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COMMUNICATION

Biomimetic protecting-group-free 2′, 3′-selective aminoacylation of nucleosides and nucleotides†

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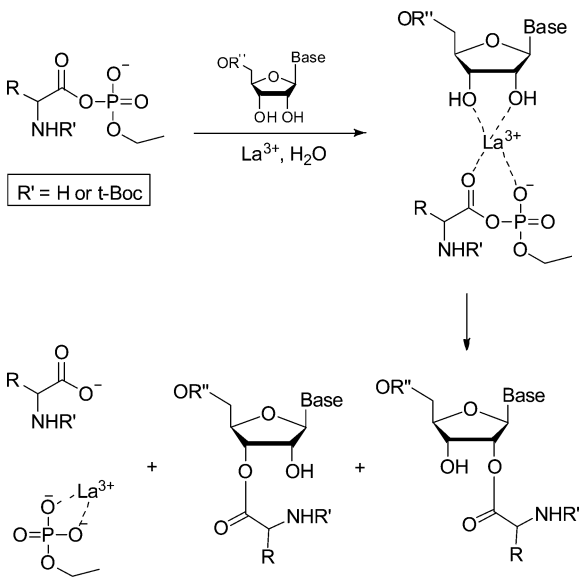
Aminoacyl phosphate monoesters can be prepared free of an amino-protecting group and used directly in lanthanum-promoted selective monoacylation of either the 2′ or 3′-hydroxyl of nucleosides and nucleotides. For example, phenylalanyl ethyl phosphate rapidly forms esters with either of the 2′ or 3′-hydroxyls of ribonucleosides and nucleotides in the presence of lanthanum ions in aqueous buffer. Oligomerization of the aminoacyl phosphate is much slower than ester formation and is not a competitive process. Competing hydrolysis of the reagent is slow. By extension, this route should provide a simplified general route to synthetically aminoacylated derivatives of tRNA.

Chemical aminoacylation of the 2′ or 3′-hydroxyls of nucleosides, nucleotides, and oligonucleotides is normally a multistep process involving protection and deprotection of numerous functional groups in the initial reaction components.[1–3] In contrast, the corresponding enzymatic reactions occur directly, utilizing enzyme-bound aminoacyl adenylates.[4–6] This biological intermediate is generated from the reaction of ATP and a specific amino acid, which activates the aminoacyl group toward substitution. It also provides a complex entity whose binding interactions with the enzyme permit precise orientation for the required reaction.[7–9] Methods for direct chemical aminoacylation have been investigated utilizing a biomimetic approach, where *N*-protected aminoacyl phosphate monoester and lanthanum ion serve as functional analogues of an aminoacyl adenylate and enzyme.[10] In this system, it is proposed that binding interactions between the enzyme and the substrate are mimicked by the formation of a bis-bidentate complex of the reactants and lanthanum. This model is based on the analysis of related reactions by Clarke and coworkers as well as the observed specificity for monoacylation of diols (Scheme 1).[11–14]

The recent report by Duffy and Dougherty of aminoacylation of the 3′-terminal of tRNA and the utilization of the deprotected product in an expression system provides an important demonstration of the feasibility of direct aminoacylation for incorporating unnatural amino acids into proteins.[15] However, the required deprotection prior to ribosomal utilization adds a step where direct utilization is clearly preferable. Furthermore, the procedure as reported required a 1000 fold excess of reagents to obtain the aminoacylated tRNA in any useful quantity.



Scheme 1 Lanthanum coordination and aminoacylation.

The use of synthetic routes that are free of protecting groups at critical stages has become an area of significant interest and has been illustrated with compelling examples.[16–18] The use of an acylating agent with a free amino group raises obvious concerns. First, is the amino group capable of reacting with the activated carboxyl of a second aminoacyl phosphate as these compounds react readily with other amines?[19] Second, will the unprotected compound selectively form an ester with a terminal diol as do their *N*-protected analogues? A promising precedent can be found in the earliest examples of synthetic aminoacyl adenylates. These were reported to be produced without protecting groups; however, the materials were not analyzed and were assumed to be unstable.[20,21] Where such species are formed transiently, they have been used to test theories of the origin of peptide formation in living systems and appear to function productively.[22–24]

We now report the preparation and characterization of protecting-group-free aminoacyl phosphate esters as well as their successful use in lanthanum-directed 2′, 3′-selective

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monoaminoacylation of nucleosides and nucleotides. The optimization of these methods now presents an opportunity to extend the process to reaction with tRNA where isolation or spectroscopic detection of products remains a challenge.

Aminoacyl phosphate monoesters were produced by deprotection of *N*-*t*-BOC-aminoacyl ethyl phosphates, compounds that are formed by the coupling of the protected amino acid and ethyl phosphate.[25] The protecting group is needed to direct the precursor to form the anhydride rather than an amide. Ethyl dichlorophosphate is converted to ethyl phosphoric acid by addition to water and then neutralized with tetraethylammonium hydroxide to obtain bis(tetraethylammonium) ethyl phosphate. *N*-*t*-BOC-phenylalanyl ethyl phosphate (BOCPheEP) was prepared by DCC-promoted coupling of bis(tetraethylammonium) ethyl phosphate and *N*-*t*-BOC-phenylalanine. The amino group was liberated by addition of a small amount of trifluoroacetic acid. The product was precipitated with added acetone to give PheEP as a white powder (30% yield). The stereochemical integrity of the sample is maintained, evidenced by ^{1}H NMR spectra indicating no exchange of the α-proton of PheEP in D_2O during the course of the reactions.[19]

We then tested the reactivity and regiospecificity of combinations of ribonucleosides (adenosine, cytidine, uridine) and ribonucleotides (5′-AMP, 5′-CMP) with PheEP in the presence of lanthanum triflate. Reactions were conducted with equimolar concentrations of reactants in 100 mM, pH 6 MES (2-(*N*-morpholino)ethanesulfonic acid) buffer at room temperature. These were quenched with a saturated solution of EDTA to remove lanthanum ions. ^{1}H NMR, high resolution MS-ESI and HPLC (C_{18} reversed phase column) were used to monitor the reaction and characterize products (Fig. 1).

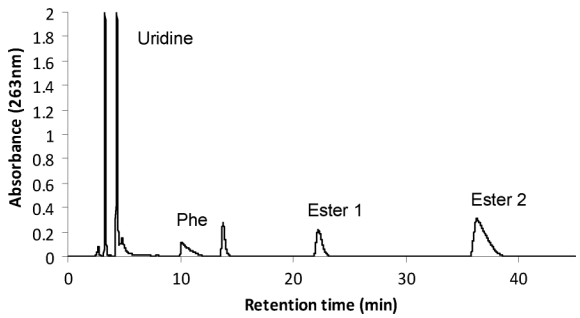


Fig. 1 Reversed phase HPLC chromatogram of lanthanum-catalyzed aminoacylation of uridine with PheEP after 1 h. Reaction conditions: [La(OTf)$_3$] = [uridine] = [PheEP] = 10 mM in MES buffer (pH 6, 100 mM) at 25 °C.

The reaction of uridine with PheEP in the presence of lanthanum triflate is complete in less than one minute, giving a mixture of monoaminoacylation products (HPLC elution times of 22 min and 37 min) as well as the hydrolysis product, phenylalanine (Phe, 12 min). The product peaks were isolated and characterized by high-resolution ESI(+)-MS as phenylalanyl monoesters of uridine (M + H$^+$ calculated m/z = 392.1452, found m/z = 392.1454, 392.1444). The yield of esters is 60–70% based on the integrated areas on chromatograms. HPLC analysis also showed that the products were formed within the first minute following addition of the reactants. The resulting aminoacyl ester is resistant to hydrolysis, being unchanged in neutral solution for hours.

Further analysis of the isolated aminoacyl esters was carried out with ^{1}H NMR (Fig. 2). The chemical shift for the signal of the 1′-proton of the ribofuranoside depends on the location of the ester: the doublet is further downfield for the 2′-ester than for the 3′-ester due to the interaction of the 2′-ester with the aminoacyl moiety. Therefore, the site of aminoacylation can be determined from the relative chemical shifts. The doublet corresponding to the 1′-proton of the ribofuranoside appears at δ 6.06 for the 2′-ester while the 3′-ester signal appears at δ 5.88. The same trend is observed for the chemical shifts of the 6′ proton of the pyrimidine ring. The signals appear at δ 8.03 and δ 7.92 for the 2′ and 3′-esters, respectively. Based on this analysis, the two peaks in the HPLC corresponding to the Phe monoesters of uridine were assigned as the 2′-ester (22 min) and 3′-ester (37 min). Integration of the chromatograms establishes that the ratio [2′-ester] : [3′-ester] is approximately 1 : 2, which reflects the lower energy of the 3′-ester. These observations are consistent with the results reported for *N*-*t*-BOC-aminoacyl ethyl phosphates.[10,25,26] In addition, the NMR spectrum of either ester after separation by HPLC results in peaks for both esters being observed. This requires that the two mono-esters equilibrate, consistent with well-known equilibration between 2′ and 3′-aminoacyl esters.[27–29]

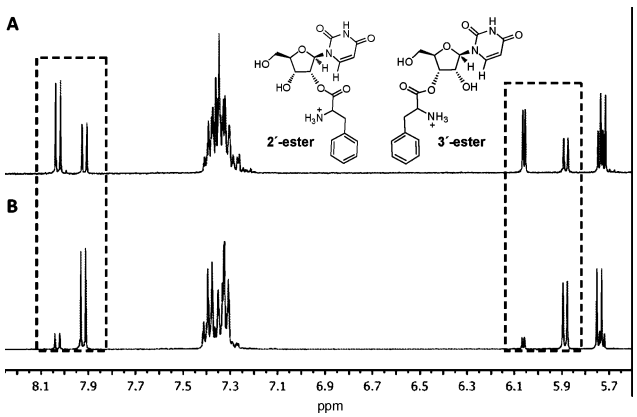


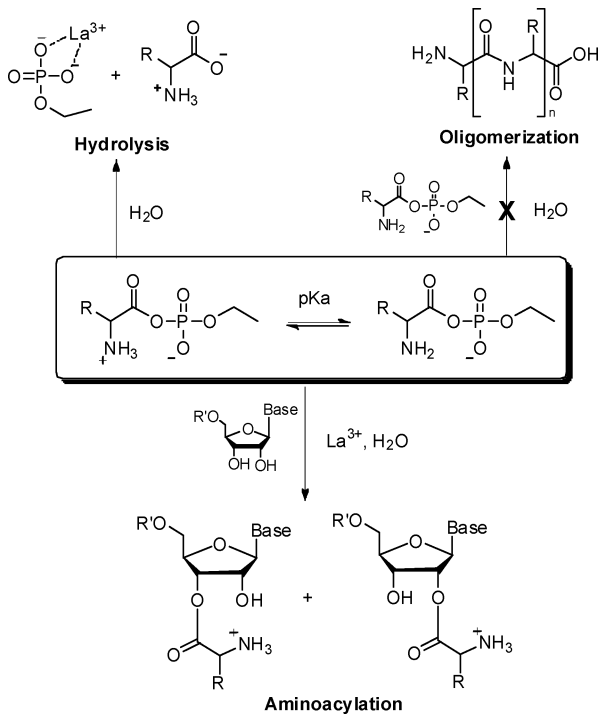
Fig. 2 ^{1}H NMR of two phenylalanine monoesters of uridine separated by reversed phase HPLC (A: Ester 1, B: Ester 2) Note that the 2′ and 3′-esters equilibrate.

Rapid mono-aminoacylation was also achieved in reactions of PheEP with adenosine, cytidine, 5′-AMP and 5′-CMP. Analysis with HPLC and MS-ESI confirmed the formation of PheEP-monoesters of adenosine (calculated m/z = 415.1724, found m/z = 415.1711, 415.1720) cytidine (calculated m/z = 391.1612, found m/z = 391.1599, 391.1605), 5′-AMP (calculated m/z = 493.1242, found m/z = 493.1197, 493.1263), and 5′-CMP (calculated m/z = 469.1129, found m/z = 469.1143, 469.1156).

Control reactions without added lanthanum ion were performed with equimolar concentrations of ribonucleosides and PheEP in MES (pH 6, 100 mM) buffer. The reactants and the hydrolysis product (Phe) were observed by HPLC with no ester or amide peaks. The requirement for the *cis*-diol, which in principle establishes selectivity for the 3′-terminal of any RNA, was tested with 2′-deoxycytidine as a reactant in the presence of lanthanum ion. After 60 min, only the starting materials and the hydrolysis products were observed *via* HPLC. This is consistent with the proposed mechanism of aminoacylation proceeding exclusively

via bis-bidentate coordination of lanthanum ion by an aminoacyl phosphate and the 2,3-diol of terminal ribosyl derivative.

An aminoacyl ethyl phosphate is unusual in that it contains both a free amino group and a reactive carboxyl group. Since a free amine would normally be a more reactive nucleophile than water or the hydroxyl groups of ribose ring, the free amino group should react as a nucleophile toward the acyl group of another aminoacyl phosphate ester to form an amide. At pH higher than the pK_A of the PheEP amino group (pK_A = 7.8),[25] this process would compete with aminoacylation or hydrolysis. Thus, PheEP polymerizes to form oligopeptides when incubated in 250 mM pH 8 HEPES buffer. However, this is suppressed where the solution's pH is below the pK_A of the amino group. At a lower pH, the *N*-protonated form of PheEP is the major species and is not a nucleophile. When the lanthanum-catalyzed reactions are carried out in pH 6 MES buffer, polymerization is very slow compared to the rapid monoacylation. In effect, the proton is a mobile protecting group (Scheme 2).



Scheme 2 Reactions of aminoacyl phosphate esters in water.

In conclusion, we have demonstrated that efficient lanthanum-promoted aminoacylation of ribonucleosides and ribonucleotides can be achieved with an aminoacyl phosphate ester with a free amino group. The methods outlined here overcome the problem of low yields as seen in previous reports. These results also predict that the protecting-group-free aminoacyl phosphate esters can be utilized in the direct and selective aminoacylation at the 3′-terminal hydroxyl of oligonucleotides and tRNA. This should assist in developing a chemical catalytic protocol for conveniently synthesizing aminoacyl-tRNA esters for use in ribosomal formation of proteins with amino acids that are not specified by the genetic code.[15,30–32] We are currently investigating this process in order to expand the scope of the methodology.

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