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5(4*H*)-Oxazolones as Effective Aminoacylation Reagents for the 3′-Terminus of RNA

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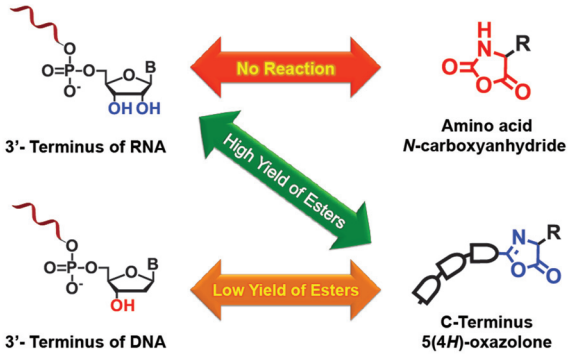

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Abstract Nucleosides and methylated nucleotide models were used as substrates to identify pathways for the chemical aminoacylation of ribonucleic acids (RNA) as a prerequisite for the evolution of translation. A selective and comparatively efficient 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 analysed.

Key words origin of translation, prebiotic chemistry, peptides, nucleotides, co-evolution

Elucidating how ribonucleic acid (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.[1,2] Imidazolides proved to be chemically competent in this process.[3] Other pathways involving ribozyme catalysis have been proposed in the context of the RNA world hypothesis starting either from α-amino acid adenylates[4] or cyanomethyl esters.[5] However, the idea that life evolved from different families of biomolecules and biopolymers[6] through a co-evolutionary process is gaining momentum because of the difficulties associated with RNA polymerization from hypothetical mixtures of ribonucleotides.[7] 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[8] by reaction of nucleotides with 5(4*H*)-oxazolones, earlier demonstrated as prebiotically relevant activated peptides.[9] Esters at the 2′- and 3′-positions of the ribose moiety were additionally formed in unexpectedly high yield at neutral pH.[8] 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, Figure 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 (NCA) 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.

The reaction of the 5(4*H*)-oxazolone **1** (Scheme 1) derived from methylated *N*-acetyl-tyrosine with methylated AMP (Me-5′-AMP)[10] was selected as a model of the aminoacylation of the 3′-terminus of RNA strands.[11] We observed essentially the formation of four isomers (Figure 2) having the composition of a stoichiometric adduct (HPLC–ESI-HRMS negative mode, calcd for $C_{23}H_{28}N_6O_{10}P^-$: 579.1610; found: 579.1605). The formation of these isomers can be accounted for by the well-known regioisomerization of acyl groups between the 2′- and 3′-hydroxyl groups of the ri-

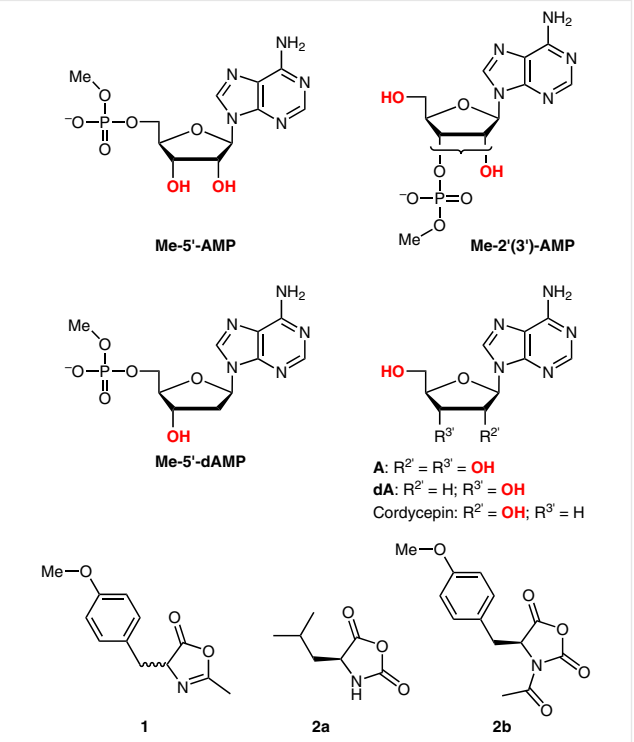
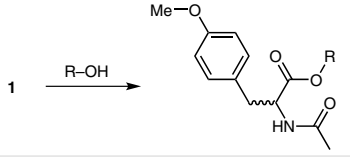


Figure 1 The different nucleosides and nucleotide models with potential acylation sites (bold red) as well as the activated aminoacylating species used in this work

bose moiety[12] 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**.[13]



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

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[14] (Figure 2). 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 2 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.[15]

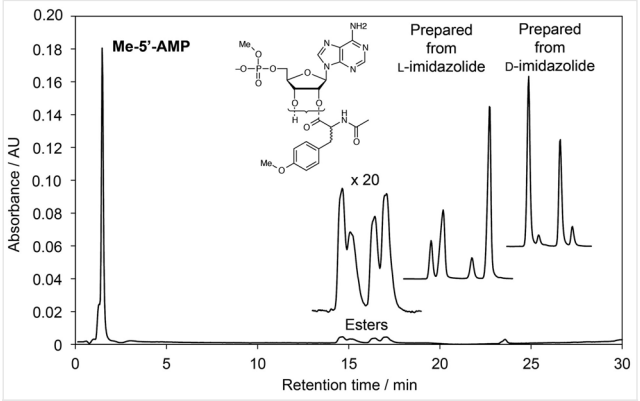


Figure 2 HPLC trace of adducts of amino acid with Me-5′-AMP after 24 h of reaction of 25 mM Ac-Tyr(Me)-OH-derived 5(4*H*)-oxazolone with 5 mM Me-5′-AMP in 100 mM MES-buffered aqueous solution (pH 6.5). Before analysis, the reaction medium was acidified and extracted twice with EtOAc to remove Ac-Tyr(Me)-OH (retention time 15.1 min), then an aliquot (50 µL) 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.

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[2] 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.

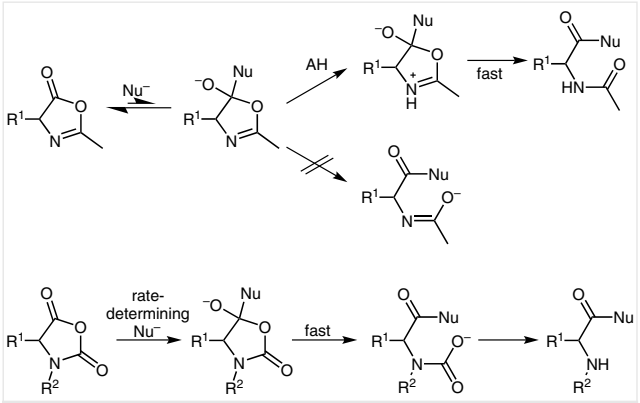
The reactions of 5(4*H*)-oxazolones with nucleotides yield aminoacylated derivatives in which the amino acid is not free but acylated. Regarding the importance of aminoacylated RNA in translation, we studied the potential of NCA as aminoacylating agents for which prebiotically relevant pathways of formation have been proposed.[16] In a first stage, the reaction of Leu-NCA **2a**[17] 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

Table 1 Reactions of Different Nucleosides/Tides with Ac-Tyr(Me)-oxazolone

	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	Bisadduct as % of the area measured at 248 nm	Ref.
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[c]	not determined	not determined	30.2[b,c]	10.8	this work
dA (2′-dA)	5.4[d]	not applicable	100%	5.4[b,d]	not detected	this work
Cordycepin (3′-dA)	16.2[e]	100%	not applicable	16.2[b,e]	not detected	this work
AMP[f]	8.4	not determined	not determined	25.6[g]	4.2	ref. 8
dAMP[gh]	1.9	not applicable	100%	27.7[g]	not detected	ref. 8
Me-5′-dAMP	2.4	not applicable	100%			this work
Me-2′(3′)-AMP	not detected			7.5[b]	not detected	this work

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

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 $NaHCO_3$ solution in which a saturated concentration of CO_2 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**[18] as an alternative model unable to undergo polymerization but in principle capable of reproducing the behaviour of free NCA 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-NH_2 as a single stereoisomer with a retention time of 10.7 min identical to that of an authentic sample.[13] 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 behaviour (Scheme 2).



Scheme 2 The different reactivity of tetrahedral addition intermediates proposed in order to account for the behaviour of 5(4*H*)-oxazolones and NCA 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 NCA (R^2 = H) or from the *N*-acylated derivatives (R^2 = Ac).

The two different pathways putatively proposed in Scheme 2 for the reactions of nucleophiles with the 5(4*H*)-oxazolones and NCA 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.[21] 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,[5] or imidazolides[3,22] could therefore be discussed on a similar basis. Interestingly, Profy and Usher observed that the reaction of an imidazolide with the 2′(3′)-sites of RNA was less sensitive to imidazole buffer concentration[22] 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 tRNA 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,[19,23] which are converted into NCA 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 NCA proved to be incompetent whereas phosphate mixed anhydride, possibly requiring acid catalysis or other forms of assistance to acyl transfer, might constitute efficient reagents. Another observation[2] confirmed by this study is the specific ability of RNA models to undergo aminoacylation, which exceeds that of DNA models by a factor of ca. 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.,[15] 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-tRNA 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[24] and ester aminolysis[25] including the peptidyl transfer of the ribosomal reaction. The study of the activity of potential prebiotic reagents for the nonenzymatic aminoacylation reactions of RNA models therefore provides valuable information on the chemistry that could have supported the first steps of life.

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Supporting Information

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(10) 5′-AMP was methylated by reaction with dicyclohexylcarbodiimide in methanol.[26]
(11) 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 MeCN solution for several weeks (see ref. 8).
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(18) The acetylated NCA **2b** was prepared by acylation of the non-substituted derivative[17,19] with acetyl chloride in the presence of *N*-methylmorpholine according to the general procedure described in ref.[20] for urethane-*N*-carboxyanhydrides. Tyr(Me)-NCA (0.3 g, 1.36 mmol) and acetyl choride (0.101 mL, 1.43 mmol) were dissolved in dry THF (5 mL) under a N_2 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 N_2. 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%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.01–6.91 (m, 2 H), 6.90–6.80 (m, 2 H), 5.01 (dd, *J* = 5.7, 2.4 Hz, 1 H), 3.79 (s, 3H), 3.60–3.20 (m, 2 H), 2.54 (s, 3 H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 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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