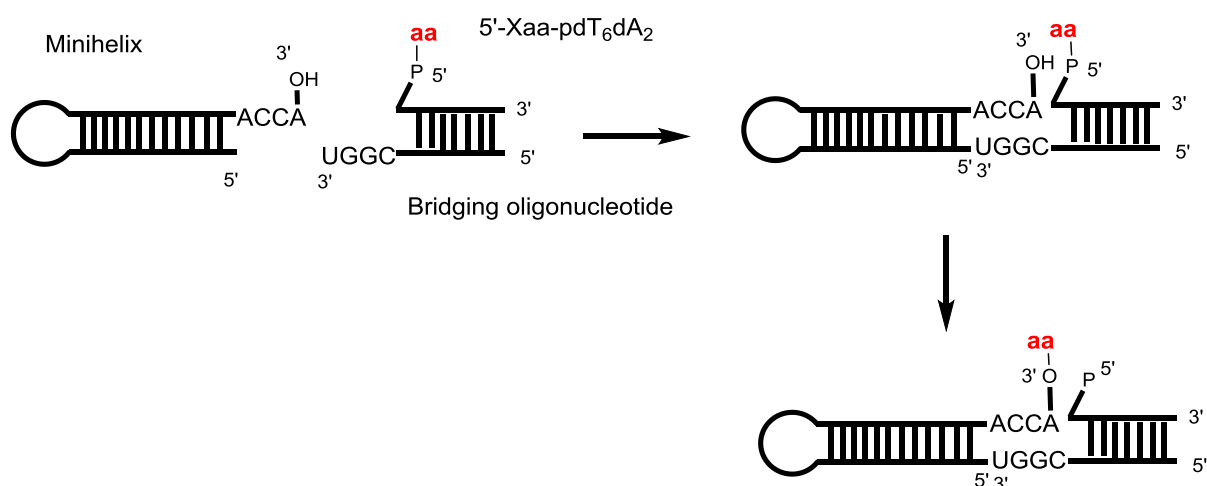
Scheme 1. The possibility of the indirect aminoacylation of 2'(3')-OH of 5'-AMP by NCAs via the intramolecular aminoacyl transfer from aa-AMP, which can compete with the breakdown of the mixed anhydride promoted by CO₂.

The preceding pathway constitutes one of the grounds on which our proposal that amino acid-nucleotide copolymers **8** (**Scheme 2**) could have played a role at early stages of evolution has been built. Therefore, the efficient intramolecular transfer observed from 5'-AMP adduct with NCAs, though in limited yield, as well as the earlier observation of similar processes from 3'-phosphorylated nucleotides (**Biron et al. 2005**) open the possibility of more sophisticated pathways with improved yields. A promising possibility to improve transfer involves longer copolymers **8** (strand of amino acids-ribonucleotides) (**Scheme 2**) able to fold into structures favorable to an intramolecular transfer or even supramolecular complexes in which reacting groups

are held at reacting distance as in the process devised by Tamura and Schimmel (**Scheme 3**) (Tamura and Schimmel, 2004).

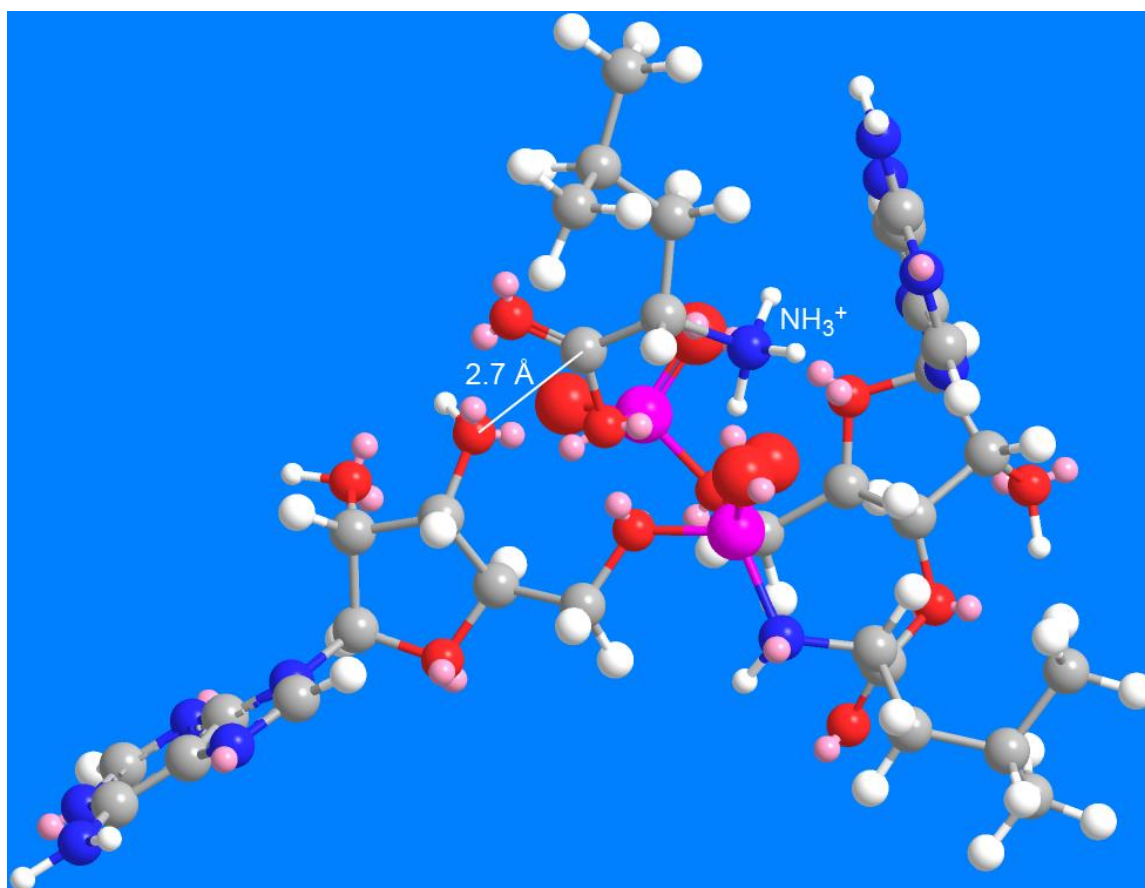
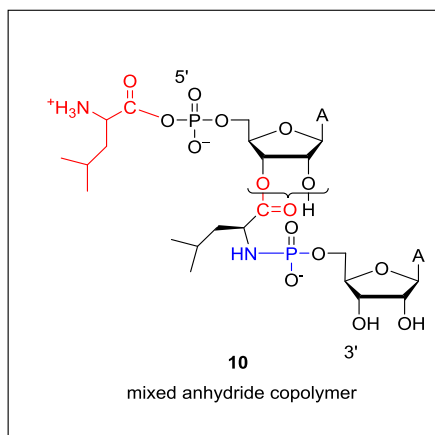


Scheme 2. Structures of a co-trimer **8** and its mixed anhydride adduct **10**.



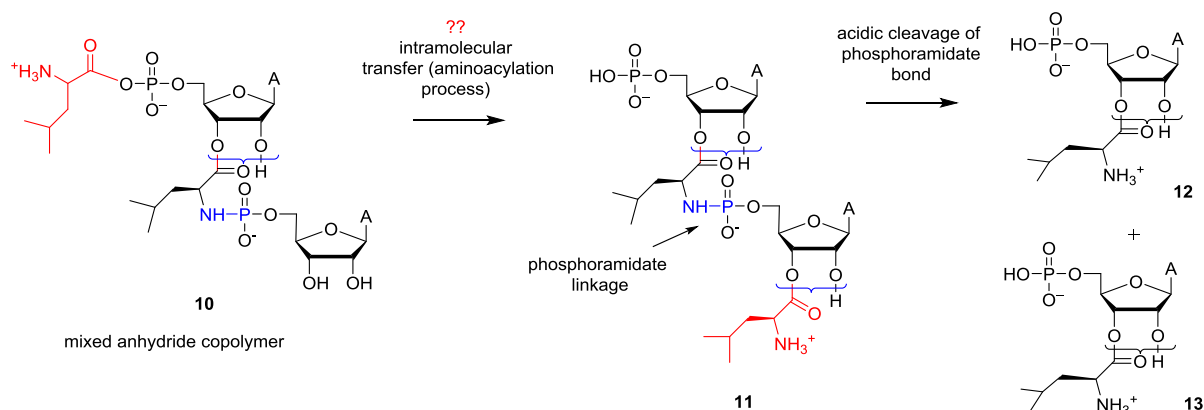
Scheme 3. Scheme for the aminoacylation with aminoacyl phosphate oligonucleotide (5'-Xaa-pdT₆dA₂) and a bridging oligonucleotide.

The simpler model in which this study could be carried out is that of the structure of the mixed anhydride of co-trimer **10** (**Scheme 2**) in which the presence of an internal aminoacyl residue bound by phosphoramidate and ester linkages could facilitate folding. Molecular modeling of this structure (using Chem3D software) indicated a possibility of a folded structure in which the acyl carbon of the mixed anhydride moiety is located at a short distance from the 3'-end hydroxyl group (2.7 Å). Moreover, it turned out that the folded structure could be substantially stabilized by electrostatic interactions involving the protonated amine and both phosphates charges (**Scheme 4**).



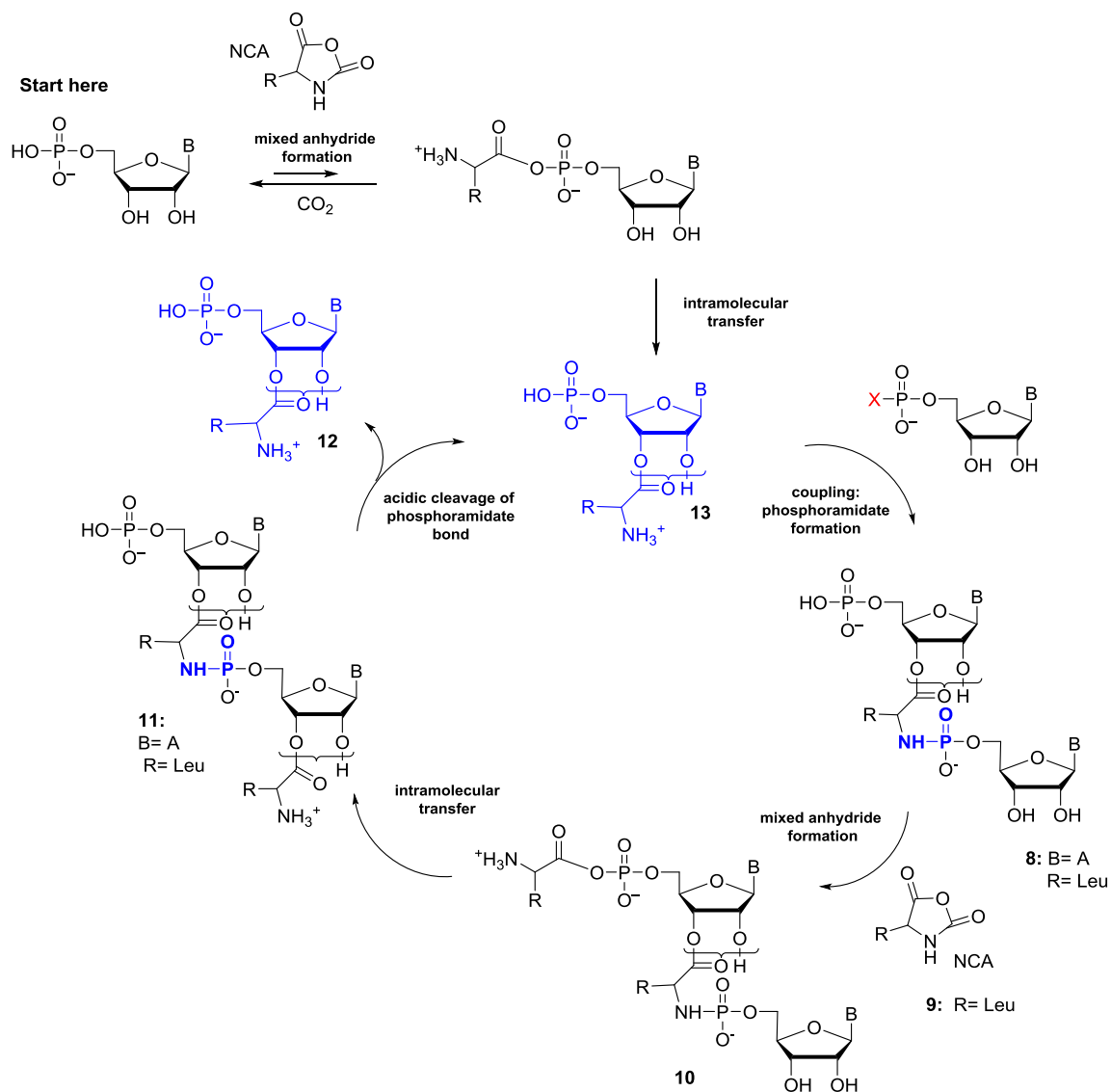
Scheme 4. 2D model and the favorable conformer of the mixed anhydride copolymer (compound **10**).

Another practical consequence of an aminoacylation process of this kind is that it could be coupled with the possibility of cleavage of the phosphoramidate moiety of co-tetramer **11** (**Scheme 5**) releasing two stoichiometric equivalents of aminoacylated nucleotide **12**, **13**.

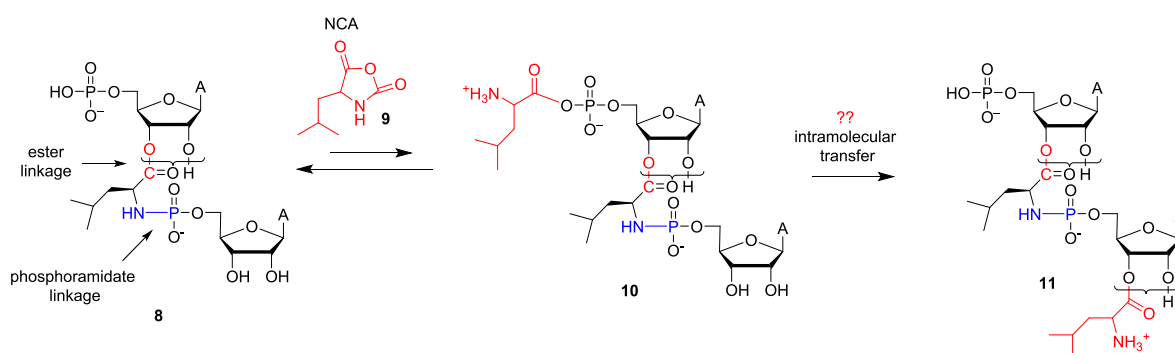


Scheme 5. Practical consequence of an aminoacylation process of this kind is that it could be coupled with the possibility of cleavage of the phosphoramidate moiety of co-tetramer **11** releasing two stoichiometric equivalents of aminoacylated nucleotide **12**, **13**.

We therefore devised a possibility of cyclic process in which aminoacylated nucleotides could be reproduced autocatalytically (**Scheme 6**) opening new perspectives in a systems chemistry approach through the specific selection abilities associated with exponential growth (**Pross et al. 2016**). We therefore decided to embark on an investigation of the possibility for intramolecular transfer of the 5' aminoacyl moiety in the mixed anhydride copolymer **10** to the 2'(3')-OH (**Scheme 7**). To this aim, the co-trimer **8** was synthesized and then its reaction with Leu-NCA **9** was investigated. The synthetic path used in the preparation of trimer **8** as well as the results of preliminary experiments carried out to check the reaction of Leu-NCA **9** are presented in the results and discussion part of this chapter. However, the results do not support a strong improvement in the yield of aminacylation with NCAs even though products with a molecular weight corresponding to that of ester **11** could be identified in limited yield but without clues that their formation result from an intramolecular process. Therefore, this chapter is dedicated to the presentation of the preliminary experiment carried out to check the possibilities of the copolymers. Though the results are not encouraging, it is important to emphasize that they represent only one of the many possibilities that are allowed by structures of that kind and that could be worthy of further investigations.



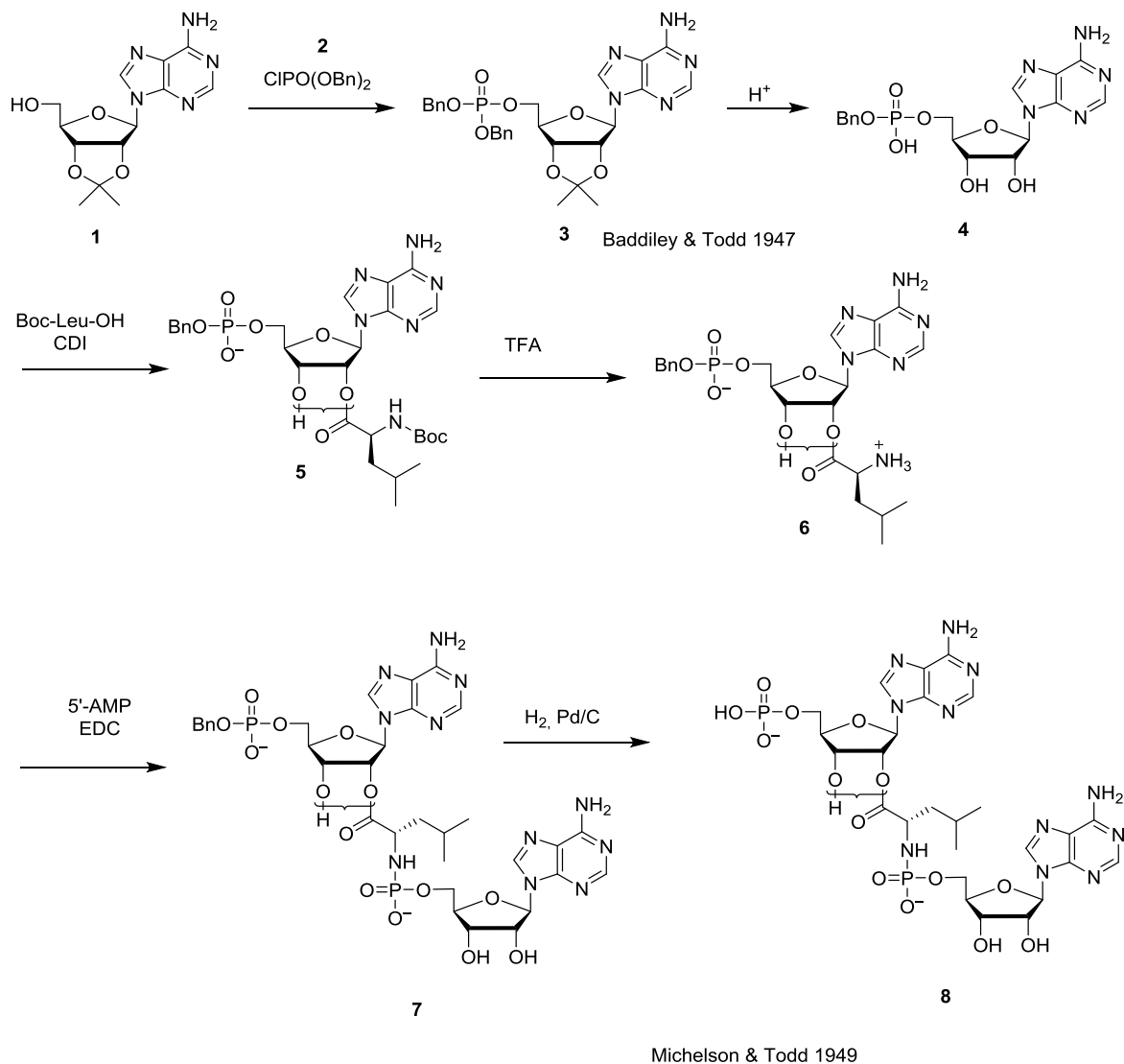
Scheme 6. A devised loop allowing the formation and the reproduction of the 2'(3')-aminoacylated nucleotide **13** based on a facilitated intramolecular transfer of the aminoacyl moiety in mixed anhydrides **10** and on the possibility of cleavage of the phosphoramidate linkage of the resulting co-tetramer **11** into two 2'(3')-aminoacylated nucleotide dimers **12**, **13**.



Scheme 7. Investigation of the possibility for intramolecular transfer of the 5'-aminoacyl moiety in the mixed anhydride copolymer **10** to the 2'(3')-OH.

Results and Discussion:

The targeted compound (**8**) was prepared in a multistep process according to scheme 8 and its reactivity with NCAs could be investigated.



Scheme 8. Chemical synthesis of compound **8**.

As depicted in scheme 8, the first step (phosphorylation of nucleoside) was made according to the procedure found in the literature (**Baddiley and Todd 1947**), the reaction was initiated by phosphorylation of 2':3'-isopropylidene adenosine **1** with freshly prepared dibenzyl phosphorochloridate to yield phosphotriester **3**. Then a selective deprotection of one of the benzyl groups coupled to that of the 2',3'-isopropylidene ketals of compound **3** by a mixture of diluted trifluoroacetic acid and ethanol giving benzylated 5'-AMP (Bn-5'-AMP) **4**. Aminoacylation of the Bn-5'-AMP **4** by the imidazolide of Boc-Leu was performed according to the literature (**Weber and**

Orgel, 1978; Liu et al., 2017) yielding the ester **5**. It was followed by the deprotection of the Boc group by TFA. The benzylated co-trimer **7** was prepared by forming phosphormidate linkage between compound **6** and activated 5'-AMP in 100 mM MES buffer (pH 6.5). The last step was the hydrogenolysis of the benzyl group of compound **7** by H₂ on Pd/C according to a procedure of the literature (**Michelson and Todd 1949**).

Reactivity Study of the Copolymer (8) with Leu-NCA (9) in 100 mM MES buffer pH 5.5: Then based on the molecular modeling results, we made some of the experiments by adding Leu-NCA (5 additions of 5 mM separated by 60 min periods of time to reduce the occurrence of NCA polymerization) to the copolymer at pH 5.5 (100 mM MES buffer). After one night, we analyzed the reaction by HPLC-MS (ESI-, method D). We observed only two small peaks having a molecular weight corresponding to that of the aminoacyl esters of the copolymer (**11**, m/z= 901.28) (**Figure 1**). The estimated yield is around 2%. So we realized that no significant improvement of the yield had occurred that could give rise to an efficient reproduction loop. Though this experiment does not provide evidence that aminoacylation occurred though the formation of a mixed anhydride followed by intramolecular aminoacyl transfer, the occurrence of a peak having the molecular weight of the desired adduct could in principle not be explained by a direct aminoacylation (**Liu et al. 2017**) and suggests that the process works but to a limited extent.

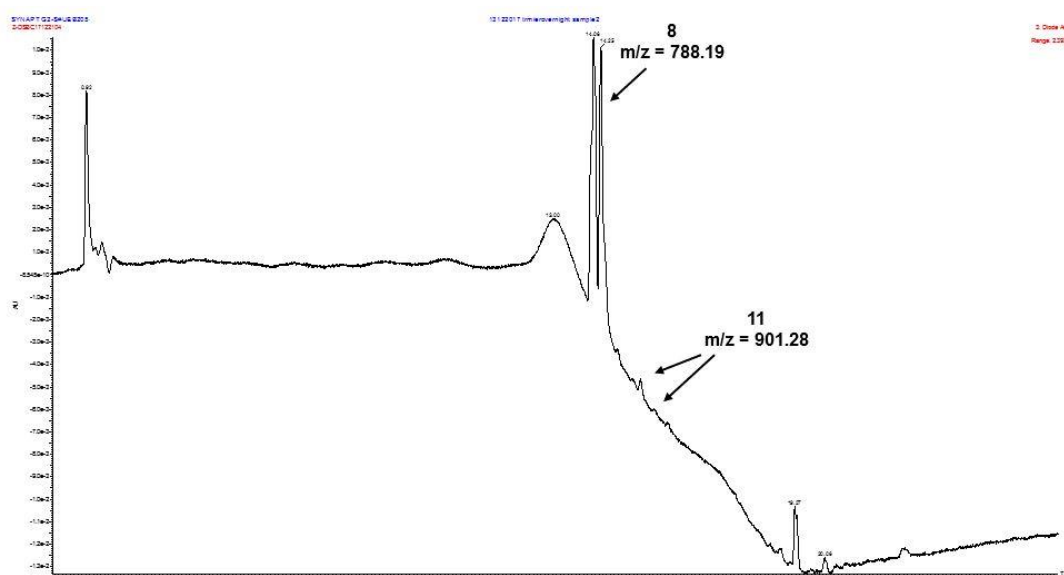


Figure 1. HPLC-MS (ESI-, method D) of the sample taken after one night from the beginning of the reaction. Reaction of copolymer (**8**) with Leu-NCA (5 additions of 5 mM separated by 60 min periods of time) in 100 mM MES buffer (pH 5.5).

Experimental procedures for chapter 5:

Reagents and solvents were purchased from Sigma-Aldrich or Bachem, and were used without further purification. All aqueous solutions were prepared using pure water (18 M Ω) produced with a Milli-Q (Merck-Millipore) apparatus. NMR spectra in either DMSO-*d*₆ or D₂O solution were recorded on a Bruker DPX 300 spectrometer (300 MHz) or on a Bruker Avance 500 spectrometer (500 MHz); chemical shifts δ_{H} are reported in ppm; coupling constants *J* are reported in Hz. In all the experiments, the pH was monitored using a Thermo Orion 3-STAR pH-meter with a VWR electrode. UPLC analyses were carried out on a Thermo Scientific Dionex UltiMate 3000 Standard system including an autosampler unit, a thermostated column compartment and a photodiode array detector, using UV absorbance detection (acquisition in the 200–400 nm range)(System A). Separations were carried out using Thermo Scientific BDS-Hypersil C18 3 μm 50 \times 2.1 mm column using gradients of solvents. **Method A:** mobile phase: A: H₂O (0.1% TFA), B: CH₃CN (0.1% TFA); flow rate 0.2 mL/min; gradient: 0 min (10% B) to 20 min (100% B). **Method B:** mobile phase made of solvent A: H₂O (0.1% TFA), B: CH₃CN (0.1% TFA); flow rate 0.2 mL/min; gradient: 0 min (5% B), 10 min (10% B), 15 min (35%), 18 min (100% B), 19 min (100% B), 21 min (100% B). HPLC/ESI-MS analyses were carried out on a Waters Synapt G2-S mass spectrometer system connected to waters Acquity UPLC H-Class apparatus equipped with in an Aquity UPLC BEH C18, 1.7 mm 2.1 \times 50 mm column (System B) or Thermo Scientific BDS-Hypersil C18 3 μm 50 \times 2.1 mm column (System C). using gradients of solvent **Method C:** System B, mobile phase: A: H₂O (0.1% formic acid), B: CH₃CN (0.1% formic acid); flow rate: 0.5 mL/min; linear gradient 0% to 100 % B over 3 min. **Method D:** System C, mobile phase solvent A: H₂O (0.1% formic acid), B: CH₃CN (0.1% formic acid); flow rate 0.2 mL/min; gradient: 0 min (5% B), 10 min (10% B), 15 min (35%) , 18 min (100% B), 19 min (100% B), 25 min (100% B).

Synthesis of starting materials and reagents:

Dibenzyl chlorophosphate, 2:

The title compound was prepared by a procedure described in the literature (**Baddiley and Todd, 1947**). To a solution of dibenzyl phosphite (2.36 g, 9 mmol) in anhydrous toluene (30 mL), *N*-chloro succinimide (1.62 g, 12.1 mmol) was added. After stirring at room temperature for 2 hr, the mixture was filtered to remove the white precipitate. The

filtrate was concentrated to give dibenzyl chloro-phosphate as a pale yellow color yield (mass obtained = 2.66 g). ^{31}P NMR (121 MHz, CDCl_3) δ 4.78.

2': 3'-isoPropylidene Adenosine-5' Dibenzyl Phosphate, 3:

The title compound was prepared by a procedure described in the literature (**Baddiley and Todd, 1947**). 2':3'-isoPropylidene adenosine (1 g, 3.25 mmol) was dissolved by warming in dry pyridine (12 mL), and the solution cooled in an acetone-liquid nitrogen bath until the liquid at sides of the flask began to solidify. Then subsequently, a solution of fresh dibenzyl chlorophosphate (2.66 g, 2.76 eq.) in toluene (2 mL) was added with shaking, and the mixture left for 3 hours in cold bath at a temperature just above the freezing point of the mixture under N_2 gas and then set aside at room temperature overnight. Water (6 mL) and sodium carbonate (1 g) was added, and subsequently, the solution was evaporated under reduced pressure. The syrup obtained was dissolved in dichloromethane (100 mL), and the solution was washed with brine solution (3 \times 100ml), then dried over Na_2SO_4 , and concentrated to give a crude syrup. The crude syrup was chromatographed on silica gel (250 mL of $\text{CH}_2\text{Cl}_2/\text{MeOH} = 97/3$, 200 mL of $\text{CH}_2\text{Cl}_2/\text{MeOH} = 95/5$), then the fraction containing the product was evaporated to give an oily product (mass obtained 550 mg). HPLC analysis (method A): retention time 13.5 min; ^1H NMR (300 MHz, CDCl_3) δ 8.23 (d, $J = 5.8$ Hz, 1H), 7.84 (s, 1H), 7.22 (dd, $J = 6.6, 4.1$ Hz, 10H), 6.03 (d, $J = 2.4$ Hz, 1H), 5.78 (s, 2H), 5.25 – 5.13 (m, 1H), 4.98 – 4.81 (m, 5H), 4.43 – 4.29 (m, 1H), 4.24 – 3.97 (m, 2H), 1.53 (s, 3H), 1.29 (s, 3H); ^{13}C NMR (75 MHz, CDCl_3) δ 155.51 (s), 153.26 (s), 149.36 (s), 139.51 (s), 135.55 (d, $J = 6.3$ Hz), 128.59 (s), 127.95 (s), 120.14 (s), 114.53 (s), 90.88 (s), 85.13 (d, $J = 8.3$ Hz), 84.16 (s), 81.33 (s), 69.50 (d, $J = 5.5$ Hz), 66.79 (s), 27.12 (s); ^{31}P NMR (121 MHz, CDCl_3) δ -0.88 (s); ESI-MS (positive mode): calcd. for $\text{C}_{27}\text{H}_{31}\text{N}_5\text{O}_7\text{P}^+$: $[\text{M}+\text{H}]^+$ 568.19, found 568.19.

Bn-5'-AMP, 4:

A procedure from the literature (**Baddiley and Todd, 1947**) was modified as follows: the compound **3** (400 mg, 0.9 mmol) was dissolved in ethanol (0.27 mL), then a 9.27 mL of 19 mM trifluoroacetic acid (1 eq.) was added, and the solution was refluxed for 90 min. Then the solvent was evaporated and then freeze-dried after the addition of water (mass obtained = 350 mg). HPLC analysis (method A): retention time 2.9 min; ^1H NMR (300 MHz, D_2O) δ 8.21 (s, 1H), 8.07 – 7.96 (m, 1H), 7.09 (dd, $J = 10.9, 7.7$ Hz, 5H), 5.92 (d, $J = 5.4$ Hz, 1H), 4.64 – 4.49 (m, 2H), 4.39 – 4.25 (m, 1H), 4.22 (s,

1H), 4.05 – 3.90 (m, 2H); ³¹P NMR (121 MHz, D₂O) δ 0.28 (s), HRMS (ESI⁻): calcd. for C₁₇H₁₉N₅O₇P⁻ [M-H]⁻ 436.1022, found 436.1034.

Procedure for preparing amino acylated mononucleotides (mixture of 2' and 3' regioisomers): 2'(3')-Leucyl adenosine 5'-benzyl phosphate, 6:

Independently, Bn-5'-AMP **4** was dissolved in water, 1.5 eq. of NaHCO₃ was added. Then the solution was freeze dried. The title compound was prepared by a procedure described in the literature (**Weber and Orgel, 1978; Liu et al., 2017**). tert-Butyloxycarbonyl (Boc) Leucine (0.31 mmol) was dissolved in dimethylformamide (DMF, 0.125 mL). 1,1'-Carbonyldiimidazole (89.2 mg, 0.34 mmol) was added to the solution and allowed to react for 5 min at room temperature. The resulting solution of the imidazolide of amino acid was added to the 5'-benzyl AMP.Na salt (0.25 mmol) in water (0.25 mL). After reacting for one night. DMF was removed under vacuum. The mono nucleotide aminoacyl ester was isolated from the aqueous phase by preparative LC C18 column with the gradient: mobile phase: A: H₂O + 5 mM triethylamine and acetic acid at pH 6.5, B: acetonitrile; flow rate: 9 mL/min; 0 min (10% B), to 21 min (60% B), and 22 min (100% B). The solvent was removed by lyophilization. The Boc protecting group was removed by trifluoroacetic acid (0.25 mL) for 10 min. The precipitate was collected by centrifugation after adding diethyl ether, washed by dichloromethane twice and diethyl ether once. The product (white powder, obtained usually 20% yield) was stored at -20 °C. HPLC analysis (method A): retention time 7.7 min, 8.18 min; HRMS (ESI⁻): calcd. for C₂₃H₃₀N₆O₈P⁻ [M-H] 549.1863; found 549.1858; **3'-regioisomer**: ¹H NMR (300 MHz, D₂O) δ 8.38 (s, 1H), 8.18 (s, 1H), 7.20 – 7.00 (m, 5H), 6.01 (d, *J* = 6.8 Hz, 1H), 5.48 (dd, *J* = 5.3, 2.5 Hz, 1H), 5.07 – 4.93 (m, 1H), 4.46 (s, 1H), 4.31 (d, *J* = 16.0 Hz, 1H), 1.80 (ddd, *J* = 34.9, 18.8, 13.5 Hz, 3H), 0.90 (dt, *J* = 11.3, 5.8 Hz, 6H). Other signals for this compound were mainly obscured; **2'-regioisomer**: δ 8.33 (s, 1H), 8.15 (s, 1H), 7.22 – 7.00 (m, 5H), 6.11 (d, *J* = 6.9 Hz, 1H), 5.60 – 5.53 (m, 1H), 5.07 – 4.93 (m, 1H), 4.33 (s, 1H), 1.80 (m, 3H), 0.90 (dt, *J* = 11.3, 5.8 Hz, 6H). Other signals for this compound were mainly obscured.

Preparation of benzylated co-trimer: (N-2'(3')- Leucyl adenosine 5'-benzyl phosphate)-5'-adenosine phosphoramidate, 7:

The 2'(3')-L-leucine ester **6** (50 mg, 0.09 mmol), 5'-AMP (63.10 mg, 0.18 mmol) and EDC (690 mg, 3.63 mmol) were dissolved in 2-(N-morpholino)ethane sulfonic acid

(MES) buffer (9.08 ml, 100 mM of MES, pH 6.5). After three days at 5 °C (in the fridge), the mixture was separated by preparative HPLC with the gradient as follows: mobile phase: A: H₂O + 5 mM triethylamine and acetic acid at pH 6.5, B: acetonitrile; flow rate: 9 mL/min; 0 min (10% B), to 12 min (15% B), to 17 min (23% B) and 18 min (100% B), 22 (100% B). The fraction containing the product was collected and the solvent was evaporated, then freeze dried after adding water (the product was contaminated by *N*-ethyl-*N'*-(dimethyl)aminopropyl urea (EDU) present as a counter ion). The white powder (35 mg, theoretically 110.3 mg for bis-*N*-ethyl-*N'*-(dimethyl) aminopropyl urea salt) was conserved at -18 °C. HPLC analysis (method A): retention time 7.8 min, 8.3 min. ¹H NMR (500 MHz, D₂O, Figure 2); Cosy (D₂O, 500 MHz, Figure 3); ³¹P NMR (D₂O, 202 MHz, Figure 4) δ 6.77 (s), 6.38 (s), 0.02 (s); ¹³C NMR (D₂O, 126 MHz, Figure 5); ESI-MS (negative mode): calcd. for C₃₃H₄₂N₁₁O₁₄P₂⁻ [M-H] 878.24; found 878.24, for C₃₃H₄₁N₁₁O₁₄P₂⁻ [M-2H] 438.62; found 438.62.

Removal of the benzyl protecting group: co-trimer, 8:

The removal of the benzyl group was done according to a procedure of the literature (**Michelson and Todd 1949**). (15 mg, 0.017 mmol) of compound **7** was dissolved in methanol (5.5 mL). An equal weight of 40 % Pd/C (per protecting group) was added. A gentle stream of nitrogen was passed through the reaction mixture for 15 mins. Then the hydrogenation proceeded overnight, and the mixture was filtered (using celite), washed with a mixture of methanol and water, and evaporated under reduced pressure. The product was used without further purification (white precipitate, mass obtained = 12 mg). HPLC analysis (method A): retention time 3 min, 4 min; HPLC analysis (method B): retention time 11.5 min, 14.4 min; HPLC-MS (negative mode): calcd. for C₂₆H₃₆N₁₁O₁₄P₂⁻ [M-H] 788.19; found 788.19, for C₂₆H₃₅N₁₁O₁₄P₂²⁻ [M-2H] 393.59; found 393.59; HRMS (ESI negative): calcd. for C₂₆H₃₆N₁₁O₁₄P₂⁻ [M-H] 788.1918; found 788.1914.

L-Leu-NCA, 9 :

The *N*-carboxyanhydride was prepared by reaction of H-L-Leu-OH with triphosgene (**Daly and Poche, 1988**). (yield 24%). ¹H NMR (400 MHz, CDCl₃) δ 6.69 (s, 1H), 4.28 (dd, J = 9.1, 3.4 Hz, 1H), 1.85 – 1.56 (m, 3H), 1.01 – 0.85 (m, 6H). ¹³C NMR (101 MHz, CDCl₃) δ 169.87 (s), 152.84 (s), 56.18 (s), 40.84 (s), 25.08 (s), 22.70 (s), 21.53 (s).

Procedures for the study of the reactivity of copolymers (8) with NCAs (9):

A 1mM of copolymer solution (**8**) was prepared in 1mL of 100 mM MES buffer pH 5.5. The mixture was continuously degassed by bubbling N₂ (from which the traces of CO₂ were removed by bubbling through 1M NaOH). Five 50 μL portions a 100 mM solution of L-Leu-NCA (**9**) in dry THF were added at regular intervals of time(4 hours) under continuous bubbling of N₂. Then 100 μL samples of the solution were withdrawn at different times (after 5 hrs from the starting time, overnight (around 20 hrs)). The samples were diluted to 1 mL in MES buffer and filtered to remove the insoluble peptides before analyses. HPLC-MS (ESI⁻) (Method D) for the sample after one night (**Figure 1, main text**).

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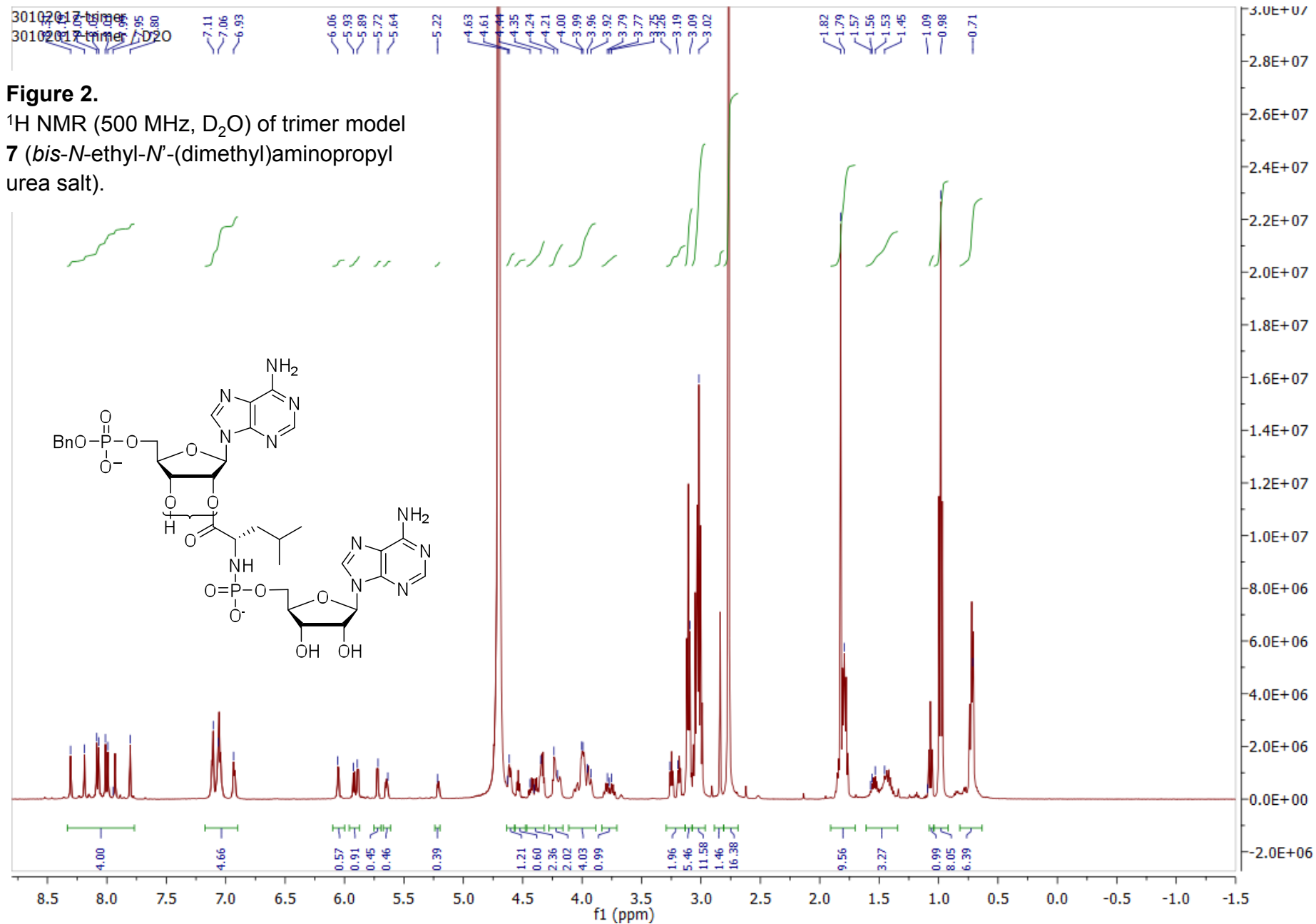
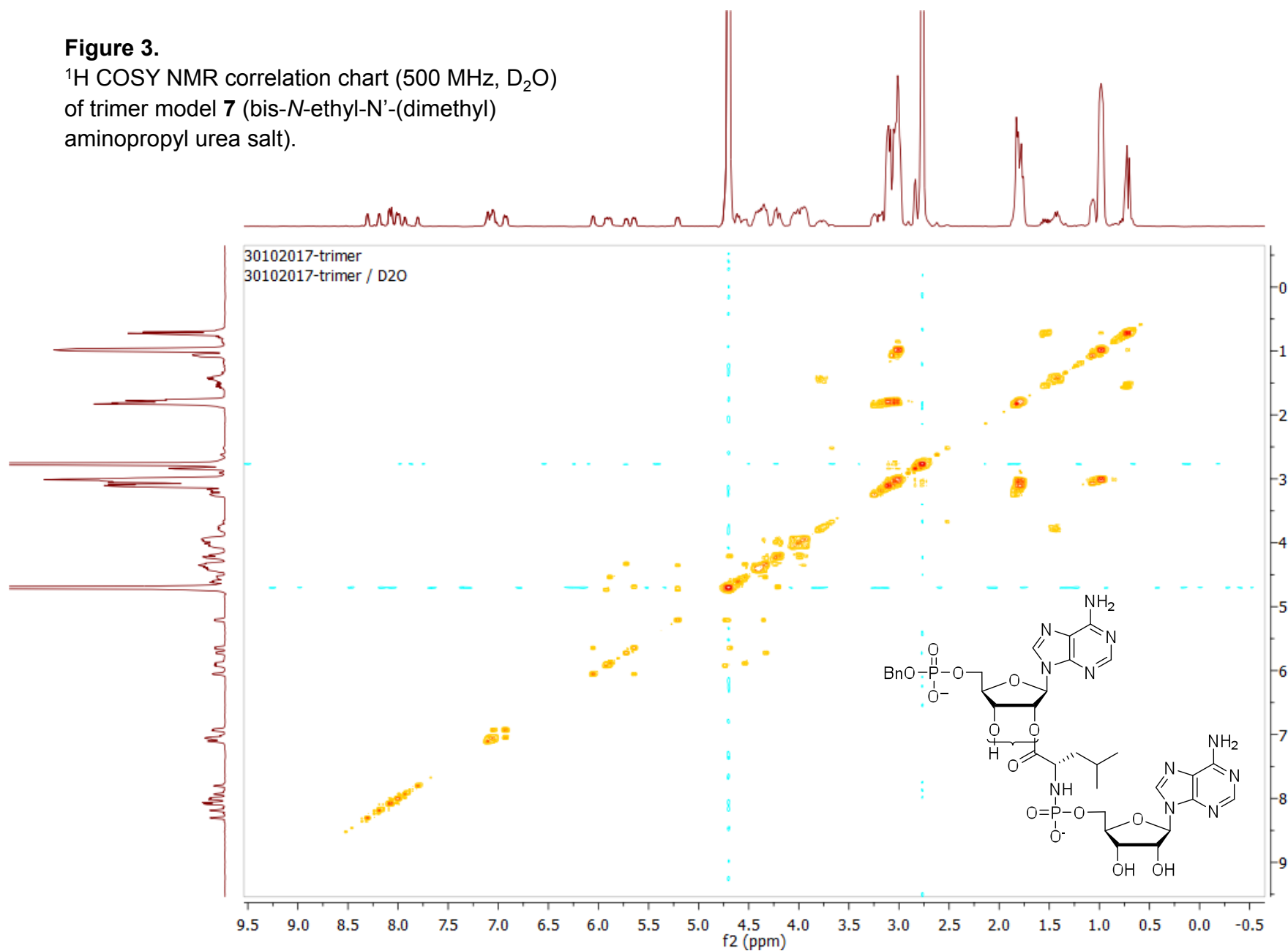
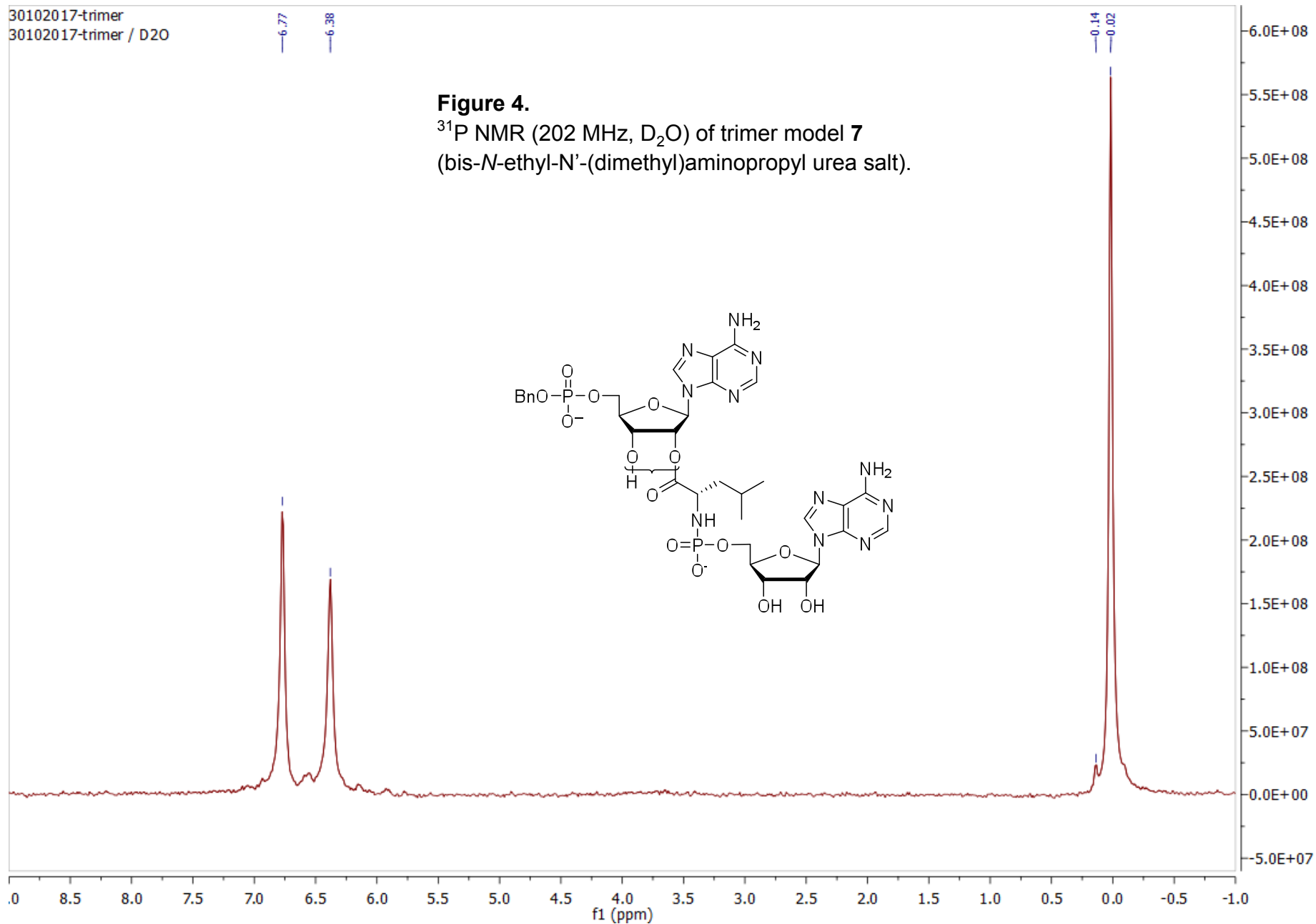


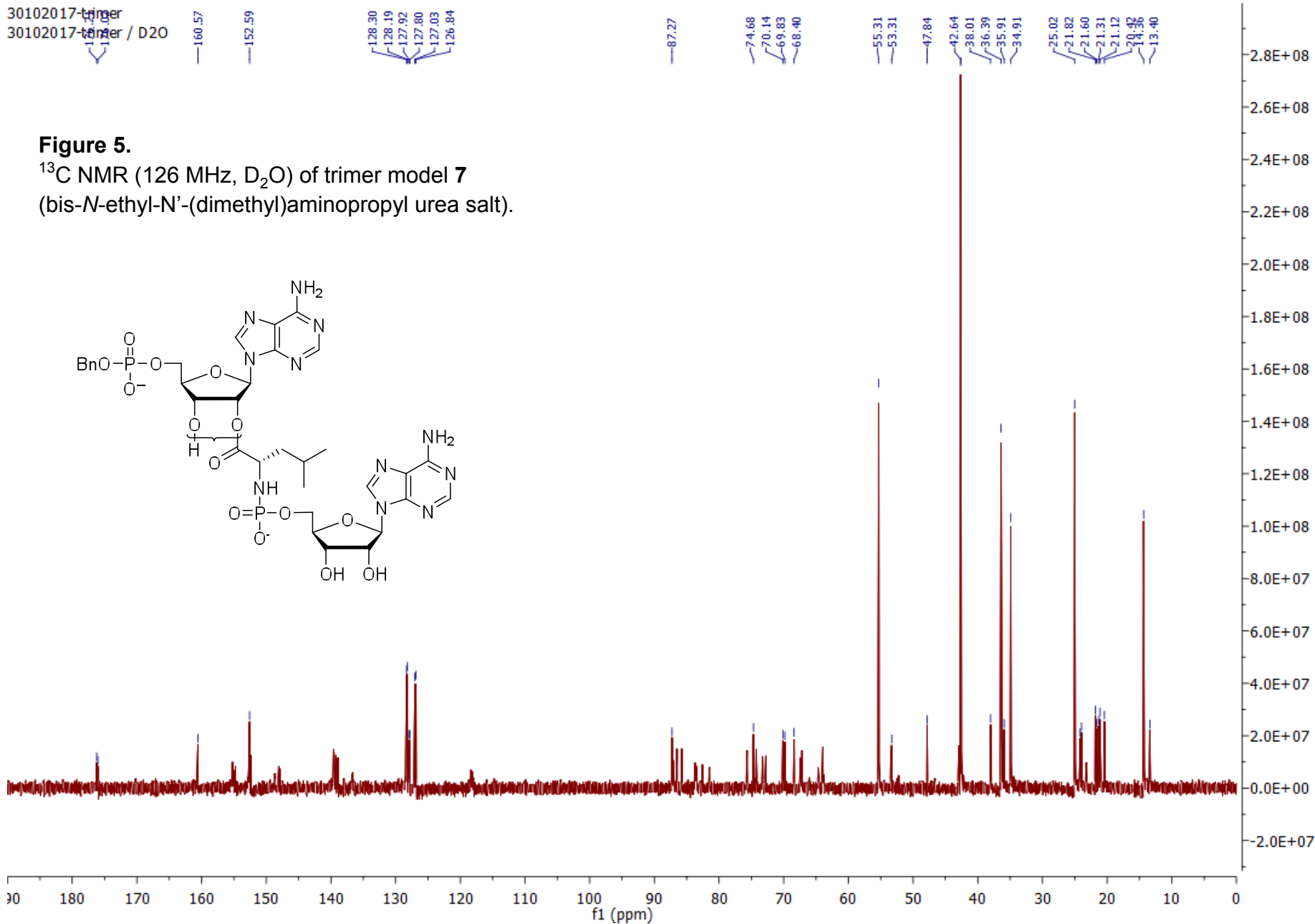
Figure 3.
 ^1H COSY NMR correlation chart (500 MHz, D_2O)
of trimer model **7** (bis-*N*-ethyl-*N'*-(dimethyl)
aminopropyl urea salt).

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30102017-trimer
30102017-trimer / D2O



General Conclusion and Perspectives

General Conclusion and Perspectives

The work presented in this thesis constitutes a fragment of a general framework of prebiotic chemistry and is more particularly dedicated to the systems chemistry approach in the context of the origin of life. Since this concept has been introduced, progress has been made and my PhD constitutes a piece of work in this direction. Though a reaction network capable of self-reproduction has not been made available yet, progress has been made in this direction. The first part of the work was aimed at contributing to efforts made to conceive chemical activation processes able to maintain such network in a far from equilibrium state (a pre-requisite for self-organization). Therefore we have investigated a series of reagents earlier proposed for the activation of the carboxyl group of amino acids. None of them could give rise to a satisfactory process for strong activation of the C-terminus of peptides, which indicates that a process for feeding a protometabolism of peptides with energy is not yet available at the notable exception of *N*-carboxyanhydrides for which prebiotically relevant pathways of formation have been demonstrated in earlier works.

Moreover, the requirement for evolvability of the previous networks is intimately linked to that of possibilities of variations. Therefore, the second part of the work was dedicated to peptides-nucleotides co-evolution processes. We have suggested that co-polymers made of both nucleotides and amino acids are promising because they solve the question of the further evolution of the systems towards current molecular biology. Much remains to be made but we consider this approach is worthy of being investigated further in the future with the aim of identifying new frameworks able of template replication, possibilities of conversion of phosphoramidates into phosphodiesteres and improved pathways for aminoacylation.

Appendix. Complete Stuffed Bibliographic References

Complete stuffed Bibliographic References

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TITLE: Energetic processes driving potential peptide protometabolisms at the origin of living systems

ABSTRACT:

The thesis addresses several issues in prebiotic chemistry in the context of the origins of life through a systems chemistry approach. **The first part** is devoted to the study of chemical activation processes that are not only important in the formation of polymers, but also to feed the system with energy in order that a far from equilibrium state is maintained, a prerequisite for self-organization. It has been suggested that 5(4*H*)-oxazolones intermediates formed by C-terminus peptide activation could be involved in self-organization of life. To this aim, we have checked the reactivity of relevant prebiotic reagents previously proposed to activate α -amino acids. None of them led to a satisfactory C-terminus activation of peptides, showing that no general process for feeding a protometabolism of peptides with energy is identified yet, with the notable exception of *N*-carboxyanhydrides (NCAs) that can be formed through prebiotically relevant pathways. Additionally, we demonstrated that carbodiimides reagents are as efficient in the activation of *N*-carbamoyl amino acids as in that of the C-terminus of peptides in diluted aqueous media. **The second part** of the dissertation discloses new results in support of a process of coevolution of peptides and nucleotides. Firstly, a study of non-enzymatic aminoacylation reagents of the 3'-terminus of RNA is presented. Secondly, we assessed co-polymers of α -amino acids and nucleotides bound by phosphoramidate and ester linkages as potential players in chemical evolution. The kinetic relevance of these structures was demonstrated as well as potential chemical processes that allow their formation.

KEYWORDS: Prebiotic chemistry; α -amino acids; Peptides; α -amino acid *N*-carboxyanhydrides; 5(4*H*)-Oxazolones; Peptides/nucleotides co-evolution; Aminoacylation; Emergence of translation; Origin of life; Evolution.

TITRE : Processus énergétiques gouvernant des protométabolismes peptidiques à l'origine des systèmes vivants

RESUME :

La thèse aborde différentes questions de chimie prébiotique dans le contexte de l'origine de la vie par une approche de chimie systémique. **La première partie** est dédiée à l'étude de processus d'activation chimique important non seulement pour la formation de polymères, mais aussi pour alimenter le système en énergie de manière à le maintenir dans un état éloigné de l'équilibre, un prérequis pour l'auto-organisation. Il a été suggéré que les intermédiaires 5(4*H*)-oxazolones formés par l'activation de l'extrémité C-terminale des peptides pourrait être impliquée dans l'auto-organisation du vivant. Dans ce but, nous avons évalué la réactivité de réactifs pertinents dans un contexte prébiotique et décrits dans la littérature comme capables d'activer des acides α -aminés. Aucun d'entre eux n'a manifesté une activité satisfaisante pour l'activation C-terminale des peptides, montrant qu'une voie possible pour alimenter un protométabolisme des peptides en énergie n'est pas identifiée à ce jour à l'exception notable des *N*-carboxyanhydrides (NCA) qui peuvent être formé par des voies prébiotiquement plausibles. Nous avons par ailleurs démontré que les carbodiimides sont aussi efficaces pour l'activation des *N*-carbamoylamino acides que pour celle du carboxyle terminal des peptides en milieu aqueux dilué. **La seconde partie** du document expose de nouveaux résultats en faveur d'un processus de coévolution peptides-nucléotides. D'abord, une étude de la réactivité d'agents d'aminoacylation de l'extrémité 3' de l'ARN est présentée. Ensuite, nous évaluons des co-polymères acides α -aminés-nucléotides liés par des enchaînements phosphoramidate et esters comme partenaires éventuels de l'évolution chimique. La pertinence cinétique de ces structures est démontrée ainsi que des voies chimiques permettant leur formation.

MOTS-CLES : Chimie prébiotique ; Acides α -aminés ; Peptides ; *N*-Carboxyanhydrides d'acides α -aminés ; 5(4*H*)-Oxazolones ; Co-évolution peptides–nucléotides ; Aminoacylation ; Emergence de la traduction ; Origine de la vie ; Evolution.

DISCIPLINE : Chimie organique, minérale et industrielle

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Équipe *Dynamique des Systèmes Biomoléculaires Complexes*