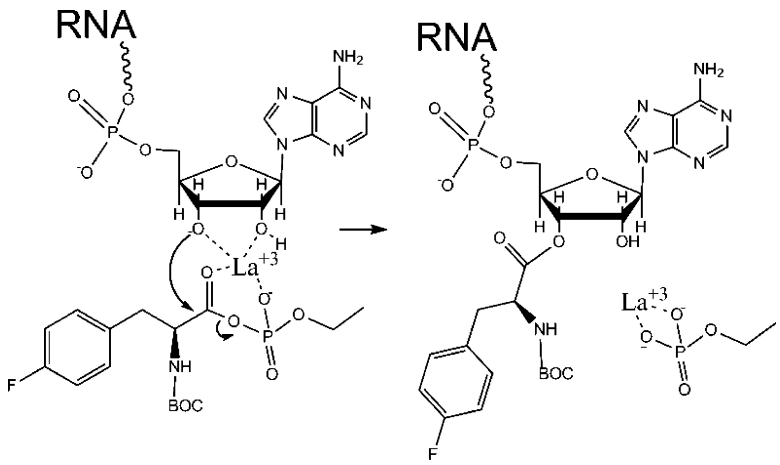
Article

Biomimetic Aminoacylation of Ribonucleotides and RNA with Aminoacyl Phosphate Esters and Lanthanum Salts

Svetlana Tzvetkova, and Ronald Kluger

J. Am. Chem. Soc., **2007**, 129 (51), 15848-15854 • DOI: 10.1021/ja073976l



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
ARTICLES
Published on Web 12/04/2007

Biomimetic Aminoacylation of Ribonucleotides and RNA with Aminoacyl Phosphate Esters and Lanthanum Salts

Svetlana Tzvetkova and Ronald Kluger*

Contribution from the Davenport Chemical Laboratories, Department of Chemistry, University of Toronto, Toronto, ON Canada M5S 3H6

Received June 1, 2007; E-mail: rkluger@chem.utoronto.ca

Abstract: Aminoacylation of tRNA in cells involves activation of the amino acid as an aminoacyl adenylate, a mixed anhydride with AMP, which reacts with tRNA. We have now established that aminoacyl phosphate esters in the presence of lanthanide ions in water will acylate hydroxyls at the 3′-terminus of RNA or a simple nucleotide. By extension, this will permit synthetically aminoacylated tRNA to be produced in a single-step biomimetic process. The reactions of Boc-4-fluorophenylalanyl ethyl phosphate were followed by HPLC separation, MS, and ^{19}F NMR analysis. In stoichiometric combination with lanthanum salts in aqueous buffer, Boc-4-fluorophenylalanyl ethyl phosphate rapidly produces 2′- and 3′-monoesters of cytidine and cytidine monophosphate. Reaction of the reagent with RNA in the presence of lanthanum and magnesium salts introduces a specifically detectable signal into the RNA, which is evidence of formation of the aminoacyl ester. When the same RNA is initially oxidized with periodate to convert the 3′-terminal vicinal diol to the cleaved dialdehyde, reaction with the aminoacyl phosphate no longer occurs as evidenced by the lack of a signal in the ^{19}F NMR spectrum. The results are consistent with a requisite chelation mechanism in which lanthanum serves as a template for both the aminoacyl phosphate *and* the 3′-terminal diol of RNA and nucleotides. The coordinated diol will then react through specific base-catalyzed intramolecular addition of the alkoxide nucleophile to the acyl group of the aminoacyl phosphate. Assessment of the method with a single tRNA was also achieved using the fluorescent reagent *N*-dansyl-glycyl ethyl phosphate. Lanthanide-promoted aminoacylation at the 3′-terminus of tRNA^{Phe} is detected by the introduction of fluorescence (detected directly and by antibody-enhanced emission). This does not occur if the 3′-terminus is converted to the dialdehyde by reaction with periodate.

Introduction

Synthetically aminoacylated tRNAs were envisioned by Hecht as the basis of a versatile method for ribosomal production of proteins that contain unnatural amino acids.[1] Schultz extended this to include procedures for *in vivo* incorporation of unnatural amino acids into proteins.[2] The reported synthetic procedures for aminoacyl tRNA require preparation of amino-protected 3′-aminoacylated diribonucleotides (derived from pCpA), followed by enzymic ligation to tRNA(-pCpA) and deprotection. In the cell, aminoacylation of tRNA is promoted by specific cytosolic enzymes, aminoacyl tRNA synthetases, in a two-part reaction. The enzymes promote an initial activation of the cognate amino acid with ATP, producing the corresponding 5′-aminoacyl adenylate (AA-AMP).[3] In a coupled step on the same enzyme, the aminoacyl moiety is transferred to the 3′-terminal hydroxyl of the cognate tRNA. Structural and mechanistic analysis suggests that the enzymic formation of the aminoacyl tRNA is achieved simply by bringing the components into reactive proximity while establishing proper alignment for reaction.

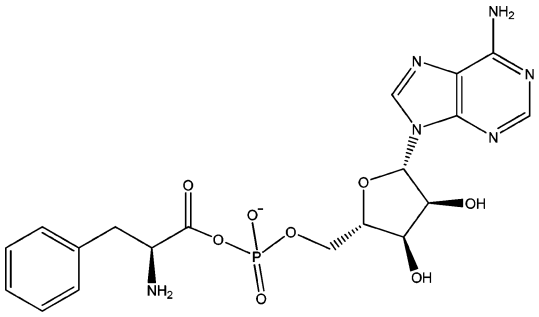
In order to make the introduction of synthetic aminoacyl units directly accessible, we sought a biomimetic process for the synthesis of aminoacyl tRNAs. We developed an aminoacyl donor that is a functional analogue of the biochemical aminoacyl-AMP intermediate, an aminoacyl alkyl phosphate, as exemplified by phenylalanyl 5′-adenylate and the simplified analogue, phenylalanyl ethyl phosphate.

Aminoacyl adenylates have been isolated in complexes with aminoacyl tRNA synthetases and studied. In some cases, the activated amino acid undergoes a spontaneous side reaction in which it forms amides derived from amino groups of the enzyme.[4] In pioneering work on this class of materials Meister prepared aminoacyl adenylates by condensation of an amino acid and AMP, establishing that they react with hydroxylamine and with proteins, as well as with ammonia, amino acids, and ribonucleic acids.[5]

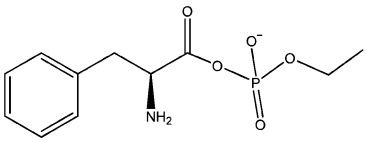
In order to direct the nonenzymic reaction specifically to the 3′-terminus of tRNA, we devised a template procedure that takes advantage of the *cis* diol functional array of the 2′ and 3′ hydroxyl groups. Chelation of the diol by La^{3+} in a bis-bidentate

(1) Heckler, T. G.; Chang, L.-H.; Zama, Y.; Naka, T.; Chorghade, M. S.; Hecht, S. M. *Biochemistry* **1984**, *23*, 1468−1473.
(2) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182−188.
(3) Mulvey, R. S.; Fersht, A. R. *Biochemistry* **1978**, *17*, 5591−5597.
(4) Kern, D.; Lorber, B.; Boulanger, Y.; Giege, R. *Biochemistry* **1985**, *24*, 1321−1332.
(5) Moldave, K.; Castelfranco, P.; Meister, A. *J. Biol. Chem.* **1959**, *234*, 841−848.

10.1021/ja073976l CCC: $37.00



Phenylalanyl 5'-adenylate



Phenylalanyl ethylphosphate

complex[6] along with the aminoacyl alkyl phosphate sets up the system for specific-base-catalyzed acylation (Scheme 1). The potential for this type of reaction selectivity is consistent with models in which esters form from the reaction of a single hydroxyl in a 1,2-diol with benzoyl methyl phosphate and lanthanide ions in water.[7,8]

We now report promising results on aminoacylation reactions of nucleosides and nucleotides with Boc-4-fluorophenylalanyl ethyl phosphate (BocFPhePEP), a fluorinated amino acid derivative whose reaction we were able to follow by ^{19}F NMR spectroscopy. The results provide strong evidence that a direct, selective reaction at the 2′ and 3′ hydroxyls is readily achieved. We also report the successful direct extension of the aminoacylation reaction to RNA.

Materials and Methods

Commercial reagents were used without further purification. RNA type VI from Torula yeast and tRNA^{Phe} from yeast were purchased from Sigma. High-resolution mass spectrometry was performed at the QStar Chemistry Mass Spectral Facility, University of Toronto. HPLC analysis utilized a C18 reversed phase preparative column (7.8 mm × 300 mm), eluting with 40% acetonitrile (HPLC grade) with 0.1% trifluoroacetic acid in deionized water. The flow rate was 3.0 mL/min, at room temperature. Eluting species were detected at 263 nm.

Boc-4-fluorophenylalanyl ethyl phosphate (BocFPheEP) was prepared from the amino acid according to the general method previously reported by Loo and Kluger.[9] Ethyl dichlorophosphate (25 mmol) was converted to the free acid by addition to 4.5 mL water over 10 min in an ice-cooled round-bottom flask and then stirred for 1 h. Hydrogen chloride that formed as a byproduct was removed by rotary evaporation with vacuum. The resulting ethyl phosphoric acid was neutralized with 2 equiv of tetraethylammonium hydroxide. The neutral solution was freeze-dried. Boc-4-fluorophenylalanine (BocFPhe, 1.62 mmol) was activated with dicyclohexylcarbodiimide (DCC, 1.12 mmol) in dichloromethane for 3 min. Tetraethylammonium ethyl phosphate (1.12 mmol) in dichloromethane was added, and the mixture was stirred at room temperature for 1 h. The product was extracted with water, freeze-dried, and used for aminoacylation without further purification. BocFPheEP, a white hygroscopic solid, was thus prepared in 80% yield.

^{1}H NMR (300 MHz, D_2O): δ 7.1 (t, 2H, Ar), 6.9 ((t, 2H, Ar), 4.3 (q, 2H, POCH_2CH$_3$), 4.0 (t, 1H, C*H*CO), 3.85 (m, 2H, A*rCH*$_2$), 3.1 (q, 8H, N$^+$(CH_2CH$_3$)$_4$, 1.2 (s, 9H, tBut), 1.1 (t, 3H, POCH$_2$CH_3), 1.0 (t, 12H, N$^+$(CH$_2$CH_3)$_4$); ^{13}C NMR (75 MHz, D_2O): δ 163.1 (*C*O−O−PO), 160.5 (p-F−*C*Ar), 157.3 (*C*O−O−*t*But), 132.2 (p-F−Ar*C*), 130.9 (Ar), 115.0 (Ar), 81.3 (*C*(CH$_3$)$_3$), 63.6 (P−O−*C*H$_2$CH$_3$), 55.9 (NH−*C*H−CO), 52.1 (N$^+$(*C*H$_2$CH$_3$)$_4$), 36.2 (Ar−*C*H$_2$), 27.6 (C(*C*H$_3$)$_3$), 15.6 (P−O−CH$_2$*C*H$_3$), 6.7 (N$^+$(CH$_2$*C*H$_3$)$_4$); ^{19}F NMR (282 MHz, D_2O): δ −117.2; ^{31}P NMR (121 MHz, D_2O): δ −6.18; MS ESI (−): found *m*/*z* 391.1203, calculated *m*/*z* 391.1196.

The isopropyl ester of *N*-Boc-4-fluorophenylalanine was synthesized according to the method of Hassner and Alexanian.[10] *N*-Boc-4-fluorophenylalanine (1.0 mmol) was dissolved in 15 mL of dichloromethane. DCC (1.1 mmol), isopropanol (1.1 mmol), and 4-pyrrolidinopyridine (0.1 mmol) were added. The solution was stirred at room temperature for 2 h. Dicyclohexylurea was removed by filtration, and the filtrate was washed with water, acetic acid, and again with water. The isopropyl ester was dried over magnesium chloride, and the solid was dried in vacuum.

^{1}H NMR (300 MHz, CD_3OD): δ 7.2 (t, 2H, Ar), 6.99 ((t, 2H, Ar), 4.97 (quintet, 1H, C*H*(CH$_3$)$_2$), 4.26 (t, 1H, C*H*NH), 2.92 (m, 2H, A*rCH*$_2$), 1.38 (s, 9H, *t*But), 1.19 (dd, 6H, CH(CH_3)$_2$); ^{19}F NMR (282 MHz, CD_3OD): δ −118.9; MS ESI (+): found *m*/*z* 348.2 (325.17+Na), calculated *m*/*z* 325.17 (+23.5).

Reactions of the aminoacyl phosphates were performed at room temperature with stirring. All reacting components were used in equimolar amounts. Lanthanide salts (triflate or trichloride) were the last component added in order to minimize the competing lanthanum-promoted hydrolysis of BocFPheEP prior to acylation.

Products were monitored by reversed phase HPLC at 263 nm and then ^{19}F NMR at 367 or 282 MHz in D_2O, with shifts recorded relative to $CFCl_3$. When ^{19}F NMR was used to follow the reactions, $LaCl_3$ was used as a catalyst since the triflate group contains fluorine atoms with signals that interfere with those of the reactants and products. In cases where the ester products were separated by HPLC, the residual TFA from the mobile phase was removed by solid-phase extraction (PL-HCO_3 MP SPE Tubes, Polymer Labs) or acetic acid was used in its place.

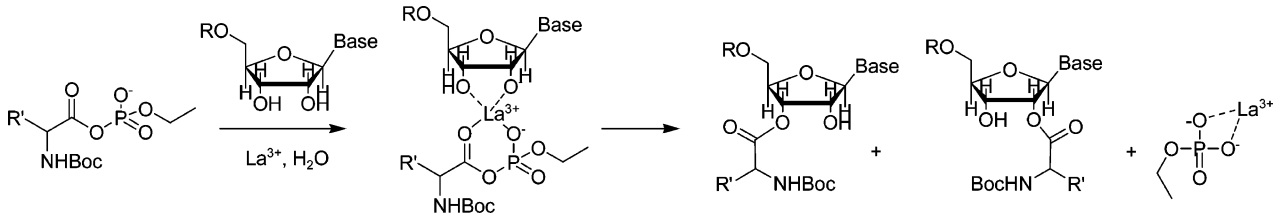
Scale-up of the reaction with RNA: The reactants were dissolved in 0.5 mM pH 8 EPPS. A 60 μL aliquot of RNA solution (20 mg/mL buffer) was first mixed with 100 μL of 100 mM $MgCl_2$ solution. BocFPheEP and lanthanum trichloride were added to give a concentration of 20 mM. The final buffer concentration was 75 mM. Distilled water was added to bring the reaction volume to 0.4 mL. Sephadex G-25 spin columns were used for isolation of the RNA-esters.

Cleavage of the terminal diol of RNA was accomplished with sodium periodate solution. RNA (9.3 mg) was dissolved in 0.5 mL of 100 mM magnesium chloride solution and 0.5 mL of 0.5 M EPPS buffer. Sodium periodate (5 mL of 0.1 M solution) was added, and the reaction was kept in the dark at room temperature for 20 min.[11] Excess sodium periodate was precipitated by addition of about 1.0 mg of potassium chloride at 0 °C.[12] The supernatant was removed, and RNA was precipitated with cold ethanol, collected, and dried.

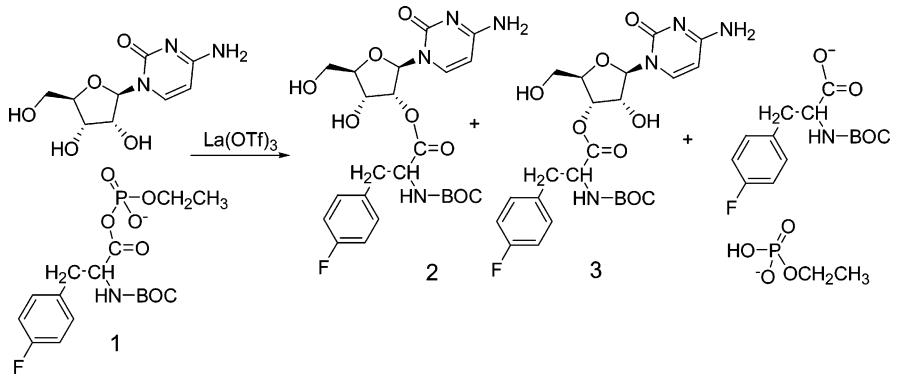
Fluorescence Studies. Commercially supplied *N*-dansyl-glycine was converted to the corresponding ethyl phosphates anhydrides according to the procedure described above for BocFPheEP. Commercially available purified yeast tRNA^{Phe} was used as the reaction substrate. Nuclease-free water (Fermentas) was used in all experiments with tRNA.

(6) Clarke, P. A.; Arnold, P. L.; Smith, M. A.; Natrajan, L. S.; Wilson, C.; Chan, C. *Chem. Commun.* **2003**, 2588−2589.
(7) Kluger, R.; Cameron, L. *J. Am. Chem. Soc.* **2002**, *124*, 3303−3308.
(8) Cameron, L.; Wang, S.; Kluger, R. *J. Am. Chem. Soc.* **2004**, *126*, 10721−10726.
(9) Kluger, R.; Li, X.; Loo, R. W. *Can. J. Chem.* **1996**, *74*, 2395−2400.
(10) Hassner, A.; Alexanian, V. *Tetrahedron Lett.* **1978**, 4475−4478.
(11) Proudnikov, D.; Mirzabekov, A. *Nucleic Acid Res.* **1996**, *24* (22), 4535−4542.
(12) Chernetskii, V. P.; Ponomareva, E. A.; Stavitskii, V. V. *Khim. Geterotsikl. Soedin.* **1970**, *6*, 987−988.

Scheme 1. La^{3+} Chelation and Aminoacylation



Scheme 2. Reaction of **1** with Cytidine and Lanthanum Triflate Produces 2′- and 3′-Esters



Polyclonal rabbit anti-dansyl antibody (anti-DNS Ab) was obtained from Molecular Probes. Aminoacylation of tRNA was carried out for 1 h at room temperature, as described for nucleotides and bulk RNA in the preceding sections. After completion of the reaction, the products were isolated and purified by passage through a G-25 column. tRNA concentrations were determined spectrophotometrically based on extinction coefficient obtained from literature.

The dansyl (DNS) group has an excitation maximum at 330 nm and an emission maximum at 550 nm. The excitation maximum of the DNS-aminoacyl phosphates was determined to be at 327 nm, and the emission maximum, at 540 nm. The instrumental settings were as follows: increment, 1 nm; excitation slit, 1 nm; emission slit, 1 nm; integration time, 1 s. Spectra were acquired from 400 to 650 nm. All measurements were performed at room temperature in nuclease-free water solutions.

Antibodies were used to test further the introduction of the fluorophore onto tRNA at a more sensitive level. An anti-DNS antibody was used in the fluorometric studies to determine the presence of a DNS-group on the tRNA after aminoacylation. It was supplied as a solution (1 mg/mL in phosphate-buffered saline pH 7.2, containing 5 mM sodium azide). Based on information supplied with the reagents, the maximum fluorescence enhancement for DNS is about 10-fold over the fluorescence of the free dye. Steric interference with the covalently bound fluorophore will lower the enhancement produced by antibody binding to the fluorophore.

Oxidation of $tRNA^{Phe}$ with $NaIO_4$ was performed as described for bulk RNA by the method of Proudnikov and Mirzabekov.[11] Fresh solutions of 0.1 M $NaIO_4$ were prepared immediately before the reaction. A 5 μL aliquot of this solution was used to oxidize 0.1 mL of 5.5×10^{-5} M tRNA. The reaction was carried out in the dark for 20 min at room temperature. Excess $NaIO_4$ was precipitated by addition of KCl at 0 °C.[12] The supernatant was removed and transferred to a vial. The oxidized tRNA was then isolated by precipitation with cold ethanol and centrifugation for 30 min at 13 000 rpm. The resulting white pellets were dried and used for the aminoacylation test reaction.

The reaction mixture for the aminoacylation of yeast $tRNA^{Phe}$ consisted of $tRNA^{Phe}$, DNS-glycyl ethyl phosphate (DNSGlyEP), and $La(OTf)_3$ in equimolar concentrations (2.47×10^{-5} M), excess $MgCl_2$ (10^{-3} M), buffered with pH 8, 3×10^{-5} M EPPS. The reaction was performed in the dark at room temperature for 1 h. The resulting tRNA/oxtRNA and DNS-aminoacyl-tRNA were isolated using the G-25 spin-column. This treatment removes unreacted DNS-aminoacyl phosphate as well as the product from its hydrolysis, the DNS-amino acid. This treatment removes Mg^{2+} and La^{3+} as well, thus avoiding any possible metal-catalyzed hydrolysis of the aminoacyl-tRNA.

Results

One of the challenges of assessing the success of the aminoacylation reaction of a nucleotide (or RNA) is detection of the product and establishment of the site of aminoacylation. We obtain this information by first introducing an NMR-detectable derivative that carries a label not normally found in RNA. Reaction of BocFPheEP with RNA (Scheme 1) is expected to give an ester that is detectable by its ^{19}F NMR signal. We assessed the specific site of the aminoacylation reaction by comparing the reaction after periodiate oxidation cleavage of the 3′-terminal diol of RNA. We find that the resulting dialdehyde does not react with the aminoacyl phosphate, confirming the necessity of the intact vicinal diol for the aminoacylation process. Because of the quantities of material necessary for NMR analysis, we used bulk RNA for this part of the study.

Aminoacylation of Nucleosides. The reaction of cytidine with BocFPheEP (**1**) in the presence of 1 equiv of lanthanum triflate occurs rapidly, producing a mixture of the 2′- and 3′-BocFPhe esters of cytidine (**2** and **3**) along with BocFPhe from the competing hydrolysis reaction of BocFPheEP (Scheme 2, Figure 1). Analysis by HPLC indicates that the products form within 30 s and remain intact thereafter (Figure 2).

The products were isolated and characterized as monoesters of cytidine (ES MSI (+) *m*/*z* calculated 508, found 509.2). Independent studies in our laboratory have revealed that the 2′-ester elutes ahead of the 3′-ester and that the concentration of the 3′-ester is about twice that of the 2′-ester. The ratio 2′-ester/3′-ester/BocFPhe was 1:2:0.5. There is no 5′-ester detected. The reaction was also studied by ^{19}F NMR (Figure 3) using lanthanum chloride in place of lanthanum triflate. The reaction was stopped with EDTA after 10 s. The signal corresponding to BocFPheEP appears at δ −117.2. In addition, a new peak at

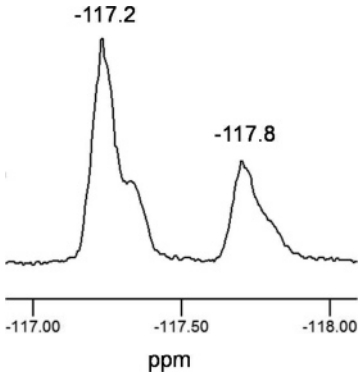


Figure 1. ^{19}F NMR (282 MHz) spectrum of BocFPheEP (δ −117.2) and its hydrolysis product, BocFPhe (δ −117.8).

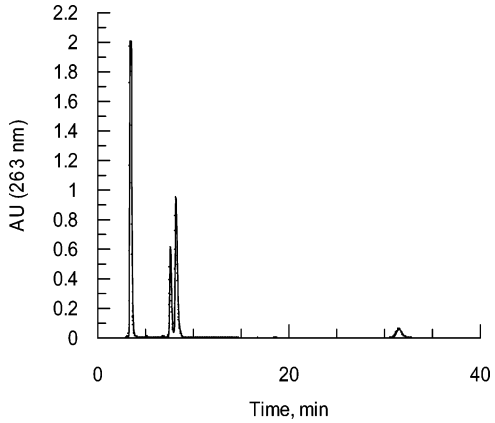


Figure 2. Aminoacylation of cytidine with BocFPheEP: Reversed phase HPLC of product after 30 s reaction. Peak contents were analyzed by MS and NMR. The initial peak is cytidine. The two subsequent peaks contain the 2′ and 3′ BocFPhe esters of cytidine (see Scheme 2). The last peak is BocFPhe.

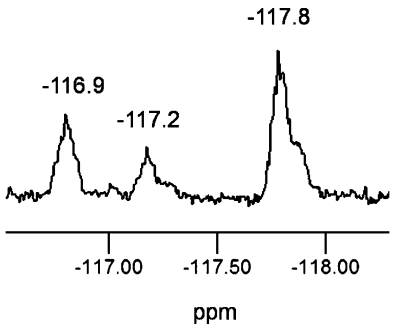


Figure 3. ^{19}F NMR (282 MHz, D_2O) spectrum of the reaction mixture of 10 mM cytidine, 10 mM BocFPheEP, 10 mM $LaCl_3$, and 100 mM EPPS pH 8, quenched with EDTA at 10 s. Peaks were identified as the 2′- and 3′-BocFPhe-cytidine esters (δ −116.9), BocFPheEP (δ −117.2), and BocFPhe (δ −117.8).

δ −116.9 appears, while the known peak of the free acid, BocFPhe, is at δ −117.8. The shoulders on the peaks of BocFPheEP and BocFPhe (Figure 1) are mainly due to aggregation during spectra acquisition or incorporation of deuterium in the compound.

Aminoacylation of Nucleotides. Reactions of 5′CMP were also evaluated. Reactions of 5′CMP in 100 mM pH 8 EPPS were complete, as determined by the complete consumption of BocFPheEP, in 40 min, and only one ester resulted. In contrast, reactions conducted at pH 6.5 were not complete after 60 min, consistent with the reaction being specific base catalyzed (Figures 4 and 5). The products were isolated by preparative

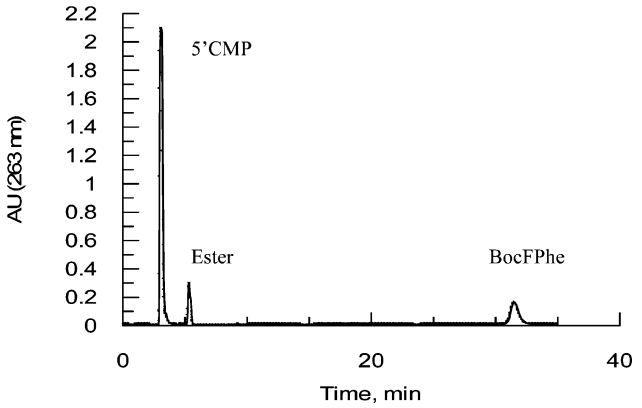


Figure 4. Aminoacylation of 5′CMP at room temperature. HPLC chromatograms at 60 min of reaction in 100 mM EPPS pH 8.

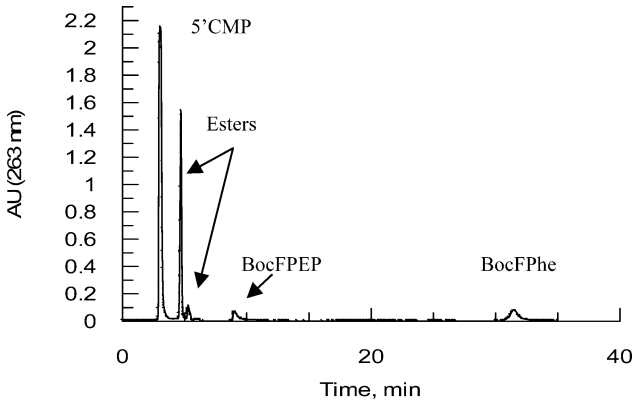


Figure 5. Aminoacylation of 5′CMP at room temperature. HPLC chromatograms at 60 min of reaction in 10 mM EPPS, initially pH 8, then lowered to pH 6.5.

HPLC and analyzed by MS ESI (−); *m*/*z* calculated = 588.15, found = 587.2, indicating that only monoesters of 5′CMP are formed. ^{1}H NMR analysis revealed that the products are the 2′- and 3′-*O*-monoesters of 5′CMP. There is a shift in the signal of the 1′ proton of the furanose ring that varies with the location of the ester (2′ vs 3′). The 2′-ester signal appears as a doublet at δ 6.14 while the 3′-ester signal appears as a doublet at δ 5.80. The esters rapidly equilibrate, with peaks from both esters observed in the ^{1}H NMR spectrum from an initial sample of either ester. The ^{19}F NMR spectrum of the reaction after 1 h indicates a new peak at δ −116.98 (Figure 6), which is distinct from the products of the hydrolysis reaction (Figure 7). ^{19}F NMR of the esters alone in the absence of La^{3+} and buffer (after HPLC purification) gives a signal at δ −117.15. Further analysis of the same product by ESI MS and ^{1}H NMR identifies the material as a mixture of the 2′- and 3′-esters of 5′CMP.

In order to estimate the likely ^{19}F NMR chemical shift of the ribose ester products, we prepared the isopropyl ester of BocFPhe. The chemical shift of the peak for the ester is at δ −118.9, which is close to those signals assigned to the esters from the nucleic acid derivatives.

Aminoacylation of RNA. Reactions with yeast $tRNA^{Phe}$ were conducted using conditions as described for nucleotides. The materials could not be analyzed by NMR due to the small amounts (concentrations in the nano- to micromolar range). In our experience, product analysis by ^{19}F NMR requires at least millimolar concentrations. In order to work at such concentrations for product analysis, we used a readily available mixture

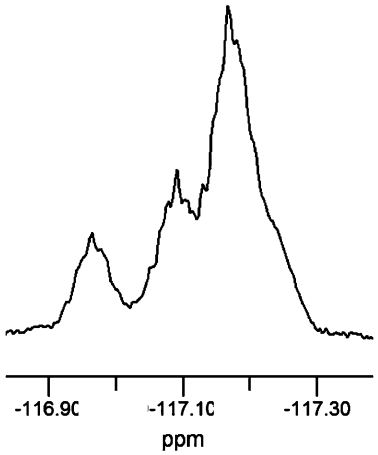


Figure 6. ^{19}F NMR (376 MHz, D_2O) of the aminoacylation reaction solution (30 mM $LaCl_3$, 5′CMP, and BocFPheEP, 34 mM buffer pH 8) after 1 h.

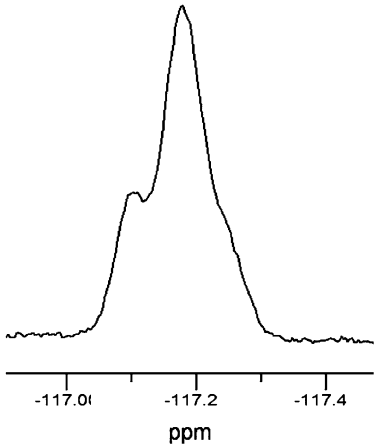


Figure 7. ^{19}F NMR (376 MHz, D_2O) of the hydrolysis reaction of BocFPheEP under the same conditions at 5 min of reaction as in Figure 6.

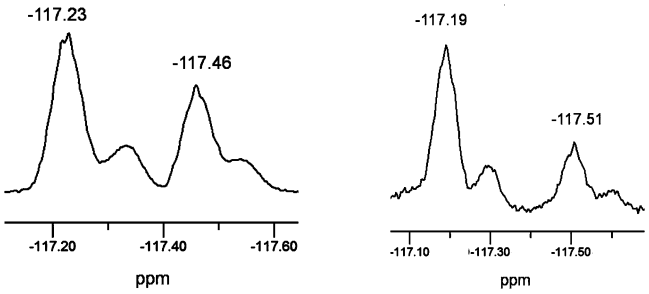


Figure 8. Aminoacylation of bulk RNA with BocFPheEP in the presence of La^{3+}: ^{19}F NMR spectrum of the reaction mixture after 1 h (left) and after purification and dilution (right).

of RNA that contains RNAs of different sizes and types, including tRNAs. As all types of RNA provide a terminal 3′-diol moiety that is required to test the aminoacylation reaction, this provides a test of the process. ^{19}F NMR spectra were recorded after reactions had proceeded for 1 h (Figure 8). Samples were passed through a size-exclusion column (Sephadex G-25) to remove salt, buffer, and starting material (Figure 8). Most of the free amino acid from hydrolysis was removed by filtration before the spectra were recorded. The spectra after purification indicate two large peaks at δ −117.19 and δ −117.51 (Figure 8) in the region where the signals from the model esters were observed, which are also present prior to

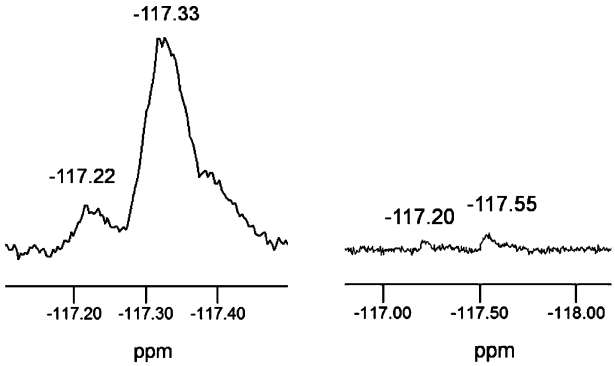


Figure 9. (Left) ^{19}F NMR spectrum of the solution from the reaction of periodate-oxidized RNA with BocFPheEP in the presence of lanthanum chloride after 1 h, prior to isolation of RNA. (Right) the ^{19}F NMR spectrum of the same amount of material after isolation of all RNA species via size-exclusion chromatography. Trace peaks result from slightly exceeding the column's capacity.

purification with the size exclusion chromatography at similar intensities. Thus, we conclude that these are the signals of products resulting from aminoacylation of RNA with BocFPheEP.

The chemical shifts of the NMR signals corresponding to the products are similar to those of the BocFPhe-derived esters of nucleosides and nucleotides. In order to test the requirement for the terminal diol for aminoacylation, the subject RNA was treated with excess $NaIO_4$ to oxidize and cleave the 3′-terminal diol. We assume that the reaction goes close to completion. The oxidized RNA was combined with BocFPheEP and lanthanum chloride in buffer containing excess magnesium chloride. The hydrolysis product was removed as a precipitate by filtration. The ^{19}F NMR spectrum of the products after 1 h of reaction time (Figure 9) was obtained by addition of 0.3 mL of D_2O to 0.2 mL of the mixture that had been purified through a Sephadex G-25 spin column.

The initial spectrum consists of peaks that correspond to residual BocFPheEP and its hydrolysis product, BocFPhe. The material analyzed after purification by size exclusion chromatorgraphy has a ^{19}F NMR spectrum that consists of only two very small peaks with chemical shifts of BocFPheEP and BocFPhe, indicating that no ester formation occurs (Figure 9). Thus, oxidation converts RNA to a species that is predictably unreactive under conditions where ester formation occurs readily with material that has not been oxidized. These results strongly support a conclusion that reaction occurs at the terminal diol in the native RNA sample.

Another aspect of this study was the effect of the specific lanthanide ion on the acylation reaction. Thus, we evaluated La^{3+}, Pr^{3+}, Nd^{3+}, and Yb^{3+} as well as Sc^{3+} and Mg^{2+} as catalysts. Table 1 represents the percent conversion of 5′CMP with each of these metal cations. While Mg^{2+} has high affinity for phosphates, it does not coordinate effectively to the diol leading to no formation of esters. Some Lewis acid properties of Sc^{3+} compare to those of lanthanides; however, it provides only a trace of ester from 5′CMP. In contrast, all lanthanides effectively promoted formation of the ester with the efficiency of the conversion decreasing with increasing atomic number (Table 1). Specifically, the early lanthanides, exemplified by La and Pr (atomic numbers 57 and 59), are better catalysts for acylation reactions than Yb (atomic number 70). It is the earlier lanthanides that have larger ionic radii (La 103 pm, Pr 99 pm,

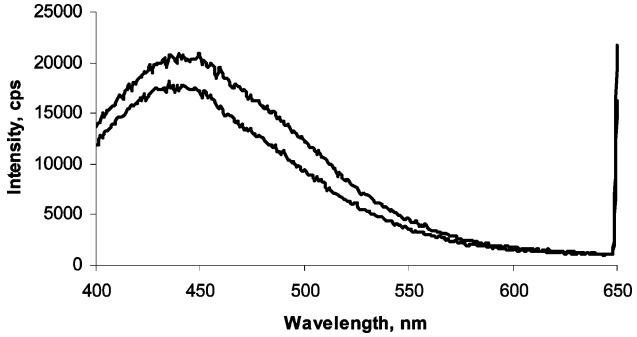


Figure 10. Emission spectrum of the product of reaction of yeast $tRNA^{Phe}$ with DNSGlyEP and lanthanum triflate at pH 8 in absence (lower) and presence (upper) of a DNS-specific antibody.

Table 1. Relative Conversion of 5′CMP to Esters of BocFPhe as Promoted by Different Metal Ions; the Amounts Are Based on HPLC Peak Integration

ion	atomic number	relative conversion of 5′CMP, %
Mg^{2+}	12	0
Sc^{3+}	21	0.2
La^{3+}	57	31
Pr^{3+}	59	30
Nd^{3+}	60	29
Yb^{3+}	70	21

Nd 98.3 pm). Established aspects of the coordination chemistry of lanthanides suggest that larger ions would coordinate more readily with the diol due to the size of the span between the hydroxyls. Later lanthanides have smaller radii (Yb 86.8 pm), and greater positive charges will make them more selective for phosphates (and not diols). Thus, where the goal is the hydrolysis of the phosphodiester, the later lanthanides are preferred as catalysts.[13]

Specific Reaction with tRNA. While the reaction patterns and analysis with ^{19}F NMR indicate that the reaction has appropriate selectivity with respect to the 3′-terminus of RNA, it is also important to conduct a reaction with a single homogeneous tRNA. The NMR method is not sufficiently sensitive to work with the quantities available for such a test. Therefore, we sought an alternative, more sensitive method. Since fluorescence detection is much more sensitive than NMR, we prepared a fluorescent derivative of an amino acyl phosphate. We reacted dansyl-glycyl ethyl phosphate (DNSGlyEP) with $tRNA^{Phe}$ under the conditions used for the NMR studies but at lower concentration levels and isolated the modified tRNA. We also oxidized the tRNA with periodate and ran the aminoacylation conditions as a control. We isolated both products and tested for the specific effect of presence of the dansylated tRNA with a dansyl-specific antibody, which serves as a fluorescence enhancer (details in the Materials and Methods). With $tRNA^{Phe}$ we observed the expected enhancement (Figure 10), whereas with $tRNA^{Phe}$ that has first been oxidized we see no significant enhancement. Since the dansylamide is stable under the reaction conditions, the source of the fluorescence is necessarily the aminoacylated tRNA. Using the same control as that in the NMR experiments, specific oxidation of the 3′-terminus with periodate converts the 1,2-diol to a dialdehyde that should not chelate

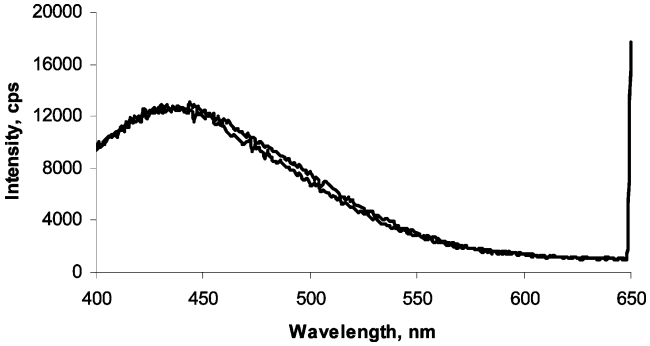


Figure 11. Emission spectra of the products from reaction of periodate-oxidized yeast $tRNA^{Phe}$ with DNSGlyEP and lanthanum triflate in the absence and presence of anti-DNS antibody.

lanthanum. Using the same reaction conditions, the lack of antibody enhanced fluorescence (Figure 11) in comparison with the untreated sample shows that the principal site of aminoacylation is the 3′-terminus of $tRNA^{Phe}$.

Discussion

These results show that lanthanum salts effectively direct aminoacylation by aminoacyl phosphate esters to the 3′-terminal of tRNA in direct analogy to the biochemical process that is accomplished by enzymic binding. Since the diol and the acyl phosphate functional groups are the coordinating entities, it appears that the method will extend to a large variety of aminoacyl phosphates and RNA. (We also find that amino group protection is not necessary for the reaction of the acyl phosphate, although the protected material will last for a considerably longer time in storage. These results are currently the subject of extended studies.)

Earlier reports that lanthanum ions in water will preferentially promote transfer of the acyl group of an acyl phosphate ester to one hydroxyl of a diol[14,15] implied that an aminoacyl phosphate would be able to react selectively with the only common diol in RNA, which is at the 3′-terminus. By exploiting this unique feature and the reagent's specificity, we have now shown that the reaction can serve as a basis for 3′-terminal specific aminoacylation of RNA.

All reactions were performed in aqueous solution, and products from hydrolysis of the aminoacylating reagent as well as esters were formed. In the presence of a lanthanum ion, the hydrolysis is very fast if no other ligand/nucleophile other than water is present. However, when nucleosides or nucleotides were introduced, mainly ester products were obtained. In the absence of the metal ion, hydrolysis is slow and acylation does not occur.

We observe that aminoacylation of nucleosides with aminoacyl alkyl phosphates and a lanthanum ion occurs rapidly. In all cases in our study, two monoacylated products were obtained in the reaction with nucleosides. Control studies in our laboratory with ribose, purines, and pyrimidines confirm that there is neither formation of an ester derived from the 5′ hydroxyl nor an amide formed from reaction of an amino group of the heterocyclic base. This is consistent with coordination of lanthanum for reaction exclusively with the terminal 2′ and

(13) Komiyama, M.; Takeda, N.; Shigekawa, H. *Chem. Commun.* **1999**, 1443−1451.

(14) Evans, C. H. *Biochemistry of Lanthanides*; Plenum Press: New York, 1990.
(15) Chargaff, E., Davidson, J. N., Eds. *The Nucleic Acids*; Academic Press: New York, 1955; Vol. 1.

3′ hydroxyl groups. The ratio of 2′- to 3′-ester (1:2) is comparable to the equilibrium ratios reported in the literature.[16] It is expected that complexes of lanthanum with uncharged sugars will not be as strong as those with phosphates.[14] Thus, the reaction occurs rapidly within the ionized bis-bidentate complex because the overall association of lanthanum with the diol is weak enough to permit the hydroxyl to alter its coordination and attack the acyl group of the coordinated acyl phosphate.

A large part of the effect of coordination to lanthanum comes from the high effective concentration of ionized hydroxyl groups that is achieved. The pK_a of the OH groups on the ribose is approximately 12.5.[15] Water bound to lanthanum has a first pK_a of about 7.[17] As well, coordination directs nucleophilic reaction of the alkoxide to the carbonyl carbon of the acyl phosphate (see Scheme 1). Consistent with the apparent importance of ionization is our observation that at pH 8 and 6.5 the regiospecificity (terminal 2′ vs terminal 3′) and the extent of ester formation from a nucleotide (5′CMP) are different. At higher pH, a greater proportion of the lanthanum-coordinated water is deprotonated so it can act as an intramolecular base catalyst. This promotes esterification as well as the hydrolysis of the aminoacyl phosphate. At lower pH, more ester products (compared to hydrolysis products) are produced.

Based on these very promising results, we envision the work extending in several directions that take advantage of the RNA terminal ester specificity of these reactions. For example, in order to be able to work at even lower concentrations, the fluorescently labelled aminoacyl phosphates will assist in detecting the incorporation of acyl phosphate derivatives of amino acids at N-termini of proteins. We also expect that there will be other applications that utilize our general method for preparing 3′-terminal esters of RNA.

Acknowledgment. We thank Steven Rathgeber for his assistance and discussions. The Natural Sciences and Engineering Research Council of Canada provided support through the Discovery Grants program.

JA073976L

(16) Griffin, B.; Jarman, M.; Reese, C.; Sulston, J.; Trentham, D. *Biochemistry* **1966**, *5*, 3638−3649.
(17) Burgess, J. *Metals in Solution*; Ellis Horwood Limited: New York, 1978; pp 259−289.