Lanthanum-mediated Biomimetic Aminoacylation

by

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Abstract

Methods are being developed to produce "designer proteins" from unnatural amino acids that are added into specific locations by the ribosome using an altered mRNA. To date, over seventy unnatural amino acids have been incorporated at specific sites in proteins by *in vitro* biosynthetic methods using chemically acylated-tRNAs and *in vivo* protein mutagenesis based on orthogonal tRNA/aminoacyl-tRNA synthetase pairs.

Lanthanum-mediated aminoacylation of cis-diols provides a general and selective method for the one-step preparation of aminoacyl-tRNA. The nature of this biomimetic process was studied for the reaction of ribonucleosides and nucleotides with *N-t*-Boc-protected aminoacyl ethyl phosphates. Successful aminoacylation was also achieved with unprotected aminoacyl ethyl phosphates. This method was extended for the aminoacylation of tRNA and analyzed by reversed-phased HPLC and MALDI-MS. These results will provide an insight to the ultimate goal of lanthanum-mediated direct acylation of tRNA and its applications in *in vitro* site-specific incorporation of unnatural amino acids.

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General Introduction

1.1. Biochemical synthesis of protein

In nature, peptides and proteins are produced using the twenty natural amino acids in genetically determined sequences. Biochemical synthesis of proteins begins in the nucleus, where the genetic information encoded as deoxyribonucleic acid (DNA) is transcribed into messenger ribonucleic acid (mRNA) as a series of three-letter codons. According to the mRNA sequence, transfer RNA (tRNA) delivers specific amino acids into the ribosome to form a polypeptide chain (Figure 1). Newly synthesized polypeptides then undergo post-translational modifications to become functional and catalyze various biochemical processes.[1]
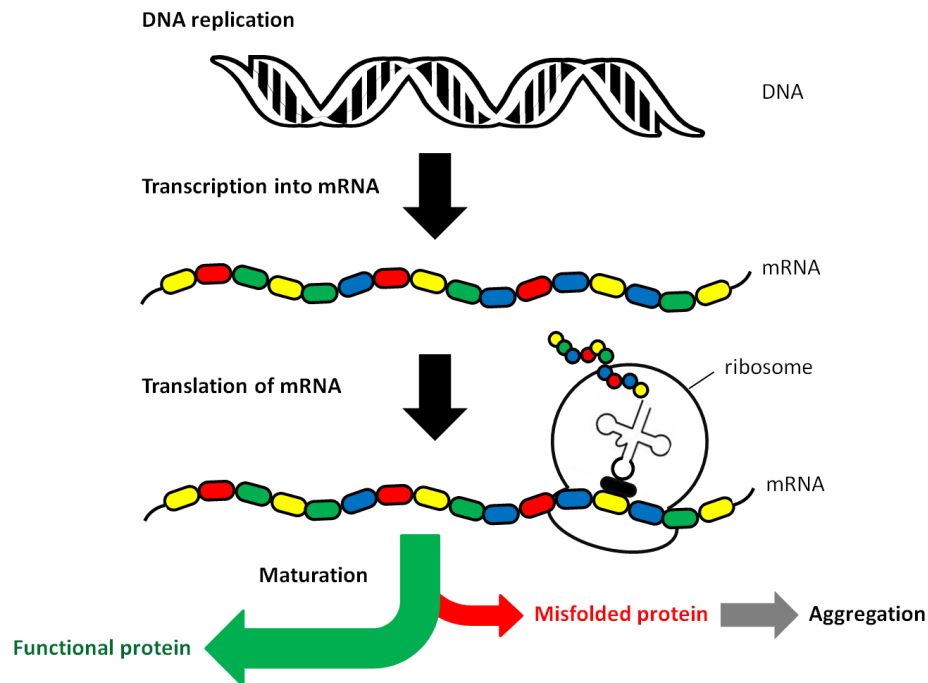
Figure 1. Biosynthesis of proteins.

1.1.1. Aminoacyl-tRNA and aminoacyl-tRNA synthetase

In the translation step of protein synthesis, the amino acid to be incorporated into the polypeptide sequence is determined by the binding of tRNA anti-codon to the corresponding 3-letter codon on mRNA.[2] Since this process does not involve any interaction between the amino acid and mRNA, the key step in maintaining the accuracy of protein translation is the formation of aminoacyl-tRNA, also known as the "charging" of tRNA.

The aminoacylation of tRNA is catalyzed by a family of enzymes known as aminoacyl-tRNA synthetases (aaRS). Each aaRS is responsible for charging a tRNA with its cognate amino acid, and can be divided into two classes that differ in their topology, binding of tRNA and the position of aminoacylation. Class I enzymes, which exist as monomers, transfer the amino acids to the 2'-hydroxyl group of the terminal ribose of the substrate tRNA. On the other hand, class II enzymes exist as dimers and aminoacylate the 3'-hydroxyl group of tRNA, with the exception of PheRS.[3,4] Transesterifcation facilitates the transfer of aminoacyl group to the 3'-hydroxyl group, which allows all aminoacyl-tRNA involved in protein synthesis to be aminoacylated at the 3'-hydroxyl position.[3]

The enzymatic aminoacylation of tRNA occurs in two steps. In the first step, the amino acid is activated by reacting with an ATP molecule to form the aminoacyl adenylate intermediate. In the second step, the amino acid is transferred to the terminal hydroxyl group of tRNA form an aminoacyl-tRNA (Figure 2).[3-5]
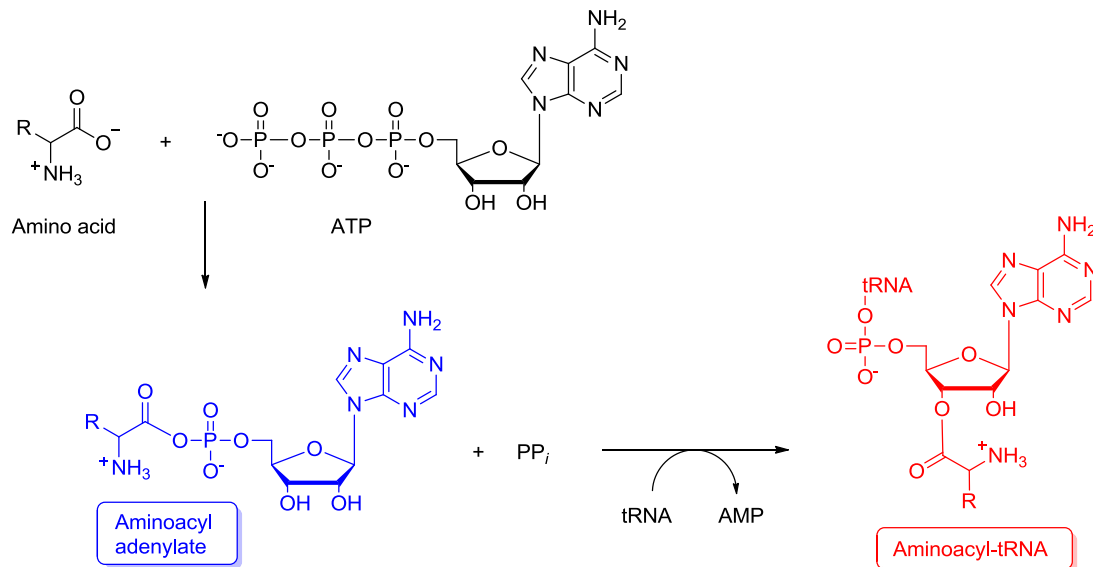
Figure 2. Formation of aminoacyl-tRNA as catalyzed by aaRSs.

Mechanistic analysis of aaRSs demonstrates that the enzyme catalyzes aminoacylation by orienting the two substrates in reactive proximities. An array of hydrogen bonding interactions aligns the enzyme-bound aminoacyl adenylate and the terminal hydroxyl group of tRNA in optimal positions for nucleophilic attack to form the aminoacyl-tRNA (Figure 3).[5]
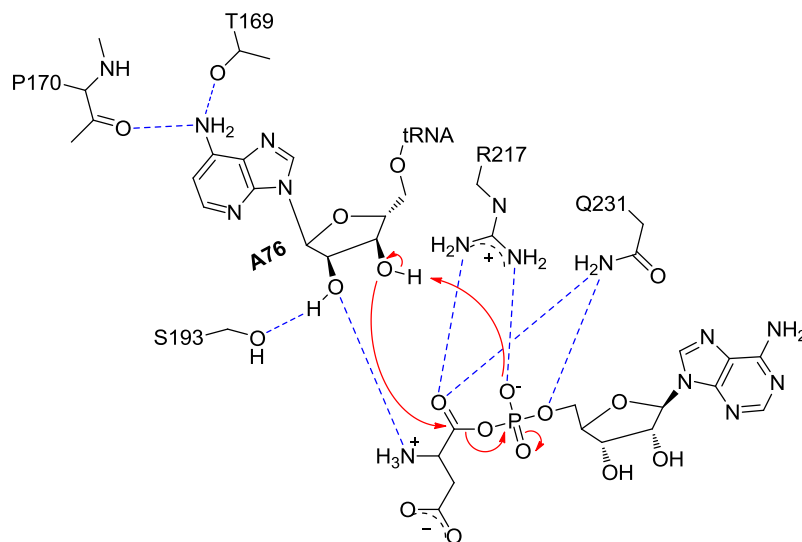
Figure 3. Proposed mechanism for the aminoacylation of tRNA in class II aaRS.

Aminoacyl-tRNA synthetases maintain high accuracy and specificity by utilizing a "double sieve" mechanism which discriminates the cognate amino acid from others that are structurally or chemically similar.[7] For most amino acids with unique side chains, recognition by aaRS is simple. However, for amino acids with similar structures, such as valine and isoleucine which differ by one methylene group, additional discrimination is required. In the "double sieve" mechanism first proposed by Fersht, it is suggested that the enzyme binds the amino acids that are similar in size or smaller than the cognate amino acid, and those larger in size will be rejected. In the second step, smaller amino acids are selectively bound and hydrolyzed in the editing domain. This two-step editing mechanism ensures that aaRSs maintain a highly accurate process of aminoacylation, and minimizes the rate of misacylation at approximately 1 in 10,000.[5]

1.1.2. Ribosomal synthesis of protein

Ribosomal protein synthesis proceeds through three steps: initiation, elongation and termination.[1] With the participation of initiation factors, the two ribosomal subunits are assembled with mRNA and fMet-tRNA to form an fMet-tRNA · mRNA · ribosome complex.[1] In this complex, fMet-tRNA is paired with the start codon (AUG) on mRNA, and occupies the P-site (peptidyl-tRNA binding site) to signal initiation. The aminoacyl-tRNA bearing the anticodon complementary to the mRNA codon then enters the A-site (aminoacyl-tRNA binding site). Formation of a peptide bond between the two amino acids results in transpeptidation, in which the peptidyl group on the P-site tRNA is transferred to the A-site tRNA (Figure 4). The free tRNA is released into the cytoplasm through the E-site (exit site), and the peptidyl-tRNA in the A-site is translocated to the P-site.[8] Elongation process continues until the protein of desired length is obtained. Binding of one of the three stop codons, amber (UAG), opal (UGA) or ochre (UAA), signals the hydrolysis of peptidyl-tRNA mediated by release factors. Once the newly synthesized polypeptide is released from the ribosome, the free tRNA and mRNA are also released to yield an inactive ribosome ready for another cycle of protein synthesis.
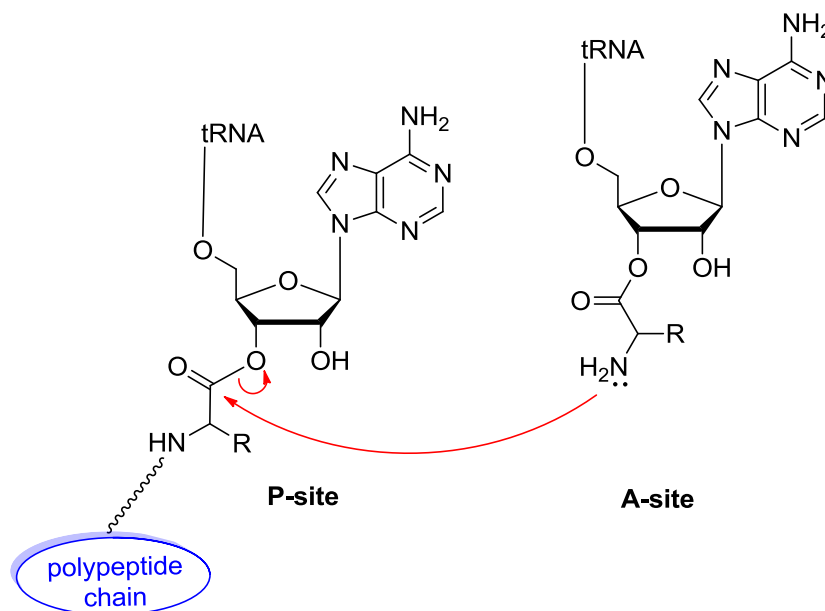
Figure 4. Peptide bond formation in the ribosome.

1.2. Incorporation of unnatural amino acids (UAA)

In nature, protein synthesis is limited by the genetic code to use only the twenty natural amino acids. However, using unnatural amino acids, which are not specified by the genetic code, it is possible to generate proteins with novel properties. Site-specific incorporation of unnatural amino acids allows the introduction of side chains that can be utilized as biophysical probes for the control of cellular functions and selective modification of proteins (Figure 5).[9]

Chemically reactive side chains   Photoreactive side chains

Fluorescent side chains   Heavy atom side chains   Metal chelating side chain
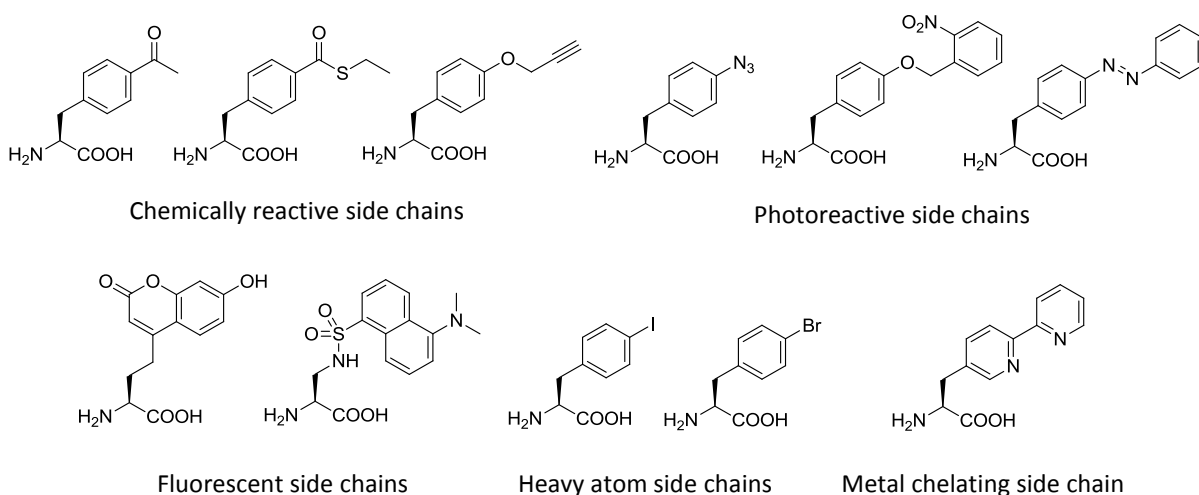
Figure 5. Selected examples of unnatural amino acids and their novel properties.

1.2.1. Chemical synthesis of proteins containing UAAs

Solid phase peptide synthesis (SPPS), which was pioneered by Merrifield in 1963, has been widely used to prepare peptides and small proteins containing both natural and unnatural amino acids.[10] In SPPS, the first amino acid is covalently linked onto a bead. Following the deprotection of the α-amino group, second pre-activated amino acid is added. This process of coupling and deprotection is repeated until the desired peptide is obtained, and the final deprotection releases the full peptide from the beads (Figure 6).
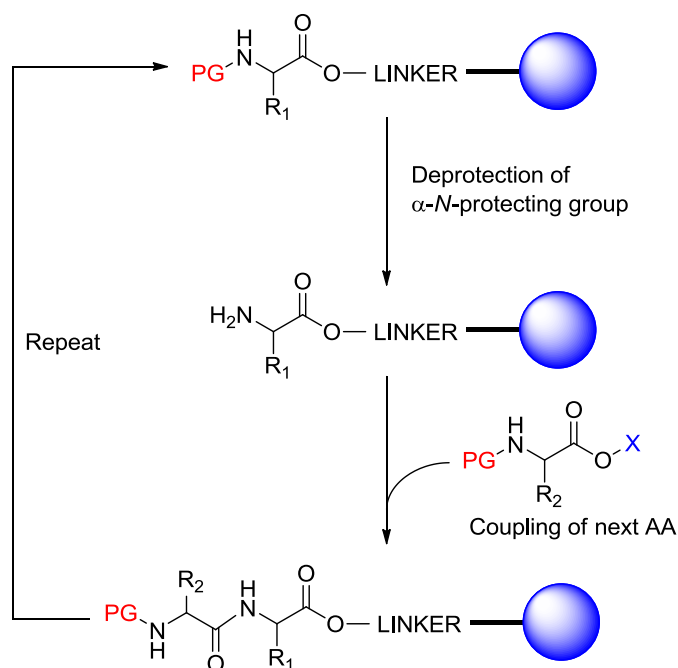
Figure 6. Simplified flowchart for the solid phase peptide synthesis.

SPPS has an added advantage in that the process can be automated. However, it is limited by the size of peptide that can be produced. Due to the solubility issues arising from longer peptides and the step-wise nature of this technique, SPPS is limited to producing peptides that are less than 50 residues in length as each cycle reduces the overall yield of the peptide. For example, if each cycle of amino acid coupling results in a 99 % yield, the overall yield drops to approximately 60 % after 50 cycles, and 37 % after 100 cycles. In order to overcome some of these limitations, approaches have been developed based on ligation of independently produced oligopeptides.[11,12]

Ligation methods allow the formation of larger proteins containing unnatural amino. In enzymatic ligation, chemically synthesized peptides are treated with proteolytic enzymes under conditions favouring aminolysis over hydrolysis. Proteases can be used to ligate a peptide with a C-terminal ester and a second peptide with an N-terminal amino group in an organic co-solvent system.[13] Mutant enzymes with reduced rates of proteolysis can be generated to improve the method to allow ligation to be used in aqueous media.[14,15]

Chemical ligation methods, such as native chemical ligation and expressed protein ligation, take advantage of the cysteine side chain to form a native peptide linkage between two

synthetic peptide chains or a peptide with a recombinant protein. A peptide with an N-terminal cysteine undergoes transthioesterification with a second peptide bearing a C-terminal thioester. Spontaneous S→N acyl transfer results in a ligated protein containing the native peptide linkage.[16] Expressed protein ligation extends this method for the formation of larger proteins by generating a recombinant protein that contains a C-terminal α-thioester (Figure 7).[17,18]
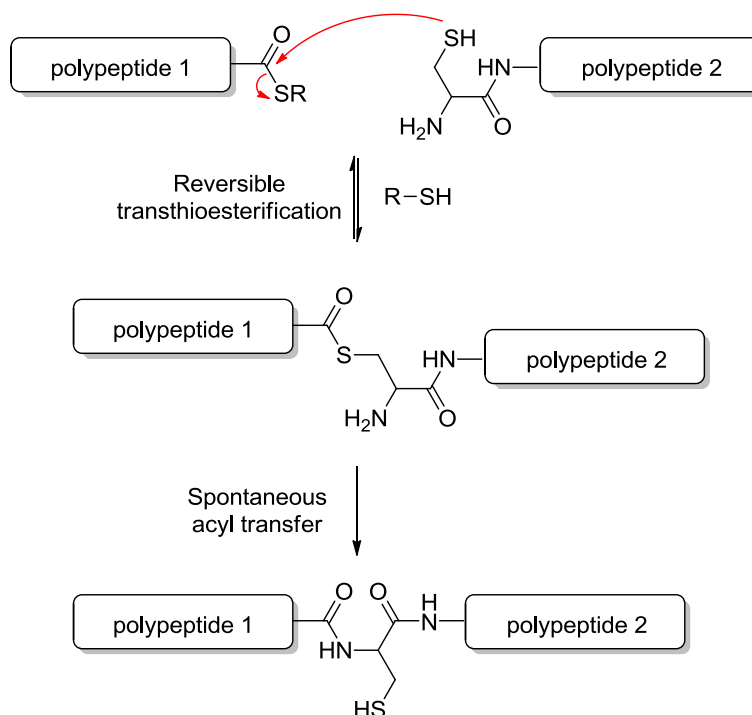
Figure 7. Schematic representation of native chemical ligation.

Although ligation methods provide an alternative approach to generating larger peptides and proteins, there are still limitations to these methods. The restriction on the site of cleavage and ligation limits utility in the site-specific incorporation of unnatural amino acids. In addition, the extracellular nature of the synthetic techniques poses difficulty for *in vivo* studies of protein structure and function.

1.2.2.   *In vitro* biosynthesis of proteins containing UAAs

Various *in vitro* (cell-free) methods have been developed in order to overcome the challenges of the synthetic preparation of proteins that contain unnatural amino acids. During the translation process, the amino acid to be inserted into the polypeptide chain is determined by the interaction between the mRNA codon and the tRNA anticodon. Since the binding interactions are independent of the identity of the amino acid attached to the tRNA, this property can be exploited to incorporate unnatural amino acids into the protein using a misacylated tRNA. Utilizing the biosynthetic machinery is advantageous since proteins of all sizes can be generated, and it provides access to the natural processing system for proper folding and post-translational modifications. However, "hijacking" the biosynthetic machinery is not trivial. To incorporate an unnatural amino acid using the biosynthetic machinery, two requirements have to be fulfilled: there must be a codon specifying the insertion of unnatural amino acid and there must be a method for aminoacylating tRNA with the unnatural amino acid.[19]

In the standard genetic code, there are 64 three-base codons of which 61 sense codons encode the addition of 20 natural amino acids into the polypeptide chain.[1] The remaining three codons are the stop codons that signal the termination of polypeptide chain by the addition of release factors.[20] Of the three degenerate stop codons, only the opal codon (UGA) occurs most frequently. Since only one stop codon is required to terminate the polypeptide chain formation, the remaining two stop codons can be used to code for unnatural amino acids. The amber codon (UAG) is the least-used stop codon in *E. coli*; therefore, it is frequently selected for unnatural amino acid incorporation.

Chemical and enzymatic approaches toward developing a method for the misacylation of tRNA have been sought. However, direct chemical acylation at the terminal hydroxyl group of tRNA is challenging due to the complex nature of tRNA with multiple reactive sites. In addition, aaRSs cannot be used directly to generate aminoacyl-tRNAs with unnatural amino acids due to their high specificity. Therefore, semi-synthetic methods involving the enzymatic ligation of acylated dinucleotide with a truncated tRNA have been developed by Hecht and co-workers.[21-23] In this method, a chemically synthesized dinucleotide (pCpA) is acylated in a multistep process with an activated α-*N*-protected amino acid. Using T4 RNA ligase, the synthesized 2'(3')-*O*-

acylated pCpA is ligated to a truncated tRNA that is lacking the terminal dinucleotide. In the final step, protecting groups are removed to obtain aminoacyl-tRNA.

Schultz and co-workers further improved this synthetic method using 2'-deoxycytidyl-adenosine-monophosphate to simplify the synthesis.[24-26] This dinucleotide is monoacylated at the 2'- and 3'-hydroxyl groups using NVOC-protected cyanomethyl ester of amino acid. Following the ligation with T4 RNA ligase and deprotection by photolysis, they obtained aminoacyl-tRNA with anticodon corresponding to the amber codon for applications in *in vitro* translation (Figure 8).
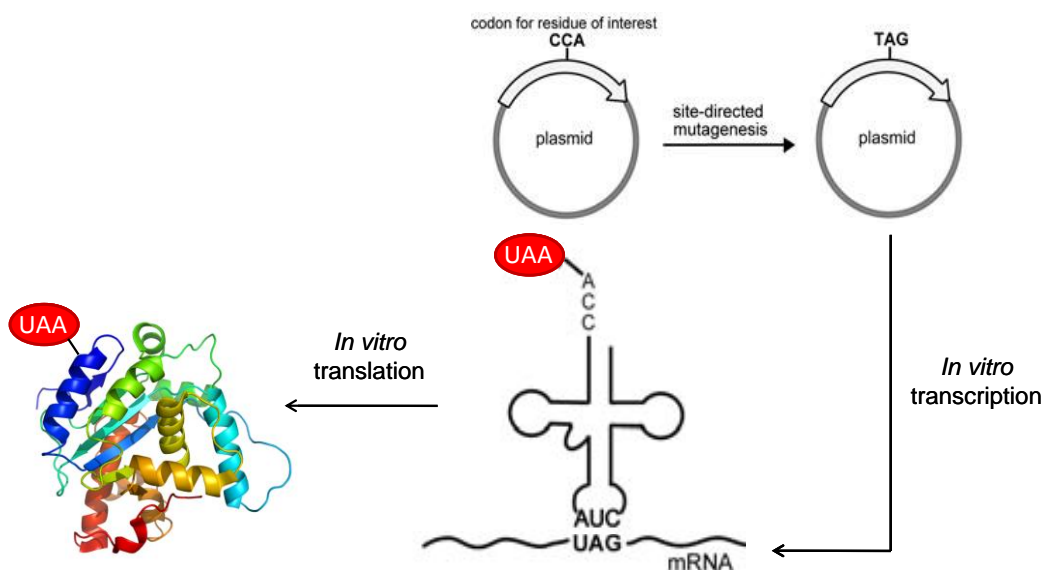
Figure 8. An *in vitro* biosynthetic method for the site-specific incorporation of unnatural amino acids (UAA) into proteins.

Unnatural amino acid mutagenesis using chemically acylated tRNA has been extended to express proteins in *Xenopus* oocytes. Dougherty and co-workers microinjected the mutant mRNA and a chemically aminoacylated tRNA into *Xenopus* oocytes.[27] The translational machinery of the oocyte is used to insert the unnatural amino acid into the site specified by the amber suppressor codon.[28] This method has been used to express integral membrane proteins containing fluorinated derivatives of tryptophan and tyrosine in the active site to study their structure-function relations.[29-32]

Using chemically misacylated suppressor tRNAs, over 50 different amino acids and analogues have been incorporated site-specifically into proteins with high fidelity.[9] Although

Hecht's pioneering work in chemical misacylation has opened possibilities for applications in peptide synthesis, there are many drawbacks to this method. First of all, the synthesis of aminoacyl dinucleotides is challenging with several protection and deprotection steps. This process is not only labour intensive, but the overall yield is also very low. The synthetic difficulties make this method cost-ineffective and inapplicable for large scale preparations. Secondly, the misacylated tRNAs are susceptible to hydrolysis by the editing domain of the endogenous aaRS. Once the misacylated tRNA is deacylated in solution, it cannot be used to incorporate unnatural amino acid; therefore, it is crucial that the misacylated tRNA is orthogonal to the organism's endogenous system. Furthermore, due to the extracellular nature of the process, it is not possible to utilize these proteins to investigate their cellular functions.[9,12]

1.2.3.   *In vivo* mutagenesis of proteins containing UAAs

As mentioned previously, the enzymatic aminoacylation of tRNA is a highly specific process. This property restricts the direct use of aaRS in the misacylation of tRNA. However, it was suggested that there is flexibility in the enzyme's ability to discriminate the natural amino acids from those that are structurally similar. Over fifty years ago, it was observed that many analogues of the twenty natural amino acids inhibit the growth of bacteria, which suggested that the amino acid analogues are incorporated into the protein by biosynthetic pathway.[33]

Tarver and co-workers tested this hypothesis by feeding ^{14}C-labeled ethionine into rats and protozoa. They detected radioactivity in the isolated peptides and proteins, which supported the proposal that the unnatural amino acids are being misincorporated into proteins.[34-37] based on this observation, Cohen and co-workers used an *E. coli* strain that is auxotrophic for phenylalanine, and cultured in a media that is deficient in phenylalanine but abundant in fluorinated phenylalanine. They obtained proteins that contain fluorinated phenylalanine at sites encoded for phenylalanine insertion. These experimental observations provided support for the potential applications of wild-type aaRS for the aminoacylation of tRNA with unnatural amino acids.[38]

Tirrell and co-workers further investigated this feature to incorporate more analogues using wild-type aaRS. By overexpressing MetRS in *E. coli* in a media that is deficient in methionine, they were able to incorporate saturated and unsaturated analogues of methionine.[39-41] They further improved this method by mutating aaRS to impair the proofreading activity. Using

the mutant aaRS, they were able to incorporate various analogues of leucine with LeuRS.[42] Using the wild-type and mutated aaRS, it was shown that biosynthetic machinery can indeed be used for *in vivo* incorporation. Nevertheless, this method is not site-specific and is only applicable to unnatural amino acids that are close analogues of the canonical amino acids.

Schultz and RajBhandary overcame this limitation by generating a unique pair of suppressor tRNA and aaRS that is orthogonal to the endogenous system.[9,43-45] The orthogonal pair of tRNA and aaRS can be used to incorporate unnatural amino acid by utilizing the biosynthetic machinery without being recognized by the host system. Several requirements must be met for this process. The tRNA cannot be recognized by the endogenous aaRS, and can deliver unnatural amino acid in response to a unique codon that does not encode any of the twenty natural amino acids. The aaRS aminoacylates the cognate tRNA but does not aminoacylate any of the endogenous tRNAs. This aaRS must also aminoacylate the cognate tRNA with only the desired unnatural amino acid and not with endogenous amino acids. Finally, the unnatural amino acid to be incorporated must be non-toxic, can be efficiently transported into the cytoplasm, and cannot be a substrate for the endogenous aaRS (Figure 9).[9]
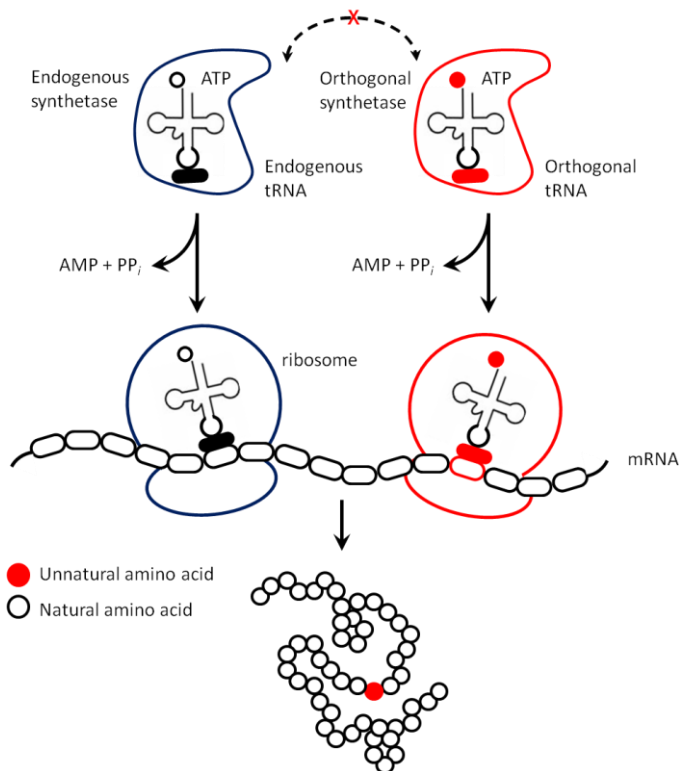
Figure 9. Illustration of unnatural amino acid incorporation by evolution of an orthogonal pair of aminoacyl tRNA and aaRS.

The tRNA and aaRS pair is imported from an archea to further reduce cross-reactivity with the host system. Random mutations to the tRNA and aaRS generate a library of suppressor tRNAs and aaRSs. Through multiple rounds of negative and positive selections, mutant tRNA that is orthogonal to the host synthetases is obtained. Similarly, the library of mutated aaRSs is subjected to a series of positive and negative selections using the unnatural amino acid and the evolved mutant tRNA. The mutant aaRS obtained through directed evolution is selective for the unnatural amino acid and orthogonal to the endogenous tRNA. This approach has been used to encode over 70 unnatural amino acids in *E. coli*, yeast and even in mammalian cells.[9,19]

1.2.4. Other methods of generating aminoacyl-tRNA for incorporation of UAAs into proteins

Another method of successfully aminoacylating tRNA for applications in unnatural protein synthesis is by utilizing engineered ribozymes. Ribozymes are the RNA molecules with well-defined tertiary structures enabling catalysis of chemical reactions. Suga and co-workers generated bifunctional ribozymes for acyl transfer via *in vitro* selection.[46-48] These ribozymes have two domains: acyl transfer (ATRib) domain and tRNA (tR) domain. The acyl transfer domain contains an internal guide sequence (UGGU) to bind both the acyl donor and tRNA at the terminal CCA nucleotides by base pairing. The tRNA domain determines selectivity by base-pairing with the anticodon loop of a specific tRNA.
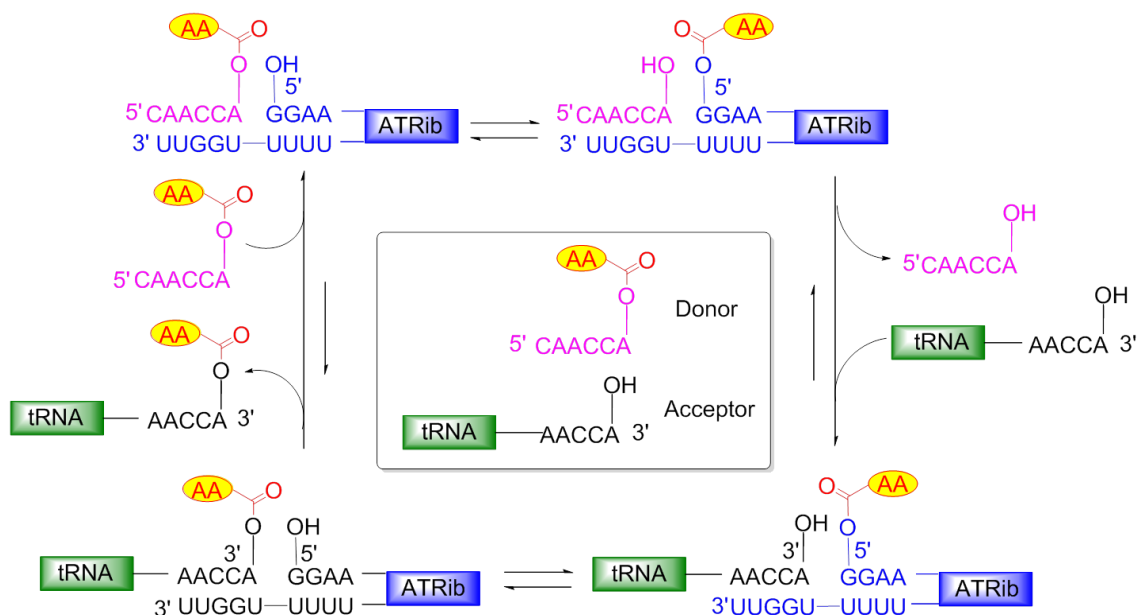
Figure 10. Ribozyme (ATrib) – catalyzed aminoacylation of acceptor tRNA.

Aminoacylation with a ribozyme occurs in a sequential manner (Figure 10). First, the acyl donor, usually a hexanucleotide, binds to the internal guide sequence by base pairing. The acyl group is transferred to the 5'-end of ribozyme to produce the acyl-ribozyme intermediate. The enzyme undergoes a conformational change to release the acyl donor and bind the tRNA terminal trinucleotide (CCA) to the acyl transfer domain. The acyl group is then transferred from the ribozyme to the 3'-terminal hydroxyl of tRNA. Another conformational change releases the aminoacyl-tRNA and binds another molecule of acyl donor to generate more aminoacyl-tRNA.
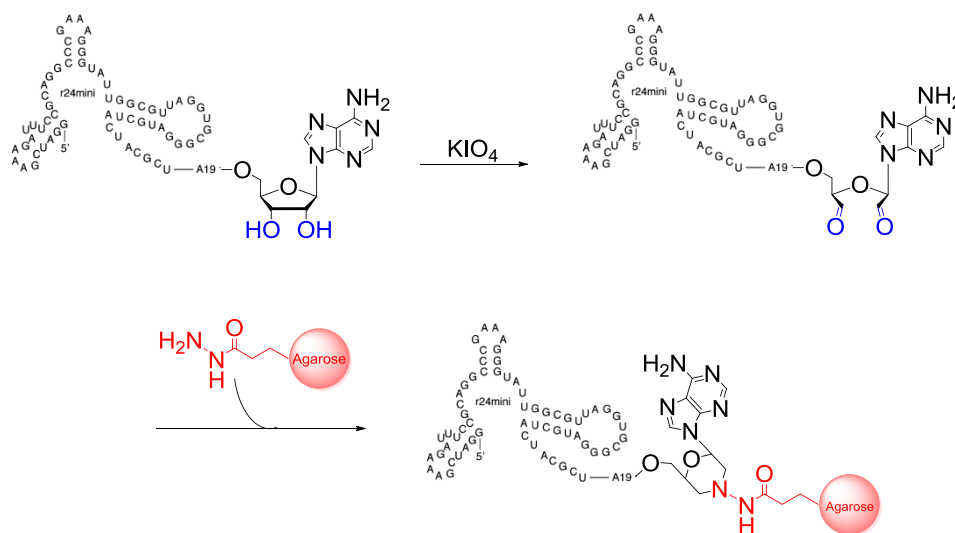
Figure 11. Solid-phase aminoacylation with immobilized ribozyme.

This method has been extended to solid phase aminoacylation to simplify purification methods. Ribozyme is first treated with periodate to oxidize the terminal diol into dialdehyde, and covalently linked to a hydraazide resin (Figure 11).[49] To the immobilized ribozyme, activated amino acid and tRNA is added, incubated and washed to generate aminoacyl-tRNA. The resin can also be recycled to obtain similar efficiencies in each cycle up to five cycles.

1.3. Our approach

Popular approaches to aminoacylating tRNA with unnatural amino acids involve complex chemical synthesis, evolving an enzyme or creating ribozymes with catalytic acyl transfer properties. These aminoacyl-tRNAs can be used to incorporate unnatural amino acids site-specifically into proteins by *in vitro* biosynthetic methods or *in vivo* protein mutagenesis utilizing orthogonal tRNA/aminoacyl-tRNA synthetase pairs.[9,12,19] While these methods have met with considerable success, they are synthetically challenging, low-yielding and are not readily generalized for application to creation of a wide variety of aminoacyl-tRNA monoesters for incorporation into proteins. The objective of our studies is to design a more general approach by chemical catalysis using a reaction that recognizes reaction sites but not side chains. This was realized using a biomimetic process based on lanthanum-catalyzed reactions of acyl phosphate monoesters, compounds that are functional analogues of biological aminoacyl adenylates.[50-52] In the biomimetic system, lanthanum ion functions as an enzyme mimic to selectively aminoacylate the 3'-terminal hydroxyl of tRNA.

1.3.1. Lanthanide-mediated selective acylation of diols with aminoacyl phosphate monoesters

The first step of enzymatic aminoacylation of tRNA is the formation of an aminoacyl adenylate. In this biological intermediate, an amino acid is activated as a mixed anhydride of a phosphate ester. The adenylate portion of the intermediate serves as a structural recognition site for binding to the enzyme and is not involved in the reaction. The aminoacyl alkyl phosphates mimic the biological intermediate by maintaining the activation of amino acid while simplifying the adenylate group with a simple alkyl chain. By utilizing the aminoacyl alkyl phosphates, the activating properties of the aminoacyl adenylate are retained without the complex chemical synthesis.[53,54]

In this biomimetic process, lanthanide ions mimic aminoacyl-tRNA synthetase to catalyze selective aminoacylation of diols.[50,52] Lanthanides are hard Lewis acids with high coordination numbers and form strong coordination with oxygen-containing ligands.[55] In the aminoacylation of diols, lanthanum coordinates to the cis-diol and the aminoacyl phosphate to form a bis-bidentate complex. The formation of this complex lowers the pKa's of the hydroxyl groups and brings the activated amino acid in reactive proximity with the diol. This facilitates the

nucleophilic attack of the hydroxyl group to afford selective aminoacylation. Cameron et al. reported successful monobenzoylation of cis-diols using lanthanide catalysts in water (Figure 12).[50,51] Tzvetkova and Kluger extended the method further to successfully aminoacylate ribonucleosides, nucleotides and RNA with *N-t*-Boc-protected aminoacyl ethyl phosphates.[52]
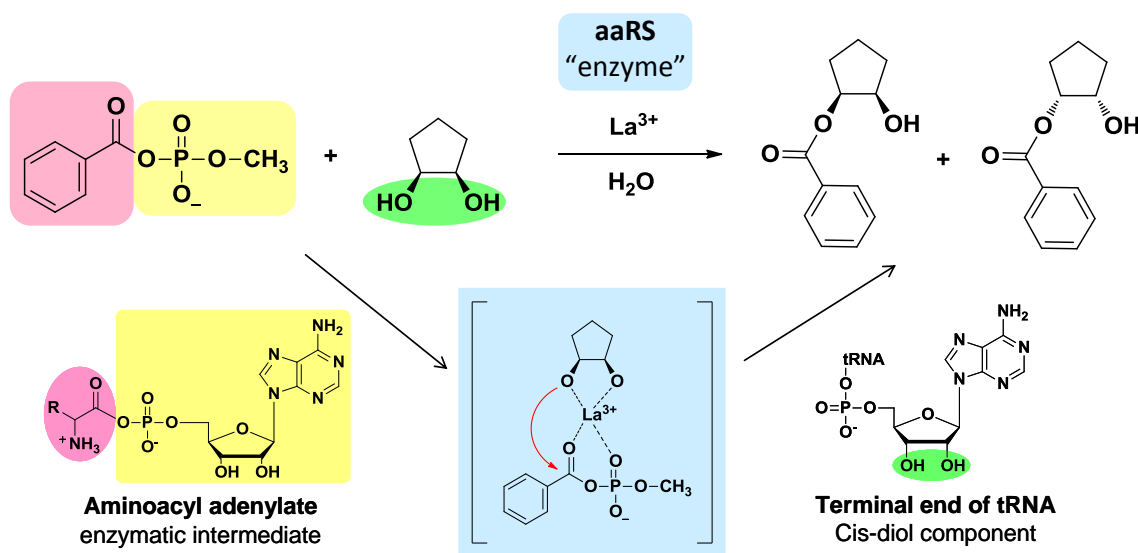
Figure 12. Lanthanum-mediated biomimetic aminoacylation of cis-diol.

1.4. Objectives

The first objective of this work was to test the factors influence lanthanum-mediated aminoacylation. The results that were obtained from this study using *N-t*-Boc-protected aminoacyl and adenosine will provide crucial information in achieving the successful aminoacylation of tRNA. Secondly, aminoacyl alkyl phosphates that are free of protecting group were used in the aminoacylation of ribonucleosides and nucleotides to investigate their applicability in the lanthanum-catalyzed aminoacylation. The use of protecting-group-free reagents would simplify the chemical preparation of aminoacyl-tRNA and allow direct application in the *in vitro* protein expression. Finally, HPLC and MALDI detection techniques were used to quantitatively analyze lanthanum-mediated aminoacylation of tRNA. Previous methods of detecting acylation of tRNA were based on qualitative observations. By applying quantitative analytical methods, it is possible to optimize the lanthanum-mediated aminoacylation of tRNA.

Chapter 2
Synthesis and Characterization of Aminoacylating Reagents

2.1. Introduction

The aminoacylating reagents used in these studies were synthesized following the general method developed by Kluger et al.[54] Phenylalanine was used as the primary choice of amino acid since the aromatic functional group allows UV detection in HPLC analysis. For the protecting group, *tert*-butyloxycarbonyl (*t*-Boc) was selected as it is readily removed without affecting the acyl phosphate. 9-fluorenylmethoxycarbonyl (Fmoc) group was also used to study the effect of bulky protecting group in aminoacylation of tRNA.

Synthesis of aminoacyl alkyl phosphates utilizes tetraethylammonium salts, which dissolves readily in both organic and aqueous solvents. This property allows dicyclohexylcarbodiimide (DCC)-mediated coupling in dichloromethane followed by extraction in aqueous layer for isolation.

The negatively charge *N*-protected and unprotected aminoacyl alkyl phosphates are stable toward hydrolysis in neutral solutions. The first order rate constants for the hydrolysis of *N*-*t*-Boc-phenylalanine ethyl phosphate and unprotected phenylalanine ethyl phosphate are 2×10^{-6} s^{-1} and 1.9×10^{-6} s^{-1} respectively at 25 °C, which correspond to a half-life of approximately 100 hours at pH 7.0.[54] Furthermore, unlike many carboxyl-activated amino acids, which undergo racemization, aminoacyl alkyl phosphates maintain their chiral integrity.[54] *N*-protected and unprotected aminoacyl alkyl phosphates are easy to prepare, resistant to hydrolysis at physiological conditions, and retain their stereochemical integrity. These characteristics make them ideal for aminoacylation reactions in aqueous solution.

2.2   Experimental

2.2.1   Materials and Methods

Unless otherwise noted, commercial reagents were used without further purification. Reagent grade dichloromethane and acetone were dried over anhydrous calcium chloride prior to use. Water was doubly distilled and deionized prior to use. Sep-Pak Plus Silica Cartridges from Waters were used for purification of aminoacyl alkyl or aryl phosphates. High-resolution mass spectrometry was performed at the Advanced Instrumentation for Molecular Structure (AIMS) Laboratory, Department of Chemistry at the University of Toronto. NMR spectra were recorded at 300 MHz (^{1}H), 75 MHz (^{13}C), 282 MHz (^{19}F) and 121 MHz (^{31}P).

2.2.2   Synthesis of bis(tetraethylammonium) alkyl or aryl phosphates

alkyl/aryl dichlorophosphate
(R = Me, Et, Ph)

bis(tetraethylammonium)
alkyl/aryl phosphate
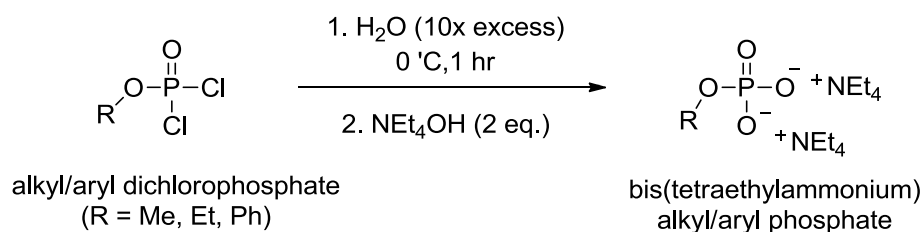
Alkyl or aryl dichlorophosphate (R = methyl, ethyl, phenyl or benzyl) was added dropwise to a ten-fold excess of water in an ice bath. The reaction was stirred for 1 hour, and the hydrochloric acid generated was removed under reduced pressure. The resulting alkyl or aryl phosphoric acid was neutralized with two equivalents of tetraethylammonium hydroxide (40 % w/w), and lyophilized to yield a white paste (85 % yield).

2.2.3   Synthesis of *N-t*-Boc-aminoacyl alkyl or aryl phosphates

N-t-Boc-amino acid

N-t-Boc-aminoacyl alkyl/aryl phosphate
(R = Me, Et, Ph)
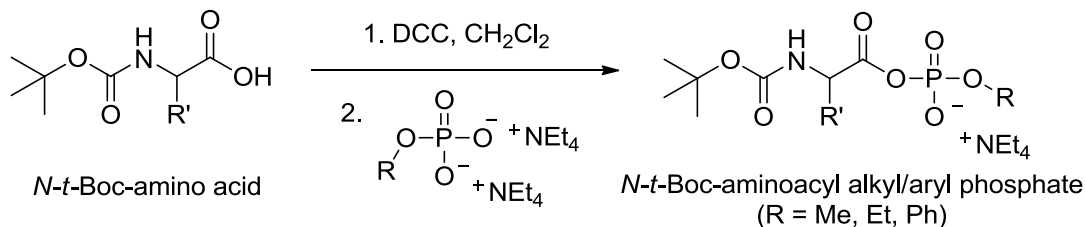
N-t-Boc-protected amino acid (1.2 eq.) was dissolved in dry dichloromethane, and activated with DCC (1.2 eq.) for three minutes. Bis(tetraethylammonium) alkyl or aryl phosphate

(1 eq.) pre-dissolved in dry dichloromethane was added to the mixture, and stirred at room temperature for one to three hours as monitored by ^{31}P-NMR. Dicyclohexylurea formed as the byproduct was removed by gravity filtration. The products were extracted with water and lyophilized. Excess inorganic salt was removed by dissolving the obtained product in dry acetone and eluting through Sep-Pak silica cartridges. *N-t*-Boc-protected aminoacyl alkyl or aryl phosphates were obtained as clear, sticky hygroscopic solids in 50 – 75 % yields.

2.2.4 Synthesis of *N*-Fmoc-aminoacyl ethyl phosphates

N-Fmoc-amino acid

N-Fmoc-aminoacyl ethyl phosphate
(R' = Phe, Ala)
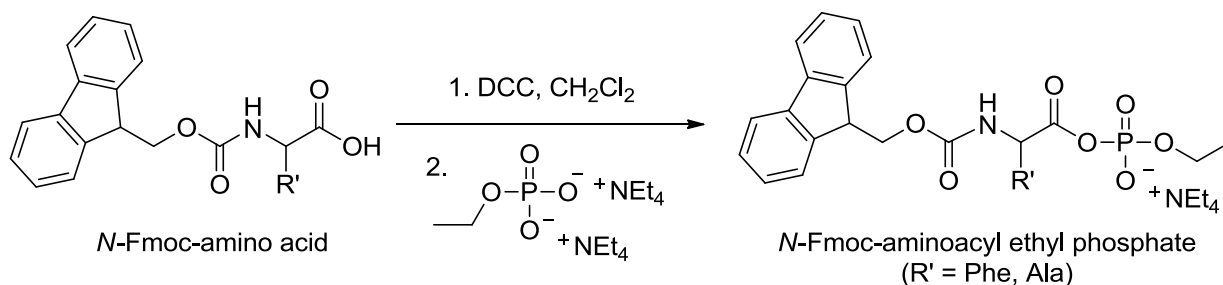
Fmoc-amino acid (1.2 eq.) was dissolved in dry dichloromethane, and activated with DCC (1.2 eq.) for three minutes. Bis(tetraethylammonium) ethyl phosphate (1 eq.) pre-dissolved in dry dichloromethane was added to the mixture, and stirred at room temperature for one hour. Dicyclohexylurea formed as the byproduct was removed by gravity filtration. The product was extracted 4 times with water. Due to the formation of an emulsion, brine was added to facilitate separation. Following lyophilization, the dried product was dissolved in acetonitrile. Brine salt was removed by gravity filtration, and the solvent was removed under reduced pressure to yield yellow, sticky hygroscopic solid (70 % yield).

2.2.5 Deprotection of *N-t*-Boc-aminoacyl ethyl phosphates

N-t-Boc-aminoacyl ethyl phosphate

Aminoacyl ethyl phosphate
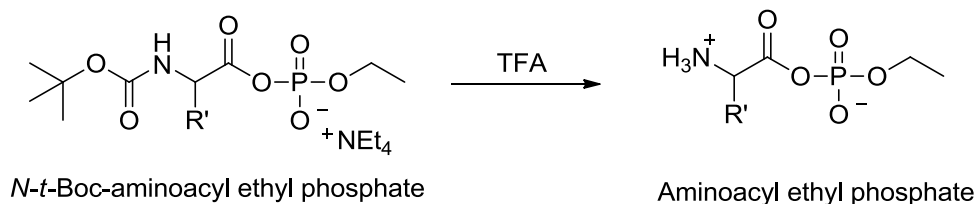
N-t-Boc-protected aminoacyl ethyl phosphate was dissolved in a minimal amount of neat TFA in a round-bottom flask. The reaction was manually stirred until *N-t*-Boc-aminoacyl ethyl phosphate had completely dissolved, and bubbling ceased. TFA was removed by rotary

evaporation at 30 ℃ for 15 min followed by vacuum pumping to form clear oil. To this, ice-cold dry acetone was added and kept at -20 ℃ to precipitate overnight. White precipitate was collected via vacuum filtration, and washed with dry acetone in 25 – 30 % yields.

2.3 Characterization

2.3.1 Bis(tetraethylammonium) alkyl or aryl phosphates

2.3.1.1 Bis(tetraethylammonium) methyl phosphate
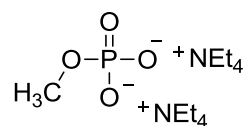
^{1}H NMR (300 MHz, D$_2$O): δ 3.45 (3H, d, OC$\underline{H}_3$), 3.18 (16H, q, $^+$N(C$\underline{H}_2$CH$_3$)$_4$), 1.19 (24H, m, $^+$N(CH$_2$C$\underline{H}_3$)$_4$)

^{31}P NMR (121 MHz, D$_2$O): δ 2.92

2.3.1.2 Bis(tetraethylammonium) ethyl phosphate
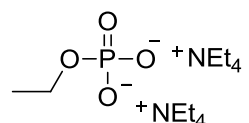
^{1}H NMR (300 MHz, D$_2$O): δ 3.74 (2H, m, OC$\underline{H}_2$CH$_3$), 2.98 (16H, q, $^+$N(C$\underline{H}_2$CH$_3$)$_4$), 1.20 – 1.10(27H, m, $^+$N(CH$_2$C$\underline{H}_3$)$_4$, OCH$_2$C$\underline{H}_3$)

^{31}P NMR (121 MHz, D$_2$O): δ 1.68

2.3.1.3 Bis(tetraethylammonium) phenyl phosphate
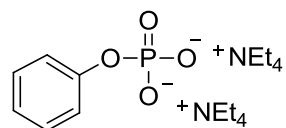
^{1}H NMR (300 MHz, D$_2$O): δ 7.20 (5H, m, Ar), 3.17 (16H, q, $^+$N(C$\underline{H}_2$CH$_3$)$_4$), 1.18 (24H, m, $^+$N(CH$_2$C$\underline{H}_3$)$_4$)

^{31}P NMR (121 MHz, D$_2$O): δ 1.47

2.3.2   *N-t*-Boc-aminoacyl alkyl or aryl phosphates

2.3.2.1   *N-t*-Boc-phenylalanyl methyl phosphate
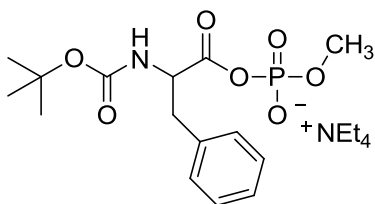
¹H NMR (300 MHz, D₂O): δ 7.27 (5H, m, Ar), 4.5 (m, 1H, CHCO), 3.55 (2H, d, ArCH₂), 3.16 (11H, m, POCH₃, ⁺N(CH₂CH₃)₄), 1.25 (9H, s, C(CH₃)₃), 1.16 (12H, m, ⁺N(CH₂CH₃)₄)

³¹P NMR (121 MHz, D₂O): δ – 6.10

MS ESI (-): calculated m/z 357.35, found m/z 358.10

2.3.2.2   *N-t*-Boc-phenylalanyl ethyl phosphate
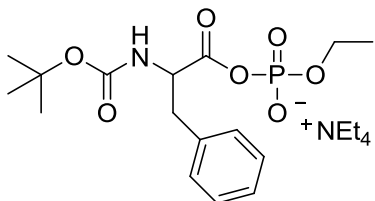
¹H NMR (300 MHz, D₂O): δ 7.2 – 7.3 (5H, m, Ar), 4.5 (m, 1H, CHCO), 4.0 (2H, quintet, ArCH₂), 3.3(10H, m, POCH₂CH₃, ⁺N(CH₂CH₃)₄), 1.3 (9H, s, C(CH₃)₃), 1.2 (15H, m, POCH₂CH₃, ⁺N(CH₂CH₃)₄)

³¹P NMR (121 MHz, D₂O): δ – 6.14

MS ESI (-): calculated m/z 372.1230, found m/z 372.1217

2.3.2.3 *N-t*-Boc-phenylalanyl phenyl phosphate
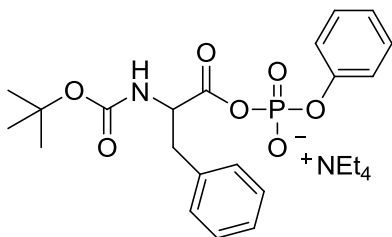
¹H NMR (300 MHz, D₂O): δ 7.28 (10H, m, Phe, Ar), 4.4 (1H, m, C<u>H</u>CO), 3.9 (2H, m, ArC<u>H</u>₂), 3.19 (8H, q, ⁺NC<u>H</u>₂CH₃), 1.25 (9H, s, C(C<u>H</u>₃)₃), 1.17 (12H, m, ⁺NCH₂C<u>H</u>₃)

³¹P NMR (121 MHz, D₂O): δ – 6.16

MS ESI (-) calculated m/z 419.42, found m/z 420.10

2.3.3   Aminoacyl alkyl phosphates

2.3.3.1 Phenylalanyl ethyl phosphate
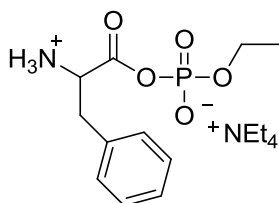
¹H NMR (300 MHz, D₂O):δ 7.3 (5H, m, Ar), 4.4 (1H, C<u>H</u>CO), 3.8 (2H, quintet, POC<u>H</u>₂CH₃), 3.2 (2H, m, ArC<u>H</u>₂), 2.1 (2H, s, N<u>H</u>₂), 1.1 (3H, t, POCH₂C<u>H</u>₃)

³¹P NMR (121 MHz, D₂O): δ -6.48

MS-ESI (+): calculated m/z 272.0693, found m/z 272.0699

Chapter 3
Aminoacylation of ribonucleosides and nucleotides with *N-t*-Boc-protected aminoacyl alkyl or aryl phosphates

3.1   Introduction

Selective monoacylation of ribonucleosides and nucleotides at the 2'- or 3'-hydroxyl group is a challenging task due to the presence of multiple reactive groups. Protection of the exocyclic amine of pyrimidine or purine is required in order to avoid undesired side reactions at these sites. Upon successful acylation, these protecting groups must be removed without affecting the newly installed acyl group. In addition, due to the low solubility of oligonucleotides in common organic solvents, tetraalkylammonium salts are frequently used to assist in dissolution. Several methods of acylating ribonucleosides have been developed. Zemlicka and Chladek generated ribonucleoside-2',3'-cyclic orthoesters via acid-catalyzed reaction of ribonucleoside with an orthoester of an *N*-protected amino acid. They obtained 2'(3')-O-acylribonucleosides by subjecting the ribonucleoside-2',3'-cyclic orthoesters to acid hydrolysis. Similar method was applied to the acylation of 5'-ribonucleotides.[56] Moffatt et al. prepared 2',3'-*O*-(dibutylstannylene)nucleosides to activate the 2'- and 3'-hydroxyl groups towards acylation using acyl chlorides or anhydride in an organic solvent.[57] Hecht et al. synthesized pCpA and *O*-aminoacylated it by reacting with *N*-protected amino acid in the presence of 1,1'-carbonyldiimidazole to afford *N,O*-diacylated dinucleotide. Selective *N*-deacylation was achieved with an inorganic acid.[23]

Generally, acylation reactions cannot be carried out in an aqueous solution due to rapid hydrolysis of activated acylating reagents. However, it is possible to overcome this difficulty by using acyl phosphate monoesters, which are stable against hydrolysis in neutral solution. Acylation of the amino groups of pyrimidine and purine rings is avoided since they are weakly basic and weakly nucleophilic in neutral aqueous solution. Furthermore, selectivity is achieved by using a lanthanum catalyst that coordinates to a cis-diol to form a bis-bidentate complex with an acyl monophosphate ester. Tzvetkova and Kluger have demonstrated successful aminoacylation of ribonucleosides, nucleotides and RNA using *N-t*-Boc-protected aminoacyl

ethyl phosphates.[52] To further understand the nature of lanthanum-mediated aminoacylation, *N-t-*Boc-protected aminoacyl ethyl phosphates were used to investigate the factors affecting these reactions.

3.2   Experimental

3.2.1   Materials and Methods

Unless otherwise noted, commercial reagents were used without further purification. Synthesis of aminoacyl alkyl or aryl phosphates were performed as outlined in Chapter 2.2. HPLC analysis was performed on a C18 reverse phase analytical (Phenomenex, Jupiter 4u Proteo 90A, 250 x 4.60 mm) or semi-preparative column (Phenomenex, Jupiter 10u Proteo 90A, 250 x 10.00 mm), and the products were detected at 263 nm. The mobile phase consisted of 40 % acetonitrile and 0.1 % TFA in water, and was eluted at a flow rate of 1 mL/min for the analytical column, and 3 mL/min for the semi-preparative column. High-resolution mass spectrometry was performed at the Advanced Instrumentation for Molecular Structure (AIMS) Laboratory, Department of Chemistry at the University of Toronto.

3.2.2   Aminoacylation of ribonucleosides and nucleotides

All stock solutions were prepared fresh prior to each reaction. Reagents were added to a 1.5 mL Eppendorf tube in the order of buffer, co-solvent, ribonucleoside/nucleotide, magnesium chloride, BocPheEP and lanthanum. Reactions were stirred at room temperature, and then quenched at various time points with a saturated solution of EDTA-Na in water. Reaction products were separated by HPLC, isolated via lyophilization, and characterized by MS-ESI.

3.3   Results

3.3.1   Aminoacylation of ribonucleosides with BocPheEP

Lanthanum-mediated aminoacylation of ribonucleosides (adenosine, cytidine, uridine) was performed using *N-t-*Boc-phenylalanine ethyl phosphate (BocPheEP). Guanosine was not tested due to the low solubility in aqueous solutions.

The aminoacylation of adenosine, cytidine and uridine with BocPheEP in the presence of lanthanum was successful (reaction conditions: [ribonucleoside] = 10 mM, [La(OTf)$_3$] =

[BocPheEP] = 50 mM, [EPPS] $_{pH\,8.0}$ = 200 mM, 40 % DMSO, 60 min). In the aminoacylation of adenosine, cytidine and uridine, two esters were formed (Figures 13 – 15). They were separated by RP-HPLC and isolated by lyophilization. Characterization by ESI-MS confirmed the formation of two monoesters of adenosine, cytidine and uridine (BocPhe-adenosyl ester ESI-MS (-) calculated m/z 513.5, found m/z 513.4; BocPhe-cytidyl ester ESI-MS (-) calculated m/z 490.4, found m/z 490.5; BocPhe-uridyl ester ESI-MS (-) calculated m/z 489.5, found m/z 489.1).
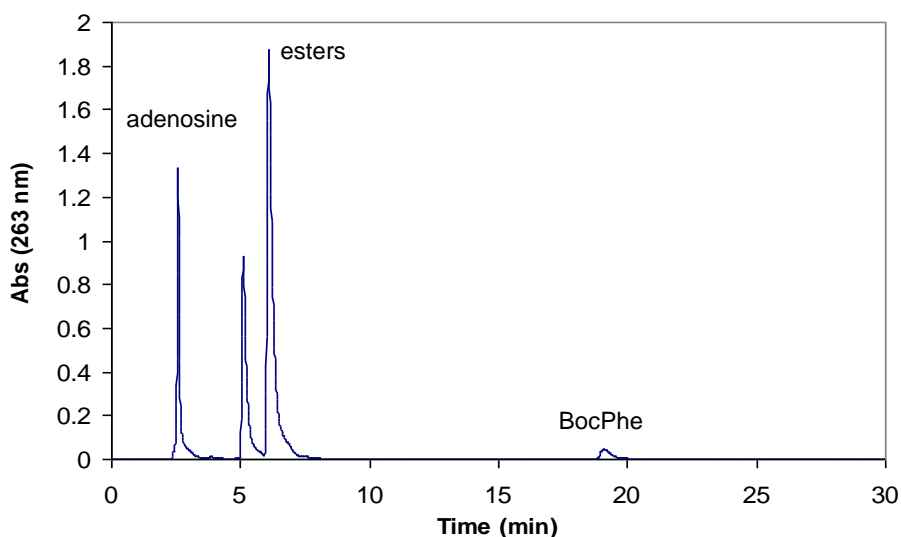
Figure 13. HPLC chromatogram for the lanthanum-mediated aminoacylation of adenosine with BocPheEP (Yield = 82 %).
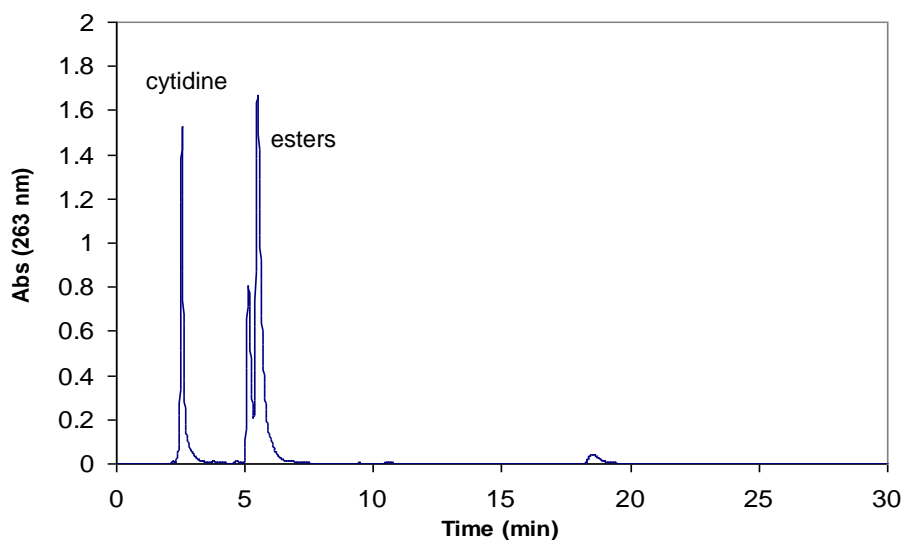
Figure 14. HPLC chromatogram for the lanthanum-mediated aminoacylation of cytidine with BocPheEP (Yield = 73 %).
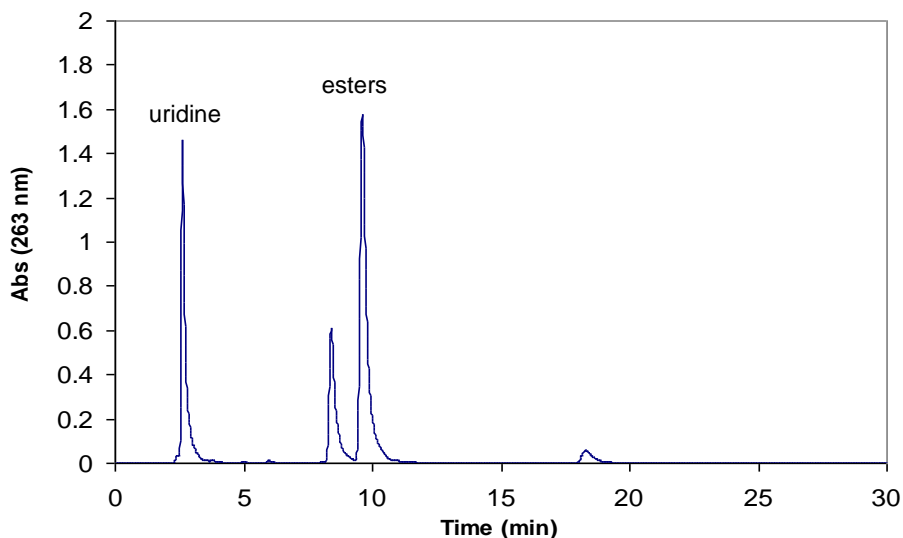
Figure 15. HPLC chromatogram for the lanthanum-mediated aminoacylation of uridine with BocPheEP (Yield = 71 %).

To confirm that the esters are formed at the 2'- or 3'-hydroxyl groups, a variety of ribonucleosides were tested for lanthanum-mediated aminoacylation. Successful ester formation was observed in the absence of 5'-hydroxyl group (5'-deoxyadenosine, Figure 16). However, aminoacylation was not observed for the ribonucleosides lacking the 2'- and 3'-hydroxyl groups (2',3'-dideoxycytidine, 2',3'-isopropylideneadenosine, Figures 17, 18).
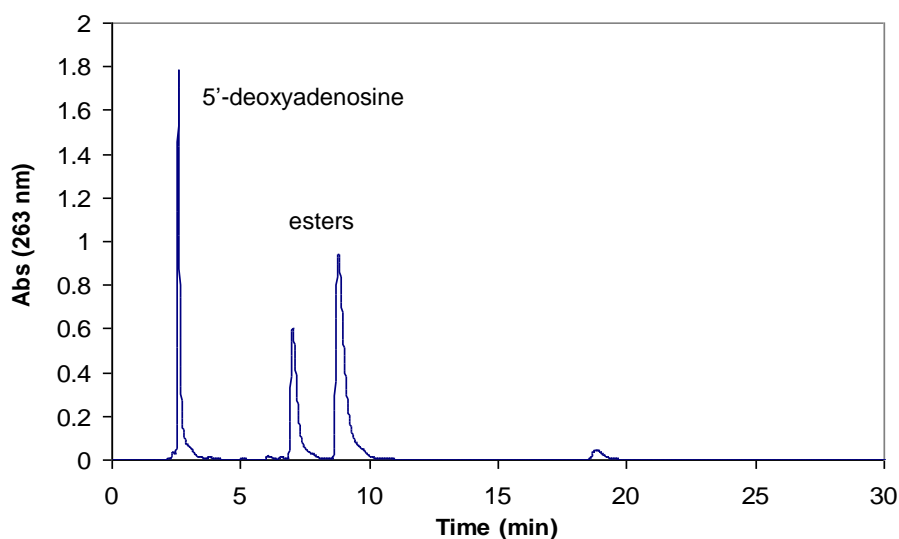
Figure 16. HPLC chromatogram for the lanthanum-mediated aminoacylation of 5'-deoxy-adenosine with BocPheEP.
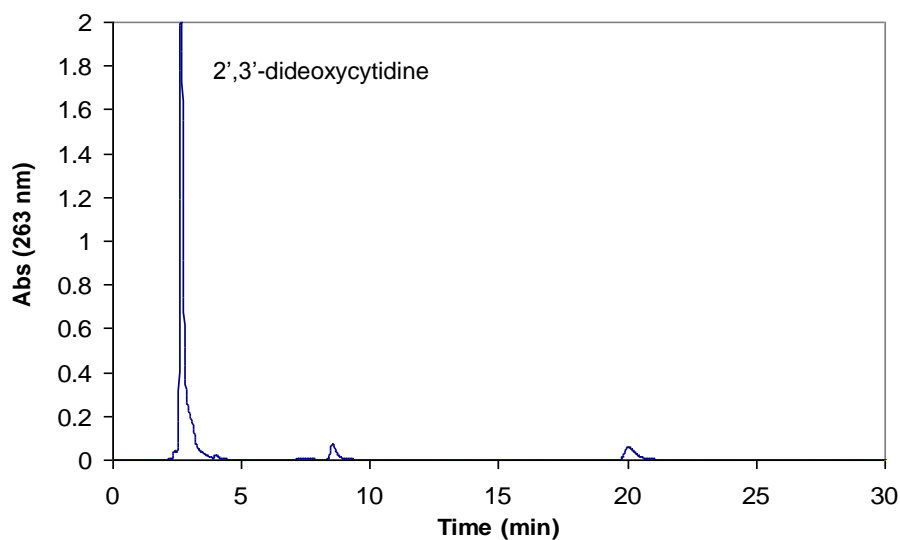
Figure 17. HPLC chromatogram for the lanthanum-mediated reaction of 2',3'-dideoxycytidine with BocPheEP. No aminoacylation was observed.
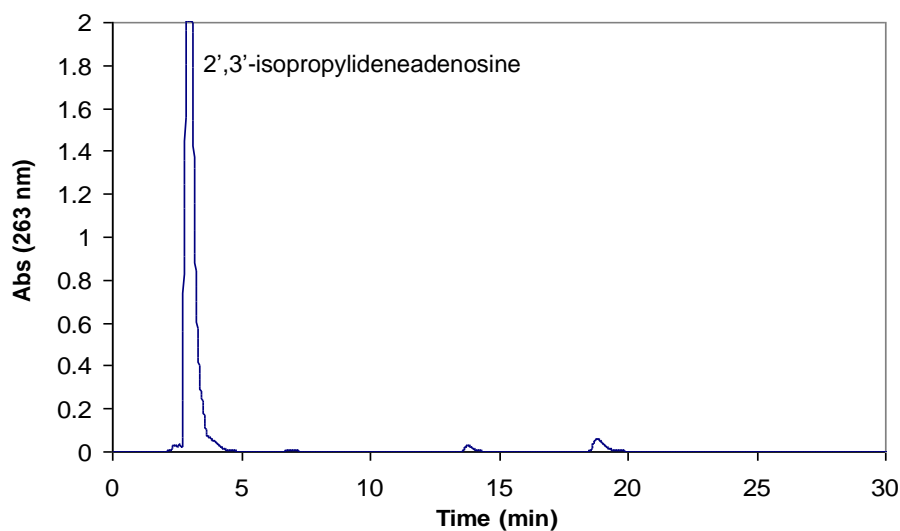
Figure 18. HPLC chromatogram for the lanthanum-mediated reaction of 2',3'-isopropylidene-adenosine with BocPheEP. No aminoacylation was observed.

3.3.2   Aminoacylation of ribonucleotides with BocPheEP

Aminoacylation of ribonucleotides (5'-AMP, 5'-CMP) was also tested in the presence of lanthanum catalyst (reaction conditions: [ribonucleotide] = 10 mM, [La(OTf)$_3$] = [BocPheEP] = 50 mM, [EPPS] $_{pH\ 8.0}$ = 200 mM, 40 % DMSO, 60 min). Successful aminoacylation of 5'-AMP and 5'-CMP was confirmed by HPLC and ESI-MS (Figures 19, 20; BocPhe-AMP ester ESI-MS

(-) calculated m/z 594.5, found m/z 594.5; BocPhe-CMP ester ESI-MS (-) calculated m/z 569.5, found m/z 569.2).
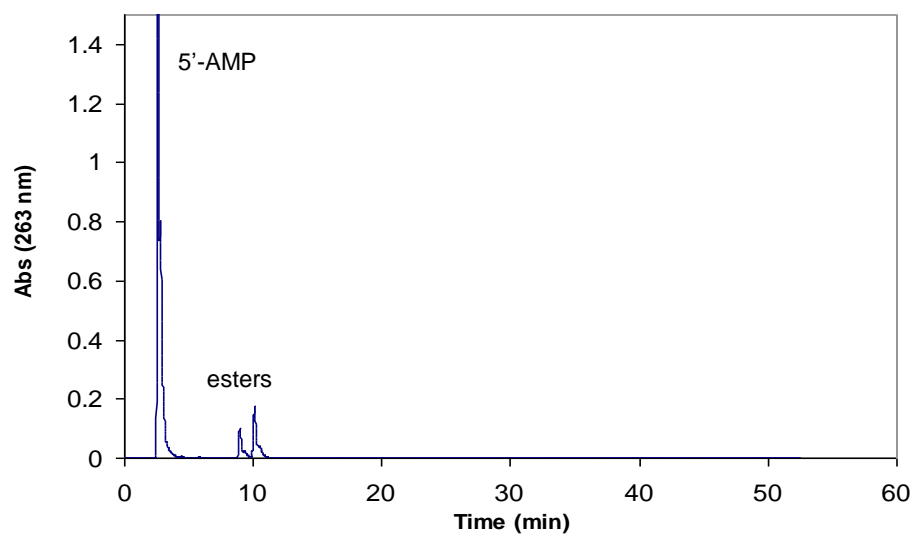
Figure 19. HPLC chromatogram for the lanthanum-mediated reaction of 5'-AMP with BocPheEP.
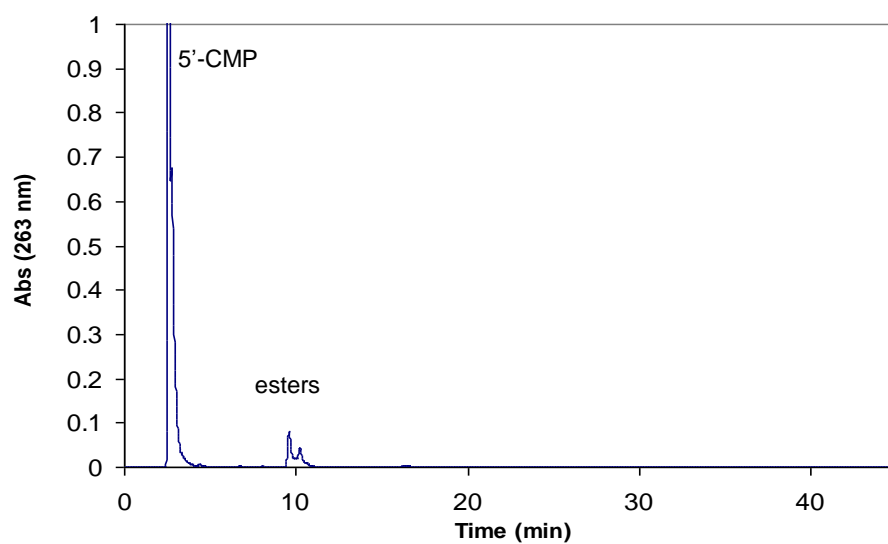
Figure 20. HPLC chromatogram for the lanthanum-mediated reaction of 5'-CMP with BocPheEP.

In order to test the need for lanthanum coordination of 2'- and 3'-hydroxyl groups for successful ester formation, the reactions of 2'-deoxyadenosine 5'-monophosphate and 2'-doexyadenosine 5'-monosphosphate were evaluated. No ester formation was observed with both ribonucleotides (Figures 21, 22).
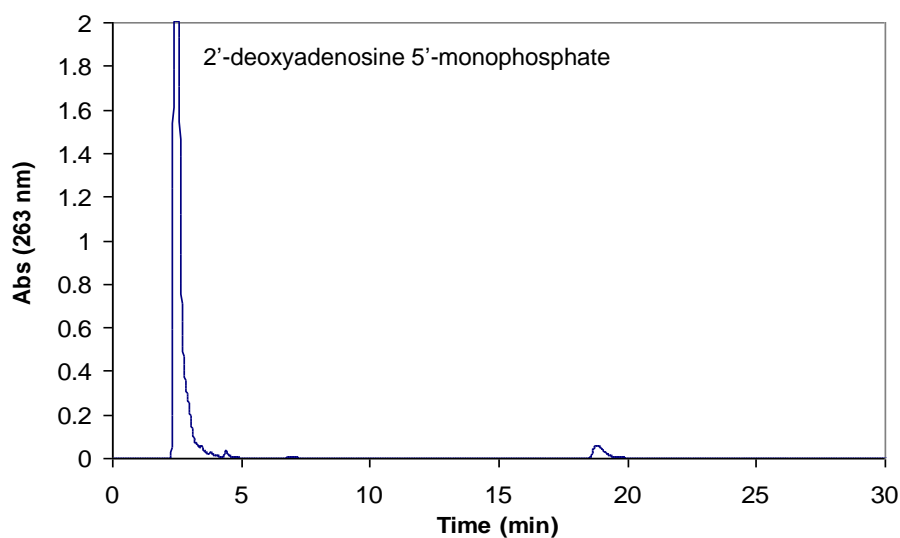
Figure 21. HPLC chromatogram for the lanthanum-mediated reaction of 2'-deoxyadenosine 5'-monosphosphate with BocPheEP. No aminoacylation was observed.
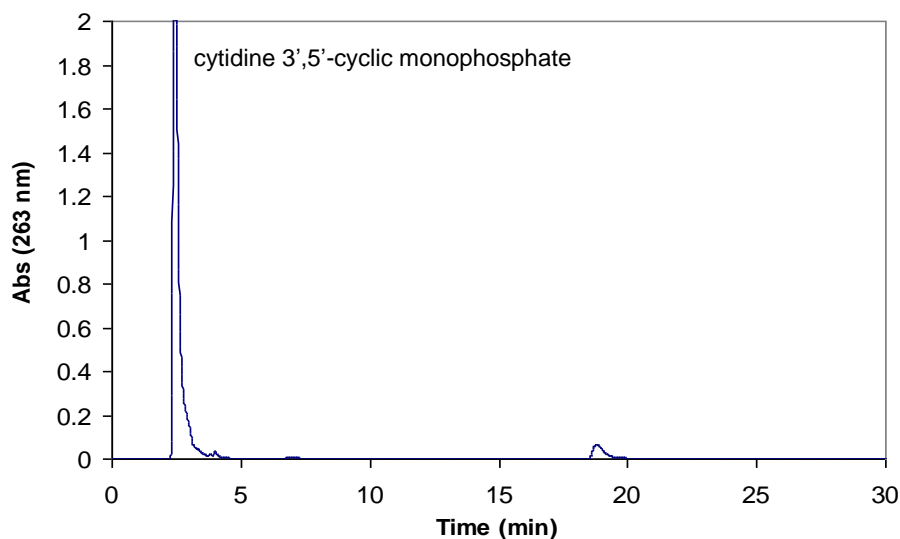
Figure 22. HPLC chromatogram for the lanthanum-mediated reaction of cytidine 3',5'-cyclic monophosphate with BocPheEP. No aminoacylation was observed.

3.3.3 Effect of phosphate R-group on aminoacylation

To determine the effect of changing the phosphate R-group on the aminoacylation of adenosine, studies were carried out with *N-t*-Boc-phenylalanyl methyl, ethyl and phenyl phosphates (BocPheMP, BocPheEP and BocPhePP, respectively). Changing the R-group on the phosphate did not have a significant effect on the yield of aminoacylation after 60 min (Table 1). However, lower yields were observed with BocPhePP in comparison to BocPheMP or BocPheEP. Highest yields were obtained with BocPheEP resulting in the formation of Boc-Phe-adenosyl ester in 81.0 %, Boc-Phe-cytidyl ester in 66.5 %, and Boc-Phe-uridyl ester in 62.6 % yields.

Table 1. Average yields of ester formation for the aminoacylation of ribonucleosides with BocPheMP, BocPheEP and BocPhePP. Reaction conditions: [ribonucleosides] = 10 mM, $[La(OTf)_3]$ = 50 mM, [BocPheM/E/PP] = 50 mM, solvent: 40 % DMSO in 200 mM, pH 8.0 EPPS buffer.

Entry	Ribonucleoside	Aminoacylating reagent	Yield (%)
1	Adenosine	BocPheMP	76.2 ± 2.4
2	Adenosine	BocPheEP	81.0 ± 7.0
3	Adenosine	BocPhePP	70.6 ± 5.0
4	Cytidine	BocPheMP	62.9 ± 6.9
5	Cytidine	BocPheEP	66.5 ± 9.5
6	Cytidine	BocPhePP	59.7 ± 3.7
7	Uridine	BocPheMP	66.9 ± 5.6
8	Uridine	BocPheEP	62.6 ± 3.2
9	Uridine	BocPhePP	52.8 ± 2.8

3.3.4   Optimization of buffer concentration and pH

To determine the optimal buffer concentration and pH for this reaction, aminoacylation of adenosine was studied under various buffer conditions. Adenosine (10 mM) was reacted with BocPheEP (50 mM) and lanthanum (50 mM) in a solvent system consisting of 40 % DMSO in 200 mM buffer of varying pH. Optimal pH was determined to be around 7.5 and 8.0 with the observed yields of approximately 86 % (Table 2).

Table 2. Average yields of ester formation for the aminoacylation of ribonucleosides with BocPheEP. Reaction conditions: [ribonucleosides] = 10 mM, [La(OTf)$_3$] = 50 mM, [BocPheEP] = 50 mM, solvent: 40 % DMSO in 200 mM, [buffer] = 200 mM, varying pH.

Entry	Buffer	pH	Yield (%)
1	MES	6.0	73.4 ± 3.6
2	MES	6.5	82.6 ± 3.2
3	HEPES	7.0	69.2 ± 6.2
4	HEPES	7.5	85.2 ± 4.9
5	EPPS	8.0	86.0 ± 5.2
6	EPPS	8.5	77.0 ± 4.8

Reaction conditions were also optimized with respect to the buffer concentration. First, the lanthanum and buffer concentrations were varied while keeping the ratio of $[La^{3+}]$: [buffer] constant at 1:4. The yield of aminoacylation increased as the lanthanum concentration increased from 10 mM to 100 mM, and the maximum ester formation was observed at 100 mM $La(OTf)_3$ and 400 mM buffer concentrations (Table 3). Optimum lanthanum concentration was also determined while maintaining the buffer concentration at 200 mM. At 200 mM concentration of buffer, the yield of aminoacylation increased to 76 % in the presence of 50 mM $La(OTf)_3$ (Table 4).

Table 3. Average yields of ester formation for the aminoacylation of adenosine with BocPheEP under varying lanthanum and buffer concentrations. Reaction conditions: [adenosine] = 10 mM, $[La(OTf)_3]$ = 10 – 100 mM, [BocPheEP] = 50 mM, solvent: 40 % DMSO in pH 8.0 EPPS buffer (40 – 400 mM), $[La^{3+}]$: [buffer] = 1:4.

Entry	$[La^{3+}]$, mM	[Buffer], mM	Yield (%)
1	10	40	23.4 ± 2.5
2	20	80	50.5 ± 3.2
3	50	200	76.0 ± 4.7
4	100	400	78.6 ± 6.4

Table 4. Average yields of ester formation for the aminoacylation of adenosine with BocPheEP under varying lanthanum concentrations. Reaction conditions: [adenosine] = 10 mM, $[La(OTf)_3]$ = 10 – 200 mM, [BocPheEP] = 50 mM, solvent: 40 % DMSO in 200 mM, pH 8.0 EPPS buffer.

Entry	$[La^{3+}]$, mM	[Buffer], mM	Yield (%)
1	10	200	20.6 ± 5.6
2	20	200	53.0 ± 4.2
3	50	200	76.0 ± 4.7
4	100	200	75.0 ± 5.2
5	200	200	76.0 ± 5.6

3.3.5   Effect of organic co-solvent system

Lanthanum-mediated aminoacylation of adenosine was performed in various solvent systems in the presence of an organic co-solvent. Among the three organic solvents tested, highest yields were obtained with DMSO and acetonitrile. When DMSO was added in 20 – 40 % (v/v) composition, yields as high as 87 % were observed (Table 5).

Table 5. Average yields of ester formation for the aminoacylation of adenosine with BocPheEP in various organic co-solvents. Reaction conditions: [adenosine] = 10 mM, [La(OTf)$_3$] = 50 mM, [BocPheEP] = 50 mM, solvent: 0 – 40 % organic solvent in 200 mM, pH 8.0 EPPS buffer.

Entry	Organic co-solvent	Percentage of organic solvent (% v/v)	Yield (%)
1	None	0	59.5 ± 4.5
2	DMSO	5	62.5 ± 5.2
3	DMSO	10	66.0 ± 7.1
4	DMSO	15	80.6 ± 5.4
5	DMSO	20	86.9 ± 5.2
6	DMSO	30	85.3 ± 6.8
7	DMSO	40	83.7 ± 4.1
8	Acetonitrile	10	71.4 ± 5.2
9	Acetonitrile	20	74.9 ± 4.9
10	Acetonitrile	30	77.4 ± 4.7
11	Acetonitrile	40	75.3 ± 6.2
12	THF	10	49.7 ± 6.8
13	THF	20	61.5 ± 5.1
14	THF	30	60.5 ± 3.9
15	THF	40	68.4 ± 6.0

3.3.6   Effect of different lanthanide salts

To investigate the effect of lanthanide metal size on the aminoacylation of adenosine, La^{3+}, Ce^{3+}, Pr^{3+}, Nd^{3+}, Eu^{3+}, Tb^{3+}, Dy^{3+} and Yb^{3+} ions were selected as catalysts for the reaction. As the atomic radius of the lanthanide ion decreased, the yield of ester formation decreased (Table 6). Highest yields (approximately 80 %) were obtained with the larger lanthanides (La^{3+}, Ce^{3+}, Pr^{3+}, Nd^{3+}) and the lowest yield (24 %) was obtained with the smallest lanthanide (Yb^{3+}). Mg^{2+} ion was tested as a negative control, and no ester formation was observed.

Table 6. Average yields of ester formation for the aminoacylation of adenosine with BocPheEP. Reaction conditions: [adenosine] = 10 mM, $[Ln^{3+}]$ = 50 mM, [BocPheEP] = 50 mM, solvent: 40 % DMSO in 200 mM, pH 8.0 EPPS buffer.

Entry	Atomic number	Lanthanide ion	Atomic radius (pm)[58]	Yield (%)
1	57	La^{3+}	116	82.8 ± 4.2
2	58	Ce^{3+}	114	77.6 ± 3.8
3	59	Pr^{3+}	113	81.4 ± 6.2
4	60	Nd^{3+}	111	77.2 ± 5.9
5	63	Eu^{3+}	107	61.5 ± 5.1
6	65	Tb^{3+}	104	57.2 ± 7.7
7	66	Dy^{3+}	103	35.3 ± 5.2
8	70	Yb^{3+}	99	23.9 ± 4.4
9	12	Mg^{2+}	72	Not detected

3.3.7   Effect of different counter-ions of lanthanum

Lanthanum ions with different counter-ions were subjected to aminoacylation of adenosine to evaluate its effect on the yield of ester formation. Three different counter-ions, $LaCl_3$, $La(OTf)_3$ and $La(NO_3)_3$, were tested under same reaction conditions. Analysis by HPLC revealed no significant difference in the yield of aminoacylation of adenosine using three different counter-ions of lanthanum (Table 7).

Table 7. Average yields of ester formation for the aminoacylation of adenosine with BocPheEP with La^{3+}. Reaction conditions: [adenosine] = 10 mM, $[La^{3+}]$ = 50 mM, [BocPheEP] = 50 mM, solvent: 40 % DMSO in 200 mM, pH 8.0 EPPS buffer.

Entry	Metal salt	Yield (%)
1	$LaCl_3$	80.9 ± 5.2
2	$La(OTf)_3$	78.4 ± 4.7
3	$La(NO_3)_3$	80.5 ± 6.2

3.4   Discussion

3.4.1   Aminoacylation of ribonucleosides and nucleotides with BocPheEP

Successful aminoacylation of ribonucleosides and nucleotides with BocPheEP was achieved. Analysis by RP-HPLC and ESI-MS confirmed the rapid formation of monoesters of ribonucleosides and nucleotides. Selectivity of the reaction was studied by using several ribonucleosides and nucleotides that lack the 2', 3'-diol moiety. Aminoacylation was observed in the presence of both the 2'- and 3'-hydroxyl groups (adenosine, cytidine, uridine, 5'-deoxyadenosine, 5'-AMP, 5'-CMP). However, where one or both hydroxyl groups is absent (2',3'-dideoxycytidine, 2',3'-isopropylideneadenosine, 2'-deoxyadenosine 5'-monophosphate, cytidine 3',5'-cyclic monophosphate), ester formation is not observed. This provides support for the efficient selectivity of lanthanum for 2'- and 3'-hydroxyl groups of ribonucleosides and nucleotides.

3.4.2   Optimization of reaction conditions

In order to optimize the reaction conditions, lanthanum-mediated aminoacylation of adenosine was studied with respect to the reagent concentrations, phosphate R-group, buffer concentration and pH, solvent composition, lanthanide metal and its counter-ion. Based on these factors, the optimized reaction conditions were [adenosine] = 10 mM, [La(OTf)$_3$] = [BocPheEP] = 50 mM in 40 % DMSO (v/v) in 200 mM, pH 8.0 EPPS at room temperature for 60 minutes.

The effect of phosphate R group was studied in reactions with adenosine, cytidine and uridine. The maximum yield of ester formation was 81.0 % for the aminoacylation of adenosine with BocPheEP. Similar yields of aminoacylation were obtained utilizing BocPheEP and BocPheMP as the aminoacylating reagent. Yields were slightly lower using BocPhePP, which may be due to the reduced solubility in aqueous solution from the introduction of the hydrophobic phenyl group.

Reactions with adenosine were also optimized in terms of the buffer concentration and pH. Reaction pH has an important impact on the aminoacylation of cis-diols. In basic solutions (pH 10 – 11), the rate of hydrolysis of aminoacyl alkyl phosphate increases, and at a lower pH (pH 4 – 5) buffer catalysis is observed.[53] Due to the properties of aminoacyl alkyl phosphates, it is crucial to control the pH of the reaction solutions. The pH of the reaction is dependent on both the buffer and lanthanum concentrations. Lanthanum is known to coordinate to water and lower its pK$_a$.[59] Taking these properties into consideration, maximum yields of aminoacyl-adenosine were obtained in 200 mM EPPS buffer at pH 7.5 – 8.0. In solutions with at pH > 8.0, the yield of aminoacylation decreases, which is likely to be due to the increased rate of hydrolysis of BocPheEP.

The effect of solvent composition on the aminoacylation of adenosine was also studied. Polar aprotic solvents that are non-nucleophilic (DMSO, THF, acetonitrile) were selected to ensure miscibility with the aqueous buffer and to avoid any side reactions with the solvent. Introducing organic co-solvents allow enhanced solubility of BocPheEP in the reaction solution, and also reduce the concentration of water, which may lower the rate of hydrolysis of BocPheEP. Highest yields were achieved with 20 – 40 % DMSO (v/v). Further investigation is required to fully understand the effect of organic co-solvent in the aminoacylation of ribonucleosides.

Finally, aminoacylation of adenosine was studied with respect to the lanthanum salt. When varying the lanthanide ion, highest yields were obtained with La^{3+}, Ce^{3+}, Pr^{3+} and Nd^{3+} , and lowest yields were obtained with Yb^{3+}. This observation is consistent with the expected result based on "lanthanide contraction."[55] Lanthanides of larger atomic radii have greater preference for coordinating to the cis-diol while smaller lanthanides prefer binding to phosphates with smaller binding angle. Reactions with different counter-ions of lanthanum did not affect the yield of aminoacylation.

3.5 Conclusions

Selective aminoacylation of ribonucleosides and nucleotides was achieved with BocPheEP in the presence of lanthanum ion. The reaction conditions were optimized by varying the reagent concentrations and the solvent composition with respect to buffer concentration, pH and organic co-solvent. With the optimized reaction conditions, yields as high as 86 % was obtained for the aminoacylation of adenosine with BocPheEP.

Chapter 4
Effect of phosphate in inhibiting lanthanide-catalyzed aminoacylation of ribonucleosides and nucleotides

4.1   Introduction

Ribonucleosides and nucleotides are the simple analogues of tRNA bearing all the key functionalities present in the macromolecule. These small molecules can be used to test lanthanum-mediated aminoacylation without introducing the three-dimensional complexity of full-length tRNA. However, in order to extend this method for the direct aminoacylation of tRNA, several factors must be considered. Since tRNA is a polymer derived from nucleotides, it contains many more phosphate groups per molecule than do simple ribonucleotides. Anionic phosphates are stronger ligands for binding lanthanides in comparison to the neutral diols. Indeed, lanthanides are known to catalyze the hydrolysis of phosphodiester bonds of RNA and tRNA by coordinating to the phosphate groups.[60-63] Furthermore, Tzvetkova and Kluger observed an inhibition of aminoacylation by phosphates where the dianionic alkyl phosphate byproduct coordinates to lanthanum inhibiting further catalysis.[64] This has also been observed in the acylation of monosaccharides in water. In this report, Dhiman and Kluger used magnesium ion as a sacrificial cation to coordinate to the phosphate byproduct, allowing the lanthanide to be catalytic overall.[65]

The ability of lanthanum to selectively coordinate to diols over phosphates is a crucial factor in the success of aminoacylation. Aminoacylation of adenosine and adenosine phosphates have been studied under different conditions in order to determine the effect of phosphates on the aminoacylation of diols.

4.2   Experimental

4.2.1   Materials and Methods

Unless otherwise noted, commercial reagents were used without further purification. Water was doubly distilled and deionized prior to use. HPLC analysis was performed on a C18 reverse phase analytical (Phenomenex, Jupiter 4u Proteo 90A, 250 x 4.60 mm) or semi-

preparative column (Phenomenex, Jupiter 10u Proteo 90A, 250 x 10.00 mm), and the products were detected at 263 nm. The mobile phase consisted of 30 % acetonitrile and 0.1 % TFA in water, and was eluted at a flow rate of 1 mL/min for the analytical column, and 3 mL/min for the semi-preparative column. Yield of ester formation is calculated based on the integrated areas of ester peaks.

4.3 Results

4.3.1 Effect of phosphate buffer on the aminoacylation of ribonucleosides and nucleotides

It is known that La^{3+} coordinates strongly to the anionic phosphate groups. To determine the effect of lanthanum coordination to phosphates on the aminoacylation of ribonucleosides and nucleotides, reactions were carried out in a phosphate buffer and compared to the reactions in a sulfonate buffer (EPPS). Aminoacylation of adenosine and 5'-AMP were conducted with 10 mM ribonucleoside/nucleotide, 50 mM BocPheEP, 50 mM $La(OTf)_3$ in 40 % (v/v) DMSO in 200 mM, pH 8.0 EPPS or phosphate buffers. When reactions were carried out in an EPPS buffer, yields of 78 % and 26 % were observed for the acylation of adenosine and 5'-AMP, respectively (Figures 23, 25). In a phosphate buffer, aminoacylation was not observed for both adenosine and 5'-AMP (Figures 24, 26).
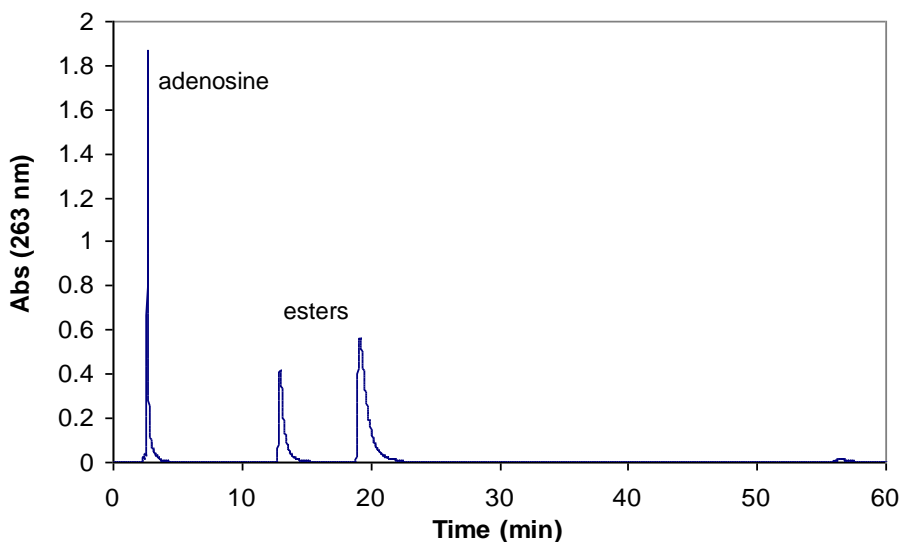
Figure 23. HPLC chromatogram for the lanthanum-mediated aminoacylation of adenosine with BocPheEP in 200 mM, pH 8.0 EPPS buffer (Yield = 78 %).
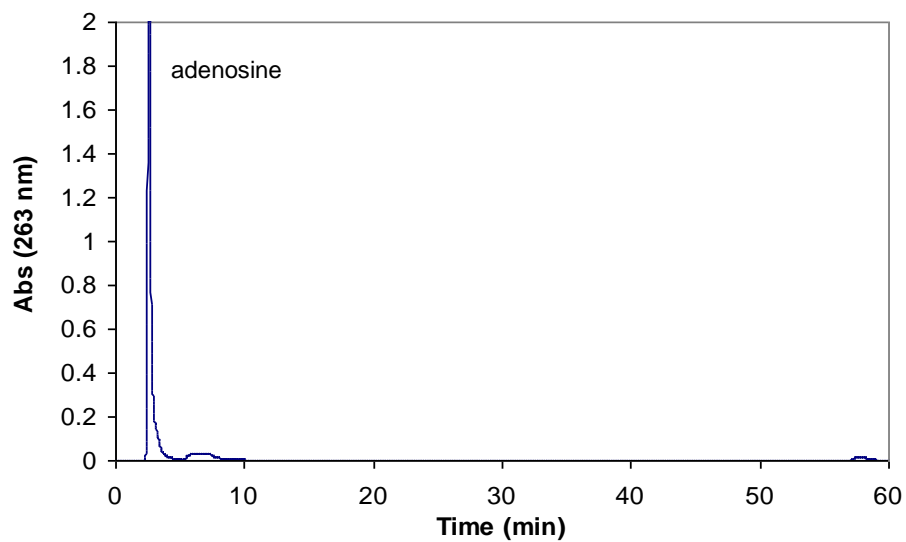
Figure 24. HPLC chromatogram for the lanthanum-mediated aminoacylation of adenosine with BocPheEP in 200 mM, pH 8.0 phosphate buffer. No aminoacylation is observed.
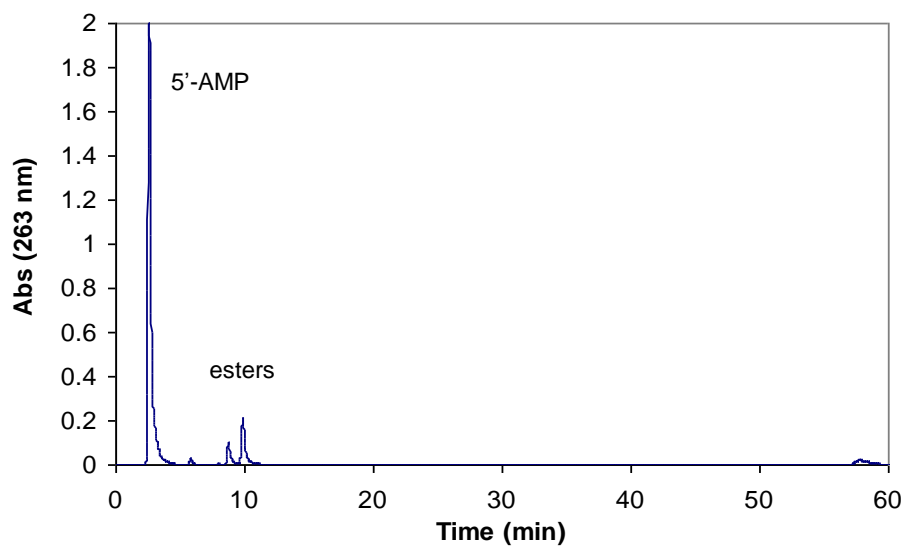
Figure 25. HPLC chromatogram for the lanthanum-mediated aminoacylation of 5'-AMP with BocPheEP in 200 mM, pH 8.0 EPPS buffer (Yield = 26 %).
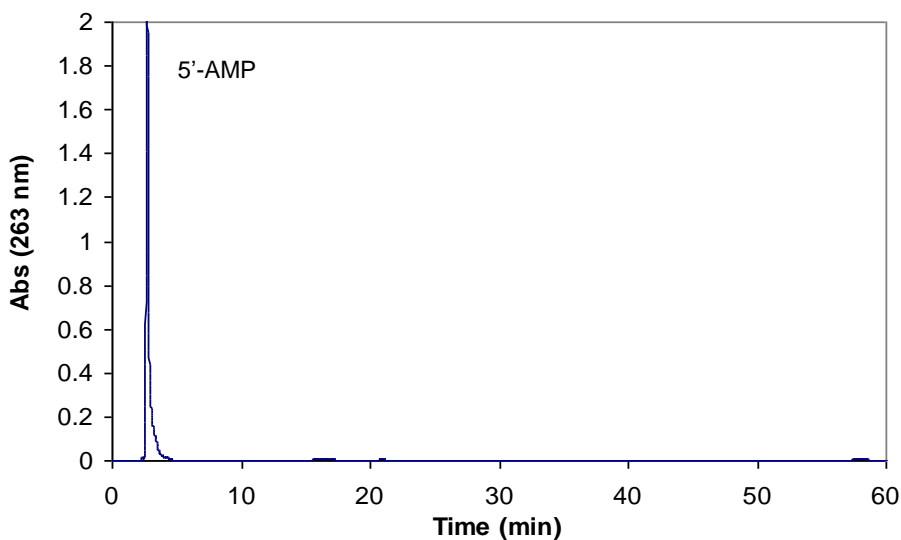
Figure 26. HPLC chromatogram for the lanthanum-mediated aminoacylation of 5'-AMP with BocPheEP in 200 mM, pH 8.0 phosphate buffer. No aminoacylation is observed.

4.3.2 Effect of phosphate anion on the aminoacylation of ribonucleosides and nucleotides

To further investigate the effect of the undesired lanthanum coordination to phosphate, varying concentrations of phosphate ions were added to the reaction containing adenosine, La(OTf)$_3$ and BocPheEP. Lanthanum concentration was also varied to see its effect in overcoming inhibition by phosphates.

Reactions were performed with 10 mM adenosine, 10 mM La(OTf)$_3$ and 50 mM BocPheEP in 40 % DMSO (v/v) in 200 mM, pH 8.0 EPPS buffer. In the presence of equimolar concentrations of adenosine and La(OTf)$_3$, a decrease in the yield of ester formation was observed with the addition of phosphate (Table 8). A significant decrease in the yield was observed when 5 – 100 mM (0.5 – 10 equiv. relative to La^{3+}) concentrations of phosphate were added. When equimolar concentration of phosphate relative to adenosine and lanthanum was added, the yield dropped to 20.9 % and even lower to 14 % in the presence of a further excess of phosphate.

Table 8. Average yields of ester formation for the aminoacylation of adenosine with BocPheEP at various concentrations of phosphate anions added. Reaction conditions: [adenosine] = 10 mM, [La(OTf)$_3$] = 10 mM, [BocPheEP] = 50 mM, [PO$_4{}^{3-}$] = variable, solvent: 40 % DMSO in 200 mM, pH 8.0 EPPS buffer.

Entry	[Lanthanum], mM	[Phosphate], mM	Equiv. of phosphate relative to lanthanum	Yield (%)
1	10	0	0	58.5 ± 4.2
2	10	1	0.1	52.4 ± 3.8
3	10	2	0.2	51.4 ± 5.6
4	10	5	0.5	31.9 ± 5.1
5	10	10	1	20.9 ± 4.7
6	10	20	2	14.8 ± 3.6
7	10	50	5	14.4 ± 3.9
8	10	100	10	13.5 ± 5.8

The addition of phosphate was also tested in the presence of excess lanthanum relative to adenosine. In the presence of 10 mM adenosine and 50 mM La(OTf)$_3$, the yield remained approximately 70 – 80 % with the addition of up to 100 mM phosphate (Table 9). However, with a further increase in the concentration of phosphate, the yield decreased significantly to 49.6 % and 25.7 % with the addition of 250 mM and 500 mM phosphate, respectively. When the lanthanum concentration was increased to 100 mM (10 equiv. relative to adenosine), the effect of inhibition by phosphate diminished further (Table 10). The yield decreased from 84.1 % in the absence of added phosphate to 70.5 % with 1 M phosphate.

Table 9. Average yields of ester formation for the aminoacylation of adenosine with BocPheEP at various concentrations of phosphate anions added. Reaction conditions: [adenosine] = 10 mM, [La(OTf)$_3$] = 50 mM, [BocPheEP] = 50 mM, [PO$_4^{3-}$] = variable, solvent: 40 % DMSO in 200 mM, pH 8.0 EPPS buffer.

Entry	[Lanthanum], mM	[Phosphate], mM	Equiv. of phosphate relative to lanthanum	Yield (%)
1	50	0	0	83.0 ± 1.2
2	50	5	0.1	83.2 ± 2.4
3	50	10	0.2	82.1 ± 3.2
4	50	25	0.5	82.6 ± 1.6
5	50	50	1	77.4 ± 4.3
6	50	100	2	68.3 ± 0.6
7	50	250	5	49.6 ± 2.6
8	50	500	10	25.7 ± 1.8

Table 10. Average yields of ester formation for the aminoacylation of adenosine with BocPheEP at various concentrations of phosphate anions added. Reaction conditions: [adenosine] = 10 mM, [La(OTf)$_3$] = 100 mM, [BocPheEP] = 50 mM, [PO$_4^{3-}$] = variable, solvent: 40 % DMSO in 200 mM, pH 8.0 EPPS buffer.

Entry	[Lanthanum], mM	[Phosphate], mM	Equiv. of phosphate relative to lanthanum	Yield (%)
1	100	0	0	84.1 ± 2.1
2	100	10	0.1	84.9 ± 3.5
3	100	20	0.2	80.8 ± 4.4
4	100	50	0.5	82.2 ± 4.1
5	100	100	1	80.2 ± 5.7
6	100	200	2	77.1 ± 4.3
7	100	500	5	75.4 ± 3.8
8	100	1000	10	70.5 ± 2.9

4.3.3 Aminoacylation of adenosine, 5'-AMP, 5'-ADP, 5'-ATP

Aminoacylation was performed with adenosine 5'-mono, di, and triphosphates in order to see the effect of increasing phosphate groups on the ribonucleotide. Reactions were carried out in 10 mM ribonucleotide, 50 mM BocPheEP and 50 mM La(OTf)3 with 40 % DMSO (v/v) in 200 mM, pH 8.0 EPPS as the solvent. The yields of aminoacylation decreased from 26 % with 5'-AMP to below 0.1 % with 5'-ADP (Figures 27, 28). When 5'-ATP was tested for aminoacylation under identical conditions, no ester formation was observed (Figure 29).
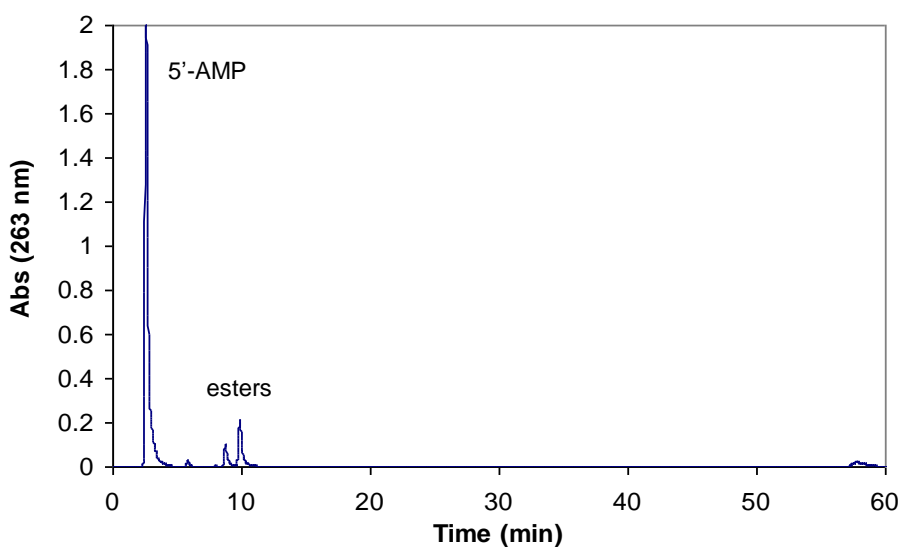
Figure 27. HPLC chromatogram for the lanthanum-mediated aminoacylation of 5'-AMP with BocPheEP in 200 mM, pH 8.0 phosphate buffer.
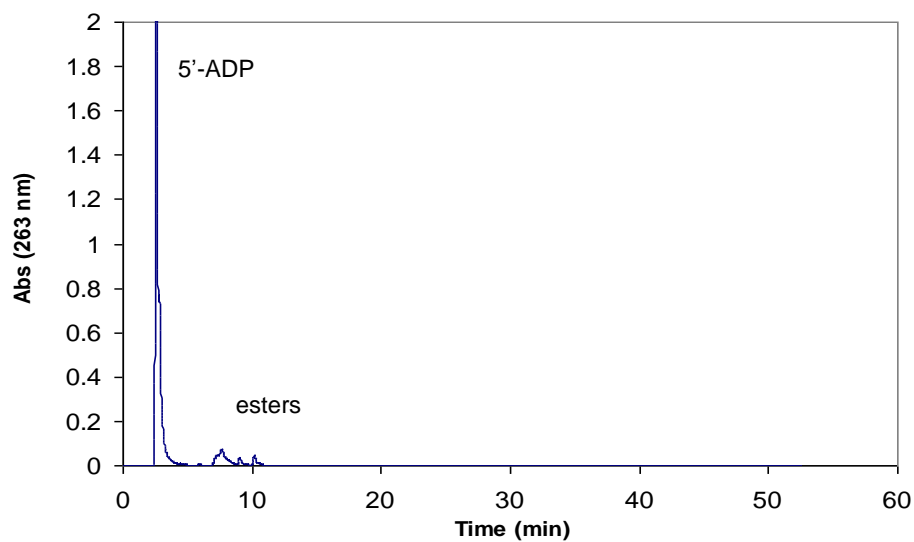
Figure 28. HPLC chromatogram for the lanthanum-mediated aminoacylation of 5'-ADP with BocPheEP in 200 mM, pH 8.0 phosphate buffer.
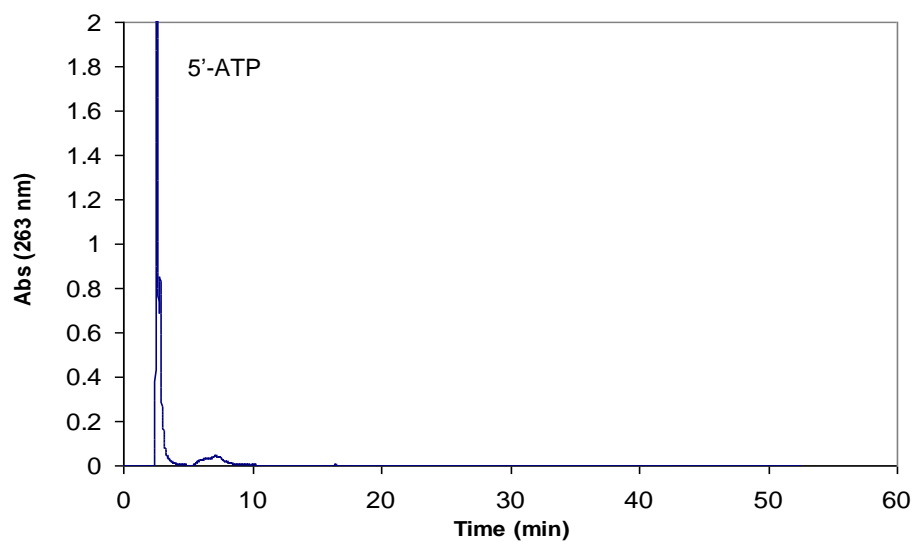
Figure 29. HPLC chromatogram for the lanthanum-mediated aminoacylation of 5'-ATP with BocPheEP in 200 mM, pH 8.0 phosphate buffer.

4.3.4   Competition studies

Lanthanum-mediated aminoacylation of adenosine was studied in the presence of a competing ribonucleoside, nucleotide or tRNA that contains a phosphate and/or a diol (Figures 31 – 38; 2',3'-dideoxycytidine, 5'-deoxyadenosine, cytidine 3',5'-cyclic monophosphate, 2',3'-isopropylidene adenosine, 2'-deoxyadenosine-5'-monophosphate, adenosine 3',5'-cyclic monophosphate, adenosine 3'-monophosphate, tRNA). Due to the overlap of the peak of the competing ribonucleoside, nucleotide or tRNA with the adenosine peak, yields were calculated based on the difference in the areas of the aminoacyl-adenosine peak in the presence and absence of competition.
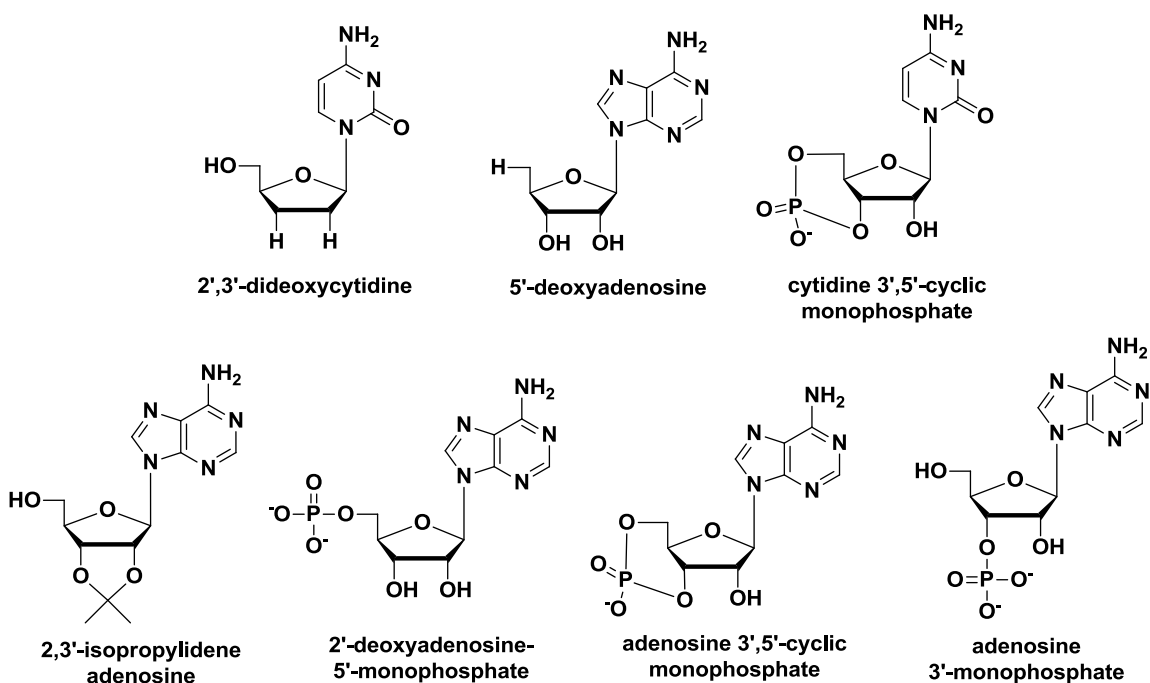
Figure 30. Ribonucleosides and nucleotides used in the competition studies of adenosine aminoacylation.
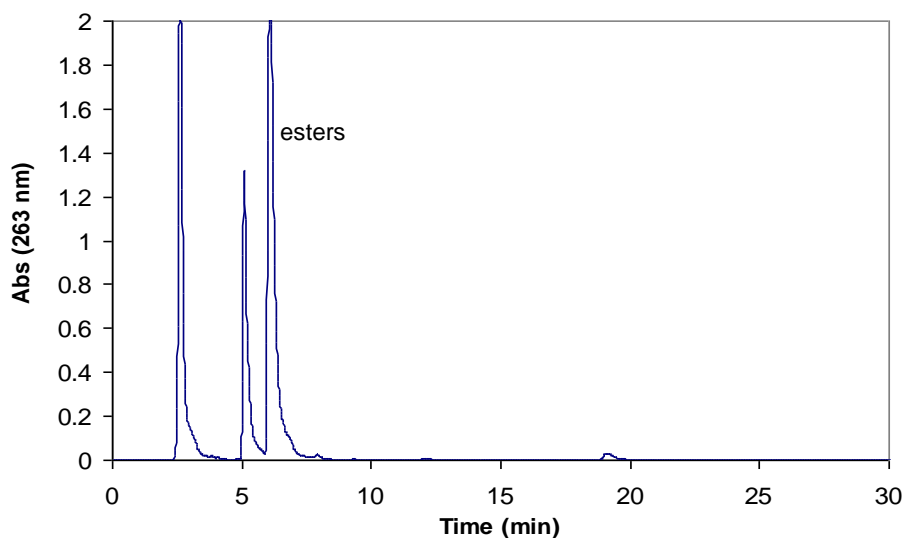
Figure 31. HPLC chromatogram for the lanthanum-mediated aminoacylation of adenosine with BocPheEP in 200 mM, pH 8.0 phosphate buffer. Reaction conditions: [adenosine] = 10 mM, $[La(OTf)_3]$ = 50 mM, [BocPheEP] = 50 mM, solvent: 40 % DMSO in 200 mM, pH 8.0 EPPS buffer.
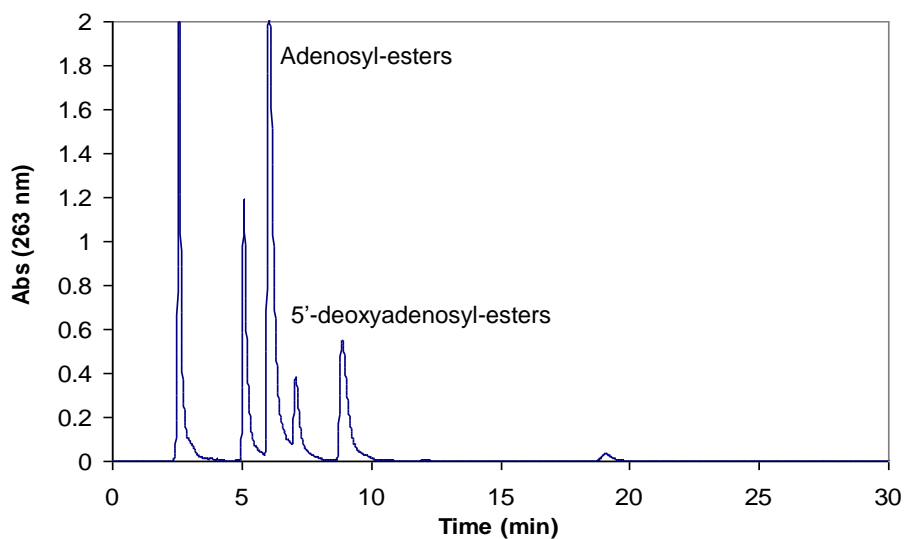
Figure 32. HPLC chromatogram for the lanthanum-mediated aminoacylation of adenosine with BocPheEP in 200 mM, pH 8.0 phosphate buffer in the presence of 5'-deoxyadenosine as competitive ribonucleoside.
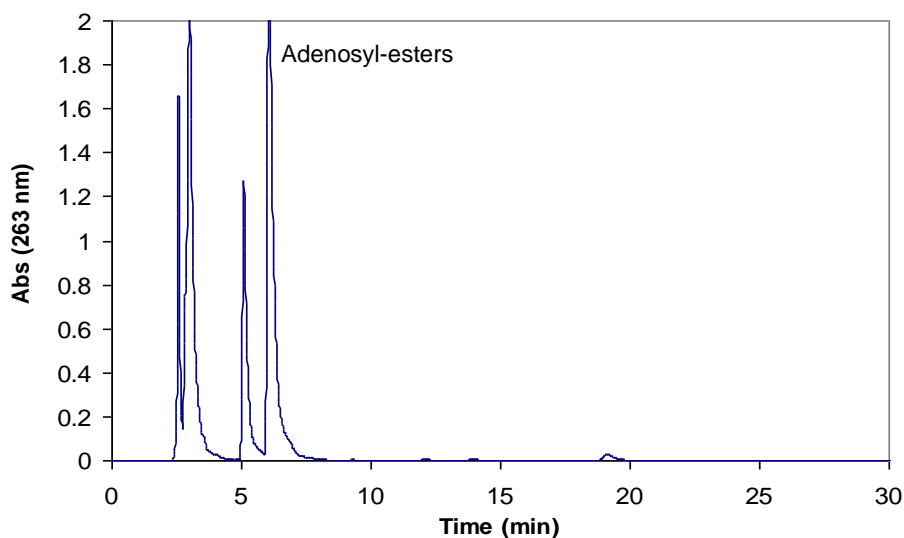
Figure 33. HPLC chromatogram for the lanthanum-mediated aminoacylation of adenosine with BocPheEP in 200 mM, pH 8.0 phosphate buffer in the presence of 2',3'-isopropylidene adenosine as competitive ribonucleoside.
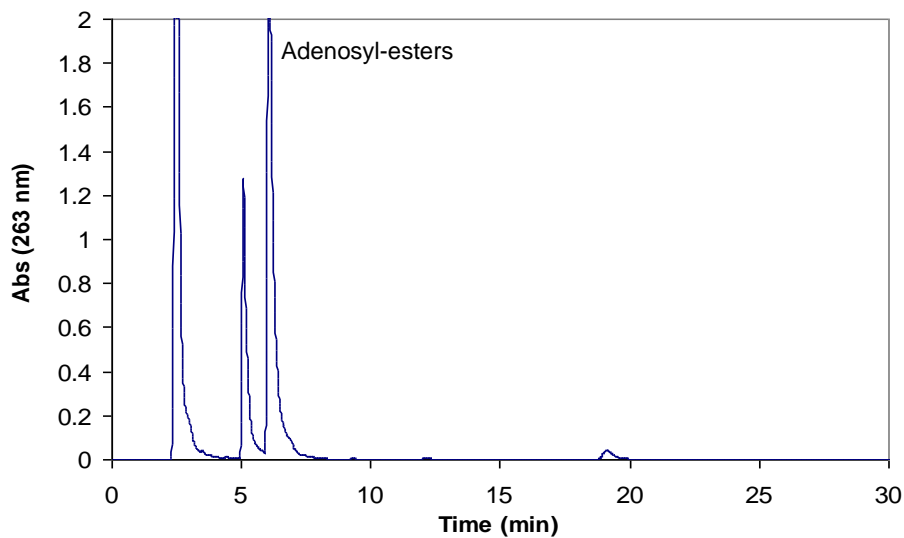
Figure 34. HPLC chromatogram for the lanthanum-mediated aminoacylation of adenosine with BocPheEP in 200 mM, pH 8.0 phosphate buffer in the presence of 2'-deoxyadenosine 5'-monophosphate as competitive ribonucleotide.
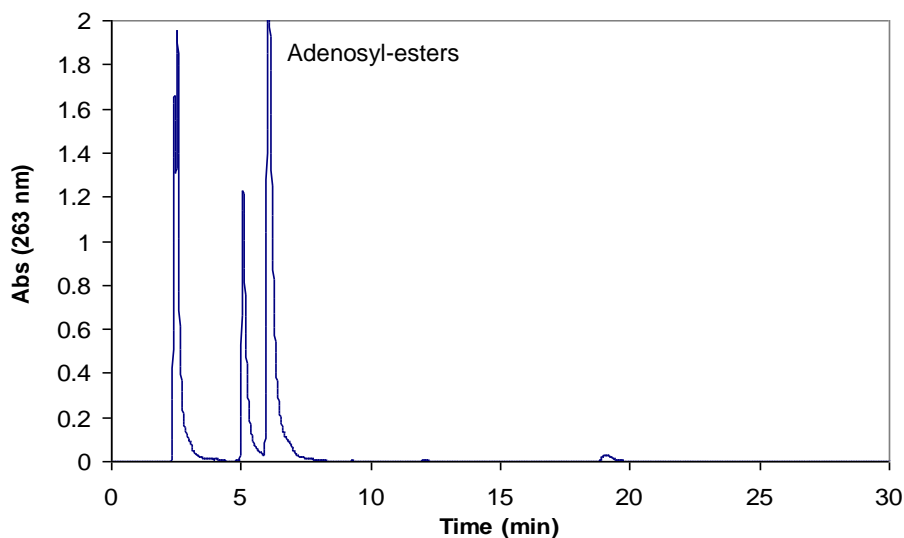
Figure 35. HPLC chromatogram for the lanthanum-mediated aminoacylation of adenosine with BocPheEP in 200 mM, pH 8.0 phosphate buffer in the presence of cytidine 3',5'-cyclic monophosphate as competitive ribonucleotide.
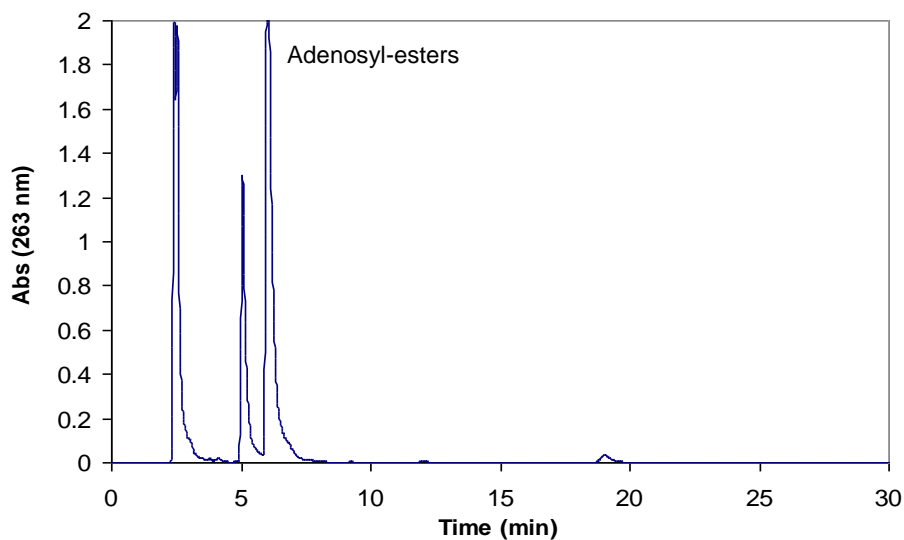
Figure 36. HPLC chromatogram for the lanthanum-mediated aminoacylation of adenosine with BocPheEP in 200 mM, pH 8.0 phosphate buffer in the presence of adenosine 3',5'-cyclic monophosphate as competitive ribonucleotide.
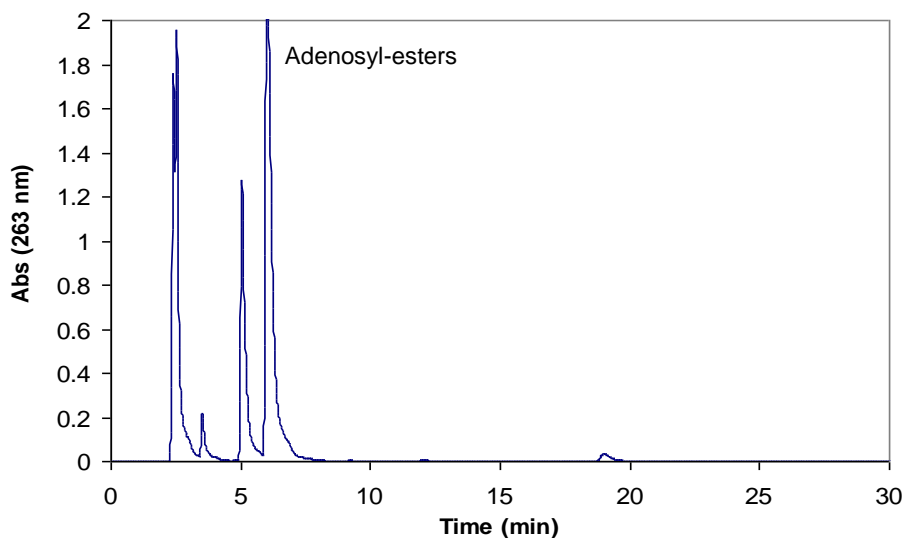
Figure 37. HPLC chromatogram for the lanthanum-mediated aminoacylation of adenosine with BocPheEP in 200 mM, pH 8.0 phosphate buffer in the presence of adenosine 3'-monophosphate as competitive ribonucleoside.
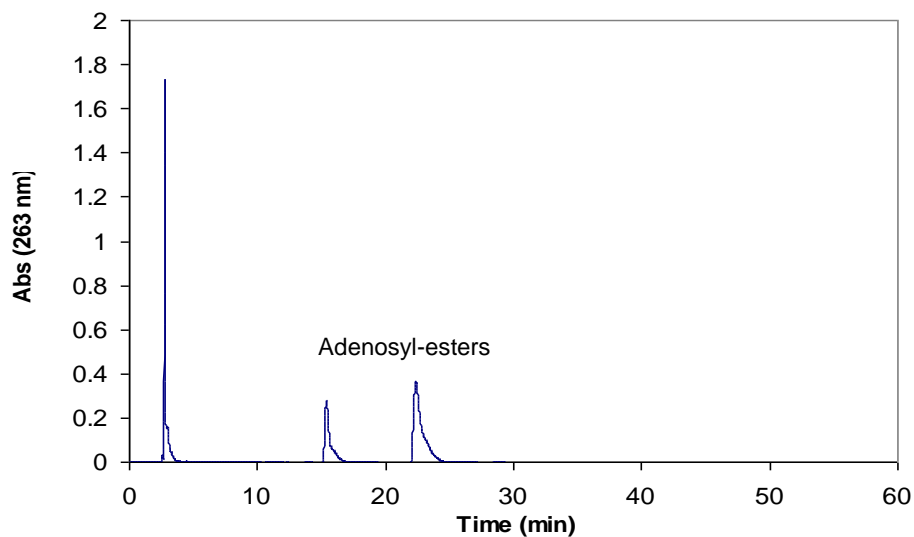
**Figure 38.** HPLC chromatogram for the lanthanum-mediated aminoacylation of adenosine with BocPheEP in 200 mM, pH 8.0 phosphate buffer in the presence of tRNA as competitive ribonucleotide. Reaction conditions: [adenosine] = 10 mM, [tRNA] = 8.8 uM, [La(OTf)$_3$] = 50 mM, [BocPheEP] = 50 mM, solvent: 40 % DMSO in 200 mM, pH 8.0 EPPS buffer.

Adenosine (10 mM) was reacted with equimolar concentrations of BocPheEP and La (OTf)$_3$ in the presence of 10 mM competing ribonucleoside or nucleotide (Table 12). Without any competition, 50.2 % yield of aminoacylation was obtained. In the presence of 5'-deoxyadenosine, we observed ester formation with both 5'-deoxyadenosine and adenosine. The introduction of 5'-deoxyadenosine resulted in a lower yield of 30.1 %. When the competing ribonucleosides containing no diol were added, 43.5% and 46.1 % yields were observed with 2',3'-dideoxycytidine and 2', 3'-isopropylideneadenosine, respectively. Phosphate groups were also introduced with the competing ribonucleotides lacking the diol functionality (2'-deoxyadenosine 5'-monophosphate, cytidine 3',5'-cyclic monophosphate, adenosine 3',5'-cyclic monophosphate, adenosine 3'-monophosphate). When equimolar concentrations of these nucleotides were added, the aminoacylation yields decreased to 20.1 %, 26.7 %, 48.4 % and 16.7 % for 2'-deoxyadenosine 5'-monophosphate, cytidine 3',5'-cyclic monophosphate, adenosine 3',5'-cyclic monophosphate, adenosine 3'-monophosphate, respectively.

Competition reactions were also studied with equimolar concentrations of adenosine and the competing nucleoside or nucleotide in the presence of 5 equivalents of La^{3+} and BocPheEP (Table 13). When excess reagents were available, there was no significant change in the yield of ester formation. 8.8 uM of tRNA was also added in the presence of equimolar concentrations of La^{3+} and BocPheEP (relative to adenosine). Additional peaks that may arise from the formation of aminoacyl-tRNA were not observed (Figure 38). The yield of aminoacyl-monoester of adenosine decreased from 50.2 % to 43.3 % with the addition of tRNA.

Table 11. Average yields for the aminoacylation of adenosine with BocPheEP in the presence of competing nucleoside or nucleotide. Reaction conditions: [adenosine] = 10 mM, [La(OTf)$_3$] = 10 mM, [BocPheEP] = 10 mM, [competing ribonucleoside/nucleotide] = 10 mM, solvent: 40 % DMSO in 200 mM, pH 8.0 EPPS buffer.

Entry	Competition ribonucleoside/nucleotide (CR)	Relative concentrations [La^{3+}]:[BP]:[adenosine]: [CR]	Yield (%)
1	None	1:1:1:0	50.2 ± 3.1
2	2',3'-dideoxycytidine	1:1:1:1	43.5 ± 2.9
3	5'-deoxyadenosine	1:1:1:1	30.1 ± 4.4
4	2',3'-isopropylidene-adenosine	1:1:1:1	46.1 ± 3.6
5	2'-deoxyadenosine-5'-monophosphate	1:1:1:1	20.1 ± 5.2
6	Cytidine 3',5'-cyclic-monophosphate	1:1:1:1	26.7 ± 6.1
7	Adenosine 3',5'-cyclic-monophosphate	1:1:1:1	48.4 ± 3.8
8	Adenosine 3'-monophosphate	1:1:1:1	16.7 ± 4.5

Table 12. Average yields for the aminoacylation of adenosine with BocPheEP in the presence of competing nucleoside or nucleotide. Reaction conditions: [adenosine] = 10 mM, [La(OTf)$_3$] = 50 mM, [BocPheEP] = 50 mM, [competing ribonucleoside/nucleotide] = 10 mM, solvent: 40 % DMSO in 200 mM, pH 8.0 EPPS buffer.

Entry	Competition ribonucleoside/nucleotide (CR)	Relative concentrations [La^{3+}]:[BP]:[adenosine]: [CR]	Yield (%)
1	None	5:5:1:0	82.3 ± 3.8
2	2',3'-dideoxycytidine	5:5:1:1	84.5 ± 4.1
3	5'-deoxyadenosine	5:5:1:1	76.7 ± 6.8
4	2',3'-isopropylidene-adenosine	5:5:1:1	82.2 ± 5.5
5	2'-deoxyadenosine-5'-monophosphate	5:5:1:1	83.9 ± 2.9
6	Cytidine 3',5'-cyclic-monophosphate	5:5:1:1	81.8 ± 6.9
7	Adenosine 3',5'-cyclic-monophosphate	5:5:1:1	84.2 ± 5.3
8	Adenosine 3'-monophosphate	5:5:1:1	82.6 ± 5.5

4.3.5 Effect of Mg^{2+} and poly-lysine on aminoacylation

Cations, such as Mg^{2+} and poly-lysine, were added to study its effect on binding to the anionic phosphate groups and minimizing the undesired lanthanum coordination to phosphates. When 0 – 100 equiv. of Mg^{2+} or 0 – 10 equiv. of poly-lysine were added relative to ribonucleotide, there was no significant effect on the yield of aminoacylation.

4.4 Discussion

Understanding the effect of lanthanum binding to phosphates is important for various reasons. First of all, in order for the reaction to be fully catalytic, lanthanum ions must be regenerated at the end of the reaction. In the previous studies on reaction inhibition by phosphates, it was observed that the addition of the ethyl phosphate byproduct results in the suppression of both the acylation and hydrolysis.[64] More importantly, understanding the binding

characteristics of lanthanum to phosphate is critical in extending the lanthanum-mediated aminoacylation method to the direct acylation of tRNA. In a 76 mer tRNA, there are 75 or 76 phosphate groups and only one cis-diol. Selective binding of lanthanum to the terminal diol will ultimately determine the efficiency of this acylation process.

With the increase in the concentration of phosphate anions, the yield of aminoacylation decreased significantly. The inhibition effect can be reduced with the addition of excess La^{3+} and BocPheEP. Similar results were observed in the competition studies with various ribonucleosides and nucleotides. The addition of competing ribonucleosides that contain no diols had no effect on the yield of aminoacylation. With the addition of competing ribonucleotides lacking the diol functionality (2'-deoxyadenosine 5'-monophosphate, cytidine 3',5'-cyclic monophosphate, adenosine 3',5'-cyclic monophosphate, adenosine 3'-monophosphate) a decrease in the yield of ester formation was observed. In the presence of 5 equivalents La^{3+}, the effect of inhibition by phosphate was alleviated. However, in the study of aminoacylation in phosphate buffers, the large excess of phosphate anions resulted in the complete inhibition of ester formation. These observations suggest that lanthanum is involved in a non-productive binding with phosphate anions, which leads to the reduced efficiency of aminoacylation. This inhibitory effect can be overcome in large excess of reagents. Inhibition of aminoacylation was also observed in comparative studies with 5'-AMP, 5'-ADP and 5'-ATP. The decrease in the observed yields of aminoacylation with increase in the concentration of competing phosphate groups further suggests that the lanthanum ions are being coordinated to the phosphate groups.

To test the possibility of reducing the undesirable binding of lanthanum to phosphates, aminoacylation was studied in the presence of Mg^{2+} or poly-lysine. Poly-lysine is a multiply charged cation that is known to irreversibly bind to tRNA's negatively charged phosphate backbone. Magnesium ions are widely used in biological systems to coordinate to the phosphate groups for stability and proper orientation of substrates.[1] When Mg^{2+} and poly-lysine were added to the aminoacylation of ribonucleosides and nucleotides, no significant effect was observed. The lack of apparent differences may be due to the stronger binding of trivalent lanthanum to the phosphate in comparison to the divalent magnesium. Also, poly-lysine has an average molecular weight of 70 – 150 kDa. It is possible that large size of poly-lysine may interfere with the selective coordination of lanthanum to diol.

4.5 Conclusions

Added phosphate derivatives inhibit lanthanum-mediated aminoacylation of ribonucleosides and nucleotides by forming an unproductive complex with lanthanum. This reduces the concentration of lanthanum that is available to bind to diols and form an ester bond. It is possible to reduce the effect of phosphate inhibition with excess lanthanum. The addition of Mg^{2+} and poly-lysine did not affect the phosphate inhibition of aminoacylation. The observation of the effect phosphates as inhibitors should prove useful for applying this technology towards the ultimate goal of direct aminoacylation of tRNA.

Chapter 5
Aminoacylation of ribonucleosides and nucleotides with unprotected aminoacyl ethyl phosphates

5.1 Introduction

Using *N-t*-Boc-aminoacyl alkyl phosphates, direct and selective monoacylation of ribonucleotides and RNA has been achieved.[52] The amino protecting group is required during the preparation of aminoacyl alkyl phosphates for selective activation of the carboxylic group.[54] However, this protecting group must be removed prior to ribosomal translation to allow elongation of the polypeptide chain. The deprotection of *t*-Boc group with trifluoroacetic acid degrades the tRNA structure. Thus, an alternate route was sought to first synthesize the aminoacyl alkyl phosphates free of protecting group, and then use this reagent in the aminoacylation reactions. Unprotected aminoacyl alkyl phosphates are straightforward to synthesize and are stable against hydrolysis in neutral aqueous solutions.[54] Since the α-amino group is not involved in the formation of bis-bidentate complex, the amino protection of the aminoacyl phosphate may not be necessary for the lanthanum-mediated acylation reaction.

Even though α-amino protection may not be necessary for efficient aminoacylation, it has been reported that α-amino group affects the stability of ester bond in aminoacyl-tRNA. At a pH lower than the pKa of α-amino group, the amino group exists mostly in the protonated form. This causes an inductive withdrawal of electron density from the adjacent carbonyl carbon resulting in an increased rate of hydrolysis of the ester linkage.[53,66-68] The aminoacyl bond is especially unstable at high pH and temperature.[69,70] The aminoacylation reaction may not be successful if the rate of deacylation is faster than the rate of aminoacylation.

In order to investigate the effects of α-amino protecting group in the lanthanum-mediated aminoacylation of tRNA, aminoacyl ethyl phosphates free of protecting group have been synthesized. These reagents have been used in reactions with ribonucleosides and nucleotides to determine their applicability in the lanthanum-mediated aminoacylation of diols.

5.2   Experimental

5.2.1   Materials and Methods

Unless otherwise noted, commercial reagents were used without further purification. Synthesis of *N*-unprotected aminoacyl ethyl phosphates were performed as outlined in Chapter 2.2. Water was doubly distilled and deionized prior to use. HPLC analysis was performed on a C18 reverse phase analytical (Phenomenex, Jupiter 4u Proteo 90A, 250 x 4.60 mm) or semi-preparative column (Phenomenex, Jupiter 10u Proteo 90A, 250 x 10.00 mm), and the products were detected at 263 nm. The mobile phase consisted of 10 % acetonitrile and 0.1 % TFA in water, and was eluted at a flow rate of 1 mL/min for the analytical column, and 3 mL/min for the semi-preparative column. Yields of ester formation are calculated based on the integrated areas of ester peaks. High-resolution mass spectrometry was performed at the Advanced Instrumentation for Molecular Structure (AIMS) Laboratory, Department of Chemistry at the University of Toronto.

5.2.2   Aminoacylation of ribonucleosides and nucleotides

All stock solutions were prepared fresh prior to each reaction. Reagents were added to a 1.5 mL Eppendorf tube in the order of buffer, ribonucleoside/nucleotide, magnesium chloride, BocPheEP and lanthanum. Reactions were stirred at room temperature, and then quenched at various time points with a saturated solution of EDTA-Na in water. Reaction products were separated by HPLC, isolated via lyophilization, and characterized by MS-ESI.

5.3   Results

5.3.1   Aminoacylation of ribonucleosides with PheEP

Lanthanum-mediated aminoacylation of ribonucleosides (adenosine, cytidine, uridine) with phenylalanyl ethyl phosphate (PheEP) lacking the *N*-protecting group was successful. Aminoacylation of ribonucleosides was rapid with the starting material completely consumed within a minute of reaction to generate a mixture of acylated products and the hydrolysis product. Products were separated by RP-HPLC and isolated by lyophilization. Characterization by ESI-MS confirmed the formation of two monoesters of adenosine, cytidine and uridine (Phe-adenosyl ester ESI-MS (+) calculated m/z 415.1724, found m/z 415.1711; Phe-cytidyl ester ESI-MS (+) calculated m/z 391.1612, found m/z 391.1599; Phe-uridyl ester ESI-MS (+) calculated m/z

392.1452, found m/z 392.1454). Control reactions without lanthanum ion showed no aminoacylation. When 2'-deoxycytidine was used as the starting material, only the hydrolysis product and starting materials were observed by HPLC.
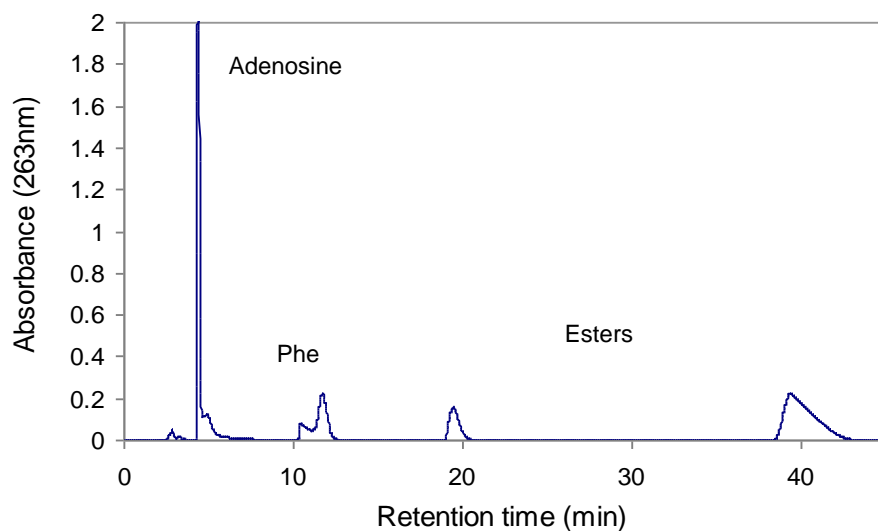
Figure 39. HPLC chromatogram for the aminoacylation of adenosine (10 mM) with PheEP (10 mM) and La(OTf)$_3$ (10 mM) in 100 mM, pH 6.0 MES buffer (Yield = 57.6 %).
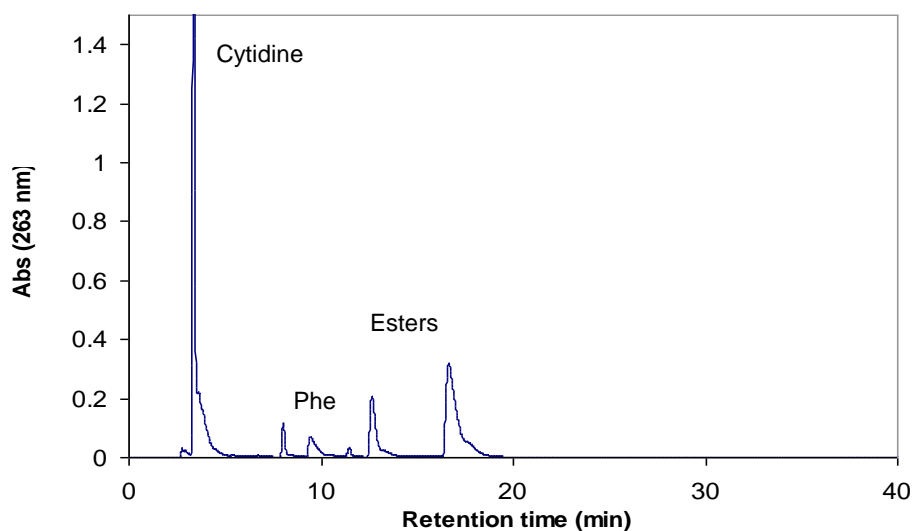
Figure 40. HPLC chromatogram for the aminoacylation of cytidine (10 mM) with PheEP (50 mM) and La(OTf)$_3$ (50 mM) in 200 mM, pH 6.0 MES buffer (Yield = 43.1 %).
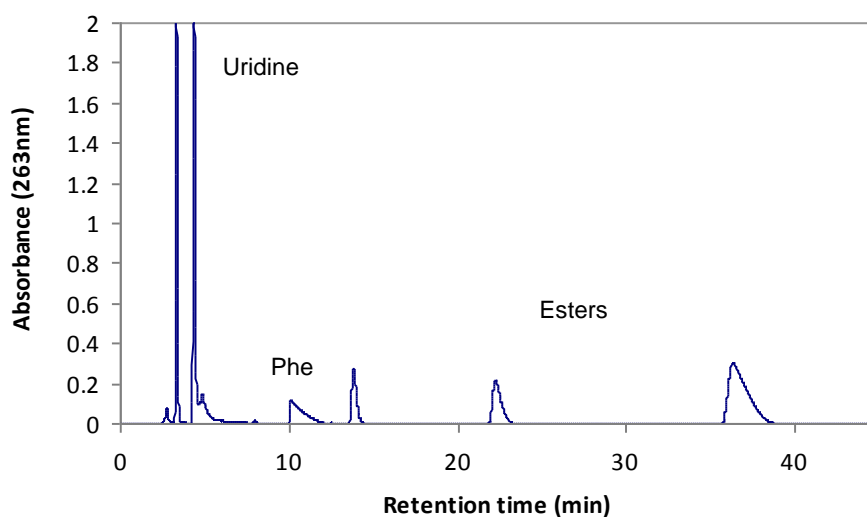
Figure 41. HPLC chromatogram for the aminoacylation of uridine (10 mM) with PheEP (10 mM) and La(OTf)$_3$ (10 mM) in 100 mM, pH 6.0 MES buffer (Yield = 58.7 %).

5.3.2 ^{1}H and ^{13}C NMR analysis of aminoacylation of uridine and cytidine with PheEP

The HPLC peaks corresponding to the two monoesters of uridine were collected, lyophilized and characterized by ^{1}H NMR to determine the site of ester formation. The chemical shift for the 1' proton of the ribofuranoside is sensitive to the location of ester: the 2'-aminoacyl ester interacts with the 1' proton to generate a doublet signal that is more downfield than the 3'-ester. The doublet corresponding to 1' proton appears at δ 6.06 for the 2'-ester and at δ 5.88 for the 3'-ester (Figure 42). The chemical shift for the 6' proton of the pyrimidine ring also shows a more downfield shift for the 2'-ester (δ 8.03) than the 3'-ester (δ 7.92). In addition, signals corresponding to both the 2'- and 3'-esters are shown in the NMR spectrum of either ester. Since the two esters were separated by HPLC prior to NMR analysis, this illustrates a rapid equilibrium of the two aminoacyl-esters.

The HPLC peaks corresponding to the two monoesters of cytidine were also collected and analyzed by ^{13}C NMR. Similar to the aminoacyl-uridine esters, the ^{13}C NMR spectrum of either cytidine esters also showed signals corresponding to both the 2'- and 3'-esters. By comparing the chemical shifts to native cytidine, it is possible to determine the site of acylation.

The observed changed in the chemical shifts of C2 and C3 carbons indicated that the esters were formed at the 2'- and 3'-hydroxyls (Table 13).
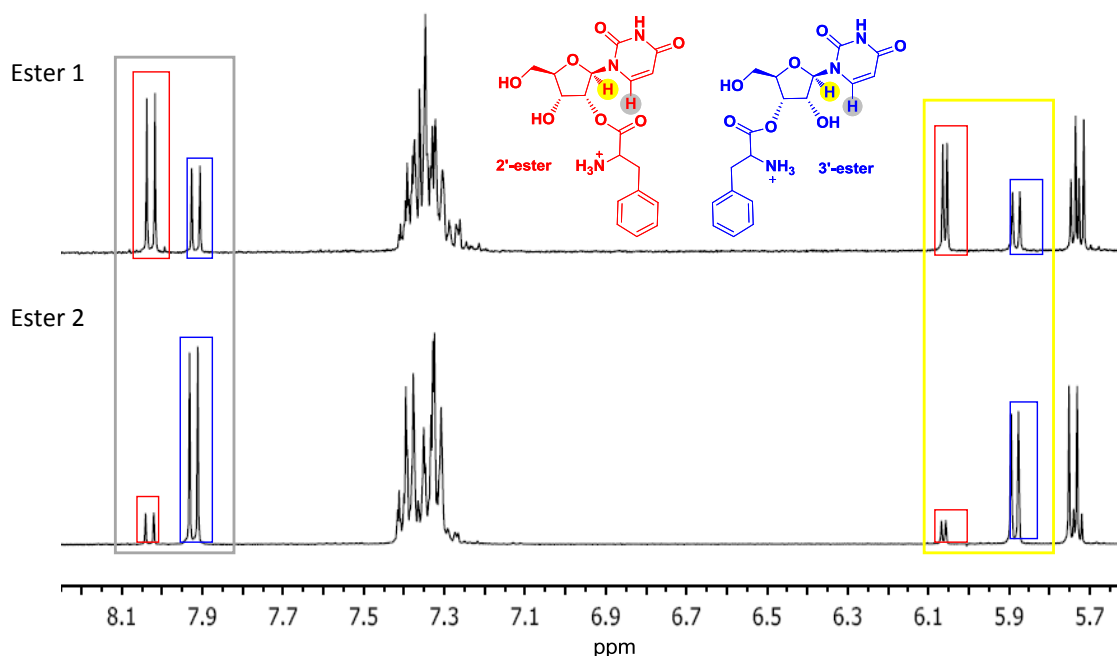
Figure 42. ^{1}H NMR spectra of 2'- and 3'-aminoacyl esters of uridine in CD$_3$OD.

Table 13. ^{13}C NMR chemical shifts for cytidine and Phe-monoesters of cytidine in CD$_3$OD*

Compound	C1	C2	C3	C4	C5
Cytidine	95.83	76.18	70.69	85.77	61.92
Ester 1	95.74	79.98 (2') 76.18 (3')	69.05 (2') 74.63 (3')	86.43 84.49	60.91 62.14
Ester 2	95.78	79.34 (2') 76.40 (3')	69.06 (2') 74.64 (3')	86.44 84.50	60.91 62.13

* (2') refers to peaks that correspond to 2'-ester of cytidine and (3') to 3'-ester of cytidine.

5.3.3   Polymerization of PheEP in water

Aminoacyl phosphate monoesters are unique in that they contain both the free amine and the activated carboxylic group. Under conditions where the free amine is not protonated (pH > pKa), it acts as a nucleophile toward the activated acyl group of another aminoacyl phosphate ester to form an amide. When PheEP was incubated in 250 mM, pH 8.0 HEPES buffer, oligomerization products were observed by ESI-MS (Figure 43). However, when the reactions

were carried out at a pH lower than the pK_a of the amino group (pKa = 7.8)[54] in the presence of lanthanum, we see suppression of oligomerization and achieve only monoacylation and along with competing hydrolysis.

Exact Mass: 294.14   Exact Mass: 312.15

Exact Mass: 459.22   Exact Mass: 606.28
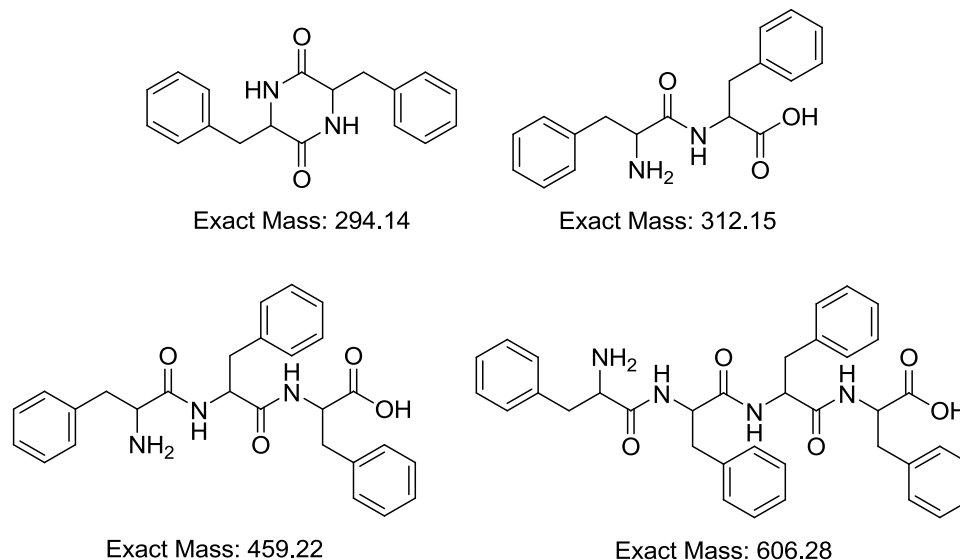
Figure 43. Oligomerization products of PheEP observed by ESI-MS.

5.3.4   Aminoacylation of ribonucleotides with PheEP

Aminoacylation of ribonucleotides (5'-AMP, 5'-CMP) with PheEP were also carried out in the presence of lanthanum. Rapid acylation was also observed. Separation by RP-HPLC and characterization by ESI-MS confirmed the identities as the Phe-monoesters of 5'-AMP and 5'-CMP (Figures 44, 45)
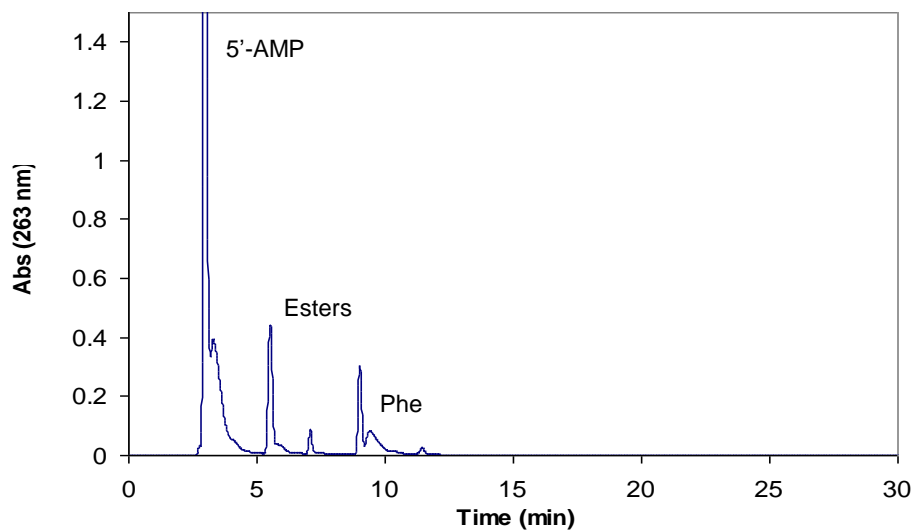
Figure 44. HPLC chromatogram for the aminoacylation of 5'-AMP (10 mM) with PheEP (50 mM) and La(OTf)$_3$ (50 mM) in 200 mM, pH 6.0 MES buffer.
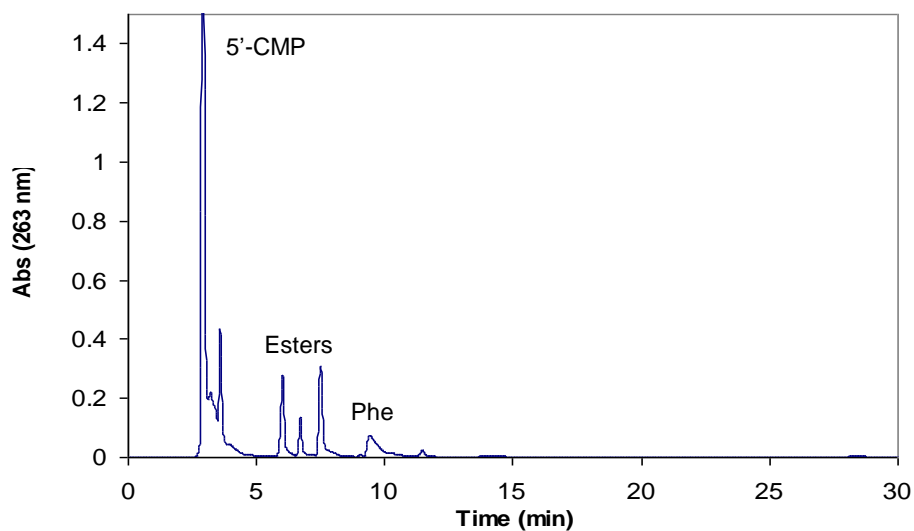
Figure 45. HPLC chromatogram for the aminoacylation of 5'-CMP (10 mM) with PheEP (50 mM) and La(OTf)$_3$ (50 mM) in 200 mM, pH 6.0 MES buffer.

5.4   Discussion

5.4.1   Aminoacylation of ribonucleosides and nucleotides with aminoacyl ethyl phosphates

Successful aminoacylation of ribonucleosides and nucleotides with PheEP demonstrated that the *N*-protecting group is not necessary for the lanthanum-mediated aminoacylation. Analysis by RP-HPLC and ESI-MS confirmed the rapid formation of monoesters of ribonucleosides and nucleotides. The resulting aminoacyl esters are stable toward hydrolysis in neutral solution. NMR analysis of the two phenylalanyl-monoesters of uridine and cytidine showed that the acylation occurs at the 2'- and 3'-hydroxyl groups. The NMR spectrum of either ester after separation by HPLC showed signals that correspond to both the 2'- and 3'-esters, which suggested that the two esters are in equilibrium. This observation is consistent with the previously reported transacylation of ribonucleosides.[71-73] Successful aminoacylation was also observed with TyrEP and FPheEP, which illustrates the potential applications of the lanthanum-mediated aminoacylation with a wide variety of amino acids.

5.4.2   Aminoacyl ethyl phosphates: versatile reagents for biomimetic reactions in water

Aminoacyl phosphate monoesters are stable toward hydrolysis in neutral aqueous solution. This property is attractive since it provides opportunities to perform reactions that would normally not be amenable in water be carried out in an aqueous solution. This reagent has been successfully utilized in the formation of a dipeptide between an *N*-protected aminoacyl phosphate and a free or *O*-protected amino acid.[74] It has also been applied to the selective aminoacylation of cis-diols.[50,52,64]

Aminoacyl ethyl phosphates free of the *N*-protecting group are unusual in that they contain both a nucleophile (free amine) and an electrophile (activated carboxyl group). At a reaction pH greater than the pK_a of the free amine ($pK_a = 7.8$),[54] the amino group is mostly in its unprotonated form. Under these conditions, the amino group undergoes nucleophilic attack at the electrophilic carbon of another molecule of aminoacyl ethyl phosphate to form an oligopeptide species. However, when the reactions are carried out in a slightly acidic condition (pH 6 – 7), the amino group remains protonated and the undesired peptide formation is suppressed. When lanthanum is introduced, selective aminoacylation of the cis-diol is achieved in addition to the

competing hydrolysis. Under these conditions, polymerization is very slow in comparison to the rapid monoacylation. This further demonstrates the unique properties and versatility of aminoacyl phosphate monoesters for a wide variety of biomimetic reactions in water.
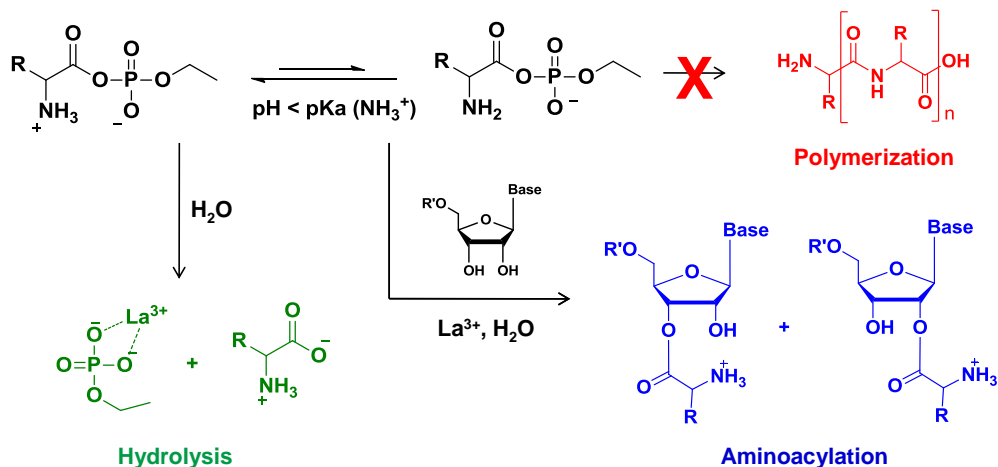
Figure 46. Reaction scheme showing the possible reactions with aminoacyl ethyl phosphate.

5.5 Conclusions

Efficient lanthanum-promoted aminoacylation of ribonucleosides and nucleotides was achieved with protecting-group-free aminoacyl ethyl phosphates. These results provide support for the application of aminoacyl phosphate monoesters in the direct aminoacylation of 3'-terminal hydroxyl of oligonucleotides and ultimately tRNA. Further studies must be undertaken with tRNA and other complex mimics of RNA in order to establish an efficient method of directly aminoacylating tRNA.

Chapter 6
Detection of aminoacylation by HPLC and efforts toward direct aminoacylation of tRNA

6.1 Introduction

Based on the promising results obtained using ribonucleosides and nucleotides, this method can be extended for applications in the direct aminoacylation of tRNA. Previous work on the lanthanum-mediated direct aminoacylation of tRNA has focused on the qualitative analysis of aminoacylation using specific amino acids that contain spectroscopic markers for detection.[52,64]

Using a fluorinated derivative of BocPheEP, aminoacylation of bulk RNA was monitored using ^{19}F NMR. Negative controls were performed using an oxidized RNA, where the terminal diol was oxidized to dialdehyde. Oxidized RNA is unable to undergo lanthanum-mediated aminoacylation as cis-1,2-diol is an imperative requirement for coordination and ultimately catalysis. By comparing the NMR spectra of the purified samples of RNA and the oxidized RNA after lanthanum-mediated aminoacylation, it was possible to detect the incorporation of fluorinated amino acid to the terminal diol of bulk RNA.[64]

To develop methods for detecting tRNA aminoacylation, an additional factor must be considered. Since tRNA reactions are carried out in micromolar (or lower) concentrations, conventional analytical techniques that require millimolar concentrations are not suitable for detection of tRNA aminoacylation. Dansylated amino acids were used to take advantage of the sensitive fluorescence technique. Reactions using dansylated aminoacyl ethyl phosphates were subjected to lanthanum-mediated reactions with tRNA and oxidized tRNA. An increase in fluorescence for reactions with tRNA in comparison to the negative control suggested successful aminoacylation.[64] Although these detection methods are sufficient for qualitative detection of aminoacylation at the terminal 3'-hydroxyl group, they do not allow optimization of reaction conditions through quantification. In addition, these techniques require specific functional groups in the amino acid side chain; therefore, is not general for analysis of various amino acids.

6.1.1   Separation of aminoacyl-tRNA from uncharged tRNA

For the quantification of aminoacylation, it is necessary to separate acylated tRNA from free tRNA. However, this separation is difficult using standard techniques due to their structural similarities. The two species differ only in the amino acid attachment; therefore, they have similar physical properties including UV absorbances and elution times. In order to overcome these difficulties, several methods have been developed that utilize radio-labeling and gel electrophoresis.[75-79]

Uhlenbeck and co-workers developed an assay utilizing an α-^{32}P-labeled tRNA to monitor the aminoacylation of tRNA.[78,79] In this method, α-^{32}P-labeled tRNA is prepared by using the enzyme tRNA nucleotidyltransferase, which catalyzes the removal and addition of the terminal nucleotides of tRNA. Radio-labeled tRNA undergoes aminoacylation, and the terminal α-^{32}P-labeled AMP is cleaved using a nuclease enzyme under acidic conditions. The digested tRNA is separated by TLC on polyethylimine (PEI) cellulose plates. The degree of aminoacylation can be quantified using a phosphorimager. This method provides a highly sensitive and accurate detection technique that is independent of the amino acid used.

In acid-urea polyacrylamide gel electrophoresis, tRNAs are separated under semi-denaturing conditions at an acidic pH.[75] These conditions results in the separation of slower-migrating aminoacyl-tRNA from faster-migrating free tRNA. The acidic environment also protects the labile ester linkage from hydrolysis. This technique is often followed by Northern blotting using [^{32}P]-labeled oligonucleotides as probes for quantitative analysis. By using this method, it is possible to separate tRNAs that differ by aminoacylation, formylation or modification at other sites. However, it is time-consuming as 18 to 24 hours are required to observe separation on gels as long as 45 cm. Additional time is required for Northern blotting, which adds complication for rapid analysis of aminoacylation.

6.1.2   Detecting aminoacylation of tRNA using HPLC

Various chromatography techniques such as ion exchange, benzoyl-DEAE-cellulose and reversed phase chromatography have been widely used for the purification of tRNA.[80-84] These methods as described in early reports are laborious and time-consuming. However, with the introduction of high-performance liquid chromatography (HPLC), it is possible to achieve highly

efficient separation of tRNA in less time. Separation of tRNA using reversed phase-HPLC involves a mobile phase that is high in ionic strength and low in pH.[85] High ionic strength of the mobile phase enhances the hydrophobic interaction between the nucleic acid and the stationary phase. Acidic conditions prevent hydrolysis of the labile ester linkage in aminoacyl-tRNA. Radiolabeled amino acids are frequently used to determine the efficiency of aminoacylation. In an attempt to optimize conditions for lanthanum-mediated aminoacylation of tRNA, reversed phase-HPLC was utilized using a previously reported method modified for a C8 column.

6.2   Experimental

6.2.1   Materials and Methods

Chemicals of reagent grade were used directly as received without further purification. Yeast tRNA^{Phe} and the enzymatically charged Phe-tRNA^{Phe} were generous gifts from Sutro Biopharma, San Francisco, California. Nuclease-free water from Fisher Scientific was used for all experiments involving tRNA. Illustra MicroSpin G-25 Columns from GE Healthcare were used for the purification of tRNA reactions. HPLC analysis was performed on a Waters system with Microsorb-MV C8 reverse phase analytical column. HPLC grade methanol was used in the eluent.

6.2.2   HPLC analysis

The eluents were 20 mM NH_4OAc, 400 mM NaCl, 10 mM MgOAc in water (pH 5.5, buffer A) and 40 % buffer A in methanol (buffer B). The eluents were filtered and degassed with helium for 15 minutes prior to use. The column was equilibrated in buffer A for at least 30 minutes and a linear gradient of buffer B (1.67 %/min) was initiated at the time of injection. The flow rate was 700 uL/min, and the eluting species were detected at 260 nm.

6.2.3   Aminoacylation of tRNA

All stock solutions (La(OTf)$_3$, MgCl$_2$, BocPheEP, PheEP, FmocPheEP) were made fresh. Calculated amounts of each reagent was added to a 1.5 mL Eppendorf tube to a total volume of 100 ul, and stirred with a micro stir bar. Reaction was quenched with 100 mM DTPA in 1.25 M NH_4OAc (pH 5.5). The quenched reaction mixture was eluted through Sephadex G-25 size-exclusion columns to remove compounds that are smaller than 10 kDa. tRNA was isolated by precipitation with ethanol, centrifugation and drying.

For reactions involving PCI extraction, acidified PCI (1 vol., phenol/chloroform/isoamyl alcohol = 25:24:1) was added, vortexed for 1 minute, and then micro-centrifuged for 2 minutes to separate the layers. The top aqueous layer was removed. To the organic layer was added 1 vol. of nuc-free H_2O, vortexed for 1 minute and micro-centrifuged for 2 minutes. The top aqueous layer was removed. To the combined aqueous layers was added 1 vol. of CI (chloroform/isoamyl alcohol = 24:1), vortexed for 1 minute and micro-centrifuged for 2 minutes. The aqueous layer that contains tRNA was separated, and tRNA was isolated by ethanol precipitation.

6.3   Results

6.3.1   HPLC separation of aminoacyl-tRNA from unacylated tRNA

An HPLC method was developed based on the published report by Cayama et al. with modifications adapted for a C8 column.[85] This method utilizes a mobile phase high in ionic strength and low in pH as a gradient with an increasing concentration of methanol. Using this method, successful separation of aminoacyl-tRNA (retention time = 31 min) from free tRNA (retention time = 24 min) was achieved. (Figure 47)
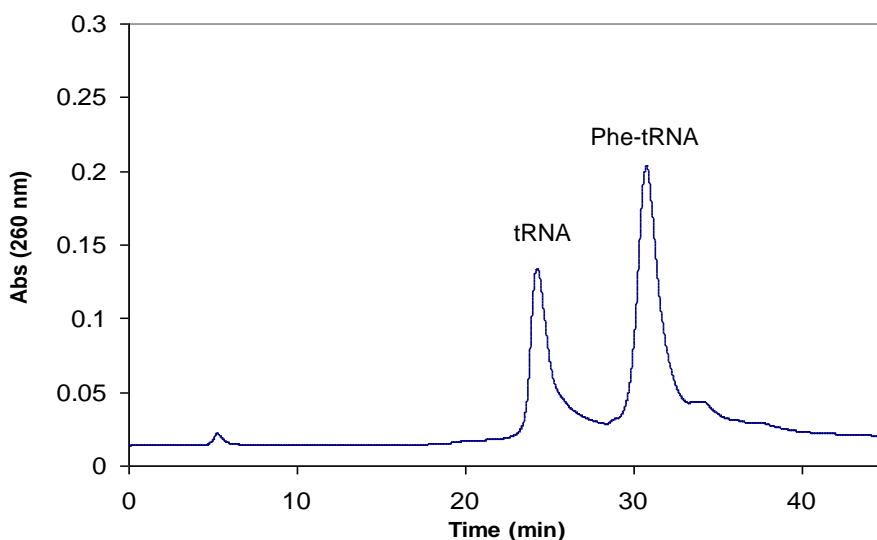
Figure 47. HPLC chromatogram illustrating the separation of aminoacyl-tRNA from unacylated tRNA using C8 reversed-phased chromatography.

6.3.2   Efforts towards aminoacylation of tRNA

Yeast tRNA^{Phe} was subjected to lanthanum-mediated aminoacylation under a variety of different reaction conditions. Crude unquenched reaction was analyzed by reversed phase HPLC (RP-HPLC). In the presence of large excess of reagents (> 1000 eq.) after one minute of reaction, a new peak appeared (retention time = 29 minutes) in addition to the peak corresponding to free tRNA (Figure 48). When the enzymatically acylated tRNA^{Phe} (Phe- tRNA^{Phe}) was co-injected with the reaction sample, two separate peaks appeared around 28 – 30 minutes (Figure 49). This peak also appeared in reactions without lanthanum metal (Figures 50), but disappeared after purification through size-exclusion column.

Aminoacylation was tested under various reaction conditions differing in buffer concentrations (0 – 500 mM), reagent concentrations (lanthanum triflate, aaEP, magnesium chloride, 0 – 5000 eq. relative to tRNA), reaction time (1 – 240 min), and solvent composition (10 – 50 % methanol, ethanol or DMSO). However, aminoacylation was not observed by RP-HPLC.

To test if the aminoacyl-tRNA was deacylated or removed during the workup process, enzymatically acylated Phe-tRNA^{Phe} (10 ug) was added to the reaction mixture and purified through a size-exclusion column. Analysis by RP-HPLC showed that Phe-tRNA^{Phe} remains acylated and is unaffected by the purification steps. (Figure 48)
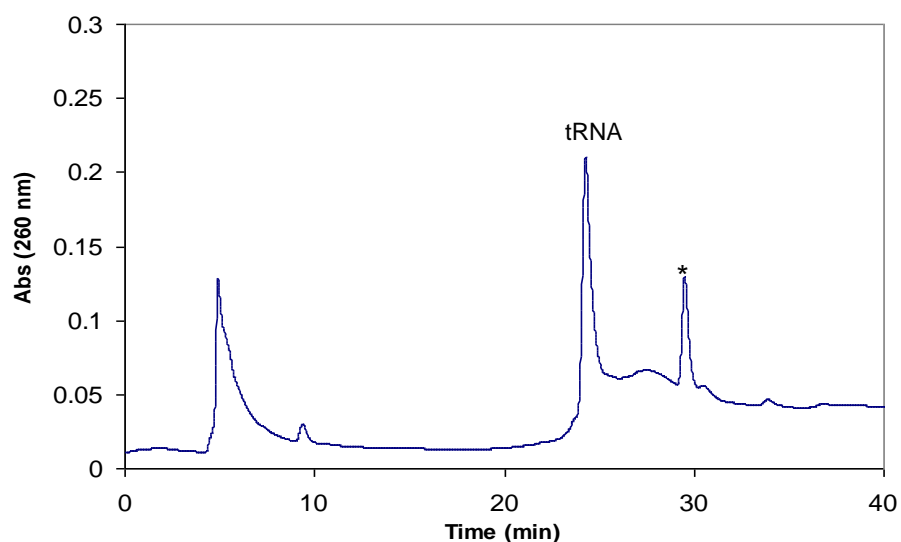
Figure 48. HPLC chromatogram for the reaction of tRNA (20 ug) with PheEP (2000 eq.), La(OTf)$_3$ (1000 eq.) and MgCl$_2$ (5000 eq.). A new peak (*) appeared at 29 minutes. Crude sample analysis before purification.
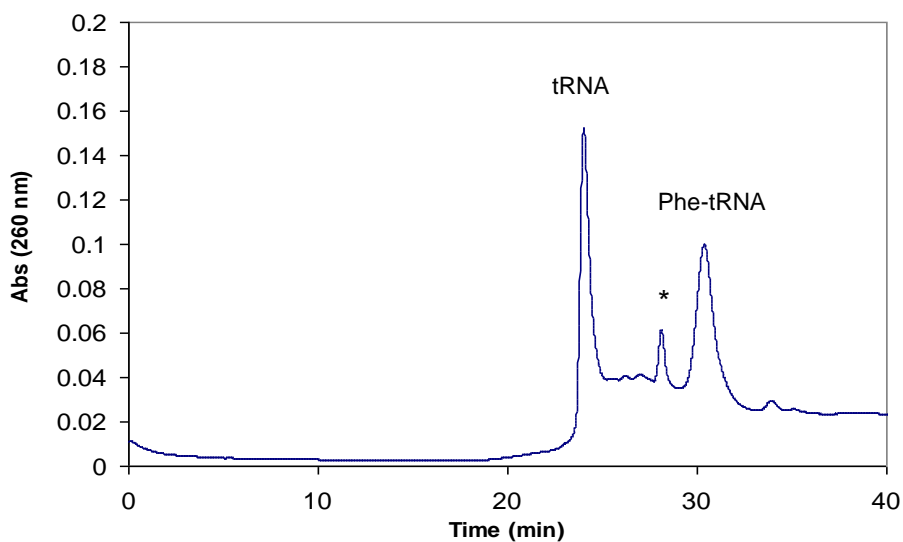
Figure 49. HPLC chromatogram for the reaction of tRNA (20 ug) with PheEP (2000 eq.), La(OTf)$_3$ (1000 eq.) and MgCl$_2$ (5000 eq.) co-injected with a pure sample of Phe-tRNA (31 min).Crude sample analysis before purification.
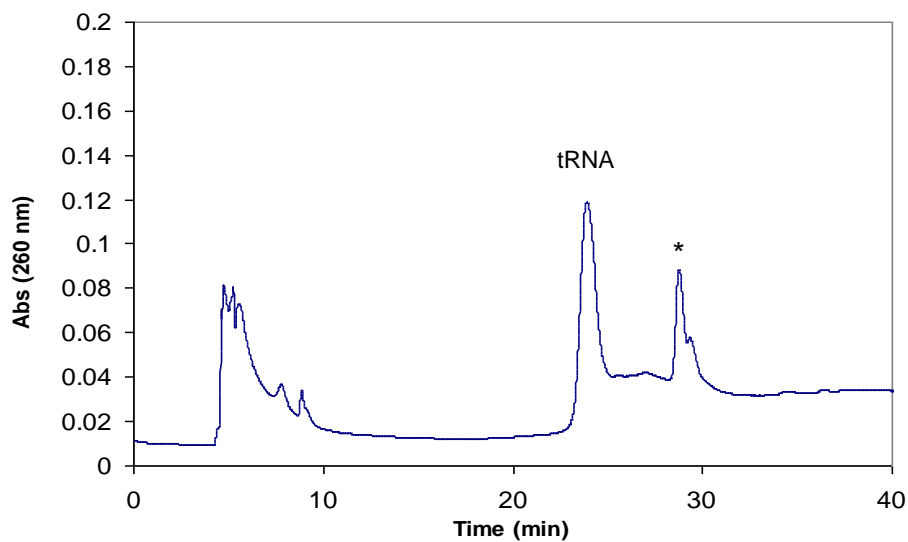
Figure 50. HPLC chromatogram for the reaction of tRNA (20 ug) with PheEP (2000 eq.). Crude sample analysis before purification. A new peak (*) appeared at 29 minutes.

6.3.3   Effect of subsequent addition of reagents

In order to ensure that sufficient reagents are available during the course of the reaction, fresh solutions of lanthanum and aminoacyl ethyl phosphate were added throughout the reaction. 50 equivalents of lanthanum and PheEP were subsequently added every 5 minutes to a total of 30 minutes, and the crude sample was analyzed. In addition to the peak corresponding to free tRNA, a new peak appeared at 28 minutes. (Figure 51) However, this peak disappeared upon elution through the size-exclusion column. (Figure 52)
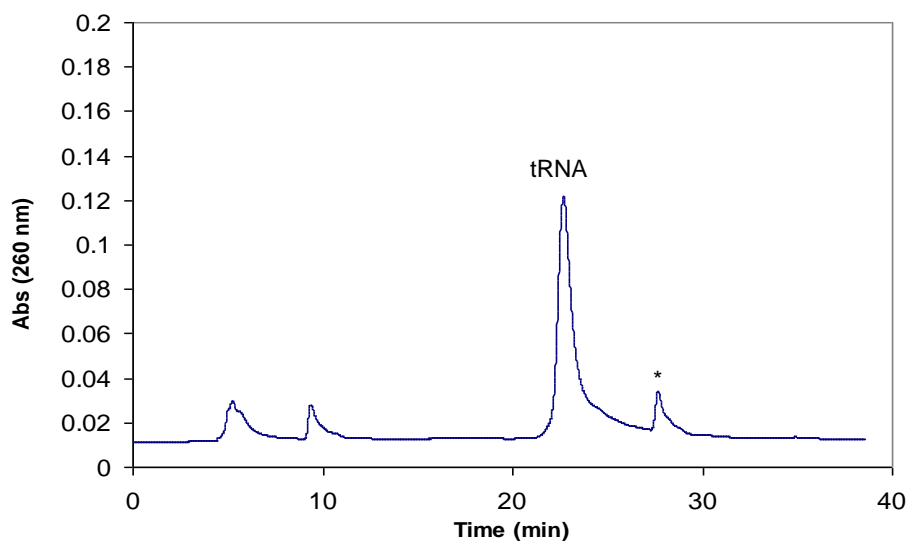
Figure 51. HPLC chromatogram for the reaction of tRNA (50 ug) with PheEP in the presence of MgCl$_2$ (1000 eq.) in HEPES (pH 7.0). Fresh solutions of PheEP (50 eq.) and La(OTf)$_3$ (100 eq.) were added every 5 minutes to a total of 30 minutes. Crude sample before purification.
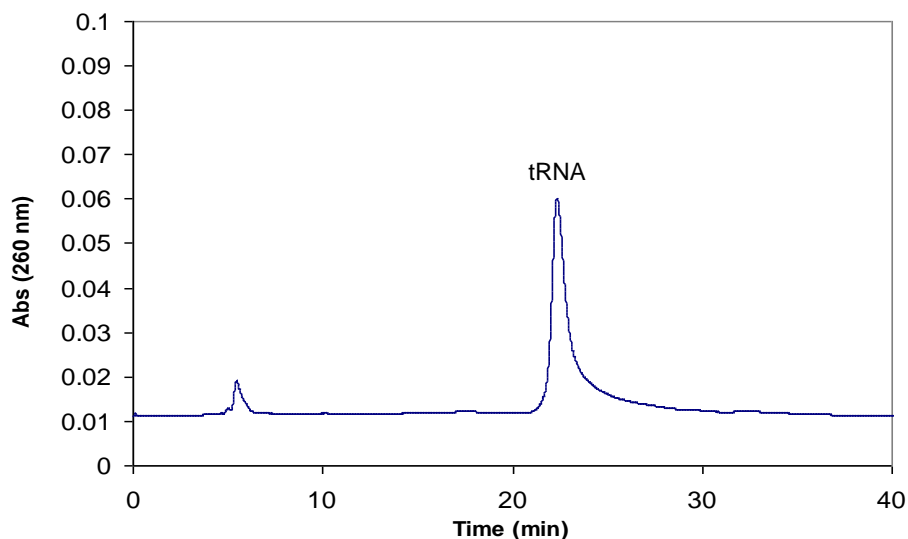
Figure 52. HPLC chromatogram for the reaction of tRNA (50 ug) with PheEP in the presence of MgCl$_2$ (1000 eq.) in HEPES (pH 7.0) after purification through size-exclusion column.

6.3.4 Increasing coordination of terminal diol with lanthanum by tRNA denaturation

Yeast tRNA^{Phe} was thermally and chemically denatured in order to make the terminal diol more accessible for coordination to lanthanum. Thermal denaturation was performed at two different temperatures. First, tRNA was first incubated at 95 ℃ for 5 minutes to unfold its secondary structure and the reagents (La(OTf)$_3$, PheEP, MgCl$_2$) were added while cooling to room temperature. After 30 minutes and purification through size-exclusion column, aminoacylation was not observed. Reactions were also performed at 60 ℃ with the constant addition of lanthanum triflate and PheEP (100 eq.) to replace the reagents that may undergo rapid hydrolysis at a higher temperature. After 30 minutes of reaction and purification through size-exclusion column, aminoacylation was not observed.

Chemical denaturation was achieved using organic co-solvents. Methanol, ethanol and DMSO were used with aqueous buffer in compositions of 10 – 50 % of organic solvents. However, aminoacylation was not observed after 30 and 60 minutes of reaction.

6.3.5 Aminoacylation with combination of lanthanides

A combination of different lanthanides was used to investigate its effects in increase the availability of lanthanum towards aminoacylation. A mixture containing equimolar concentrations of lanthanum and a smaller lanthanide (Pr^{3+}, Nd^{3+}, Tb^{3+}, Yb^{3+}) was used in the aminoacylation reactions. In all reactions, no aminoacylation was observed (Table 14).

Table 14. Reaction conditions studied with different lanthanide ions.

Entry	Lanthanide metal	Reaction conditions
1	La(OTf)$_3$ only	20 ug tRNA, 80 mM MgCl$_2$, 80 mM La(OTf)$_3$, 80 mM PheEP in HEPES (pH 7.0)
2	La(OTf)$_3$ / PrCl$_3$	20 ug tRNA, 80 mM MgCl$_2$, 40 mM La(OTf)$_3$, 40 mM PrCl$_3$, 80 mM PheEP in HEPES (pH 7.0)
3	La(OTf)$_3$ / Nd$_2$(SO$_4$)$_3$	20 ug tRNA, 80 mM MgCl$_2$, 40 mM La(OTf)$_3$, 40 mM Nd$_2$(SO$_4$)$_3$, 80 mM PheEP in HEPES (pH 7.0)
4	La(OTf)$_3$ / Yb(OTf)$_3$	20 ug tRNA, 80 mM MgCl$_2$, 40 mM La(OTf)$_3$, 40 mM Yb(OTf)$_3$, 80 mM PheEP in HEPES (pH 7.0)
5	La(OTf)$_3$ / EuCl$_3$	20 ug tRNA, 80 mM MgCl$_2$, 40 mM La(OTf)$_3$, 40 mM EuCl$_3$, 80 mM PheEP in HEPES (pH 7.0)

6.3.6   Aminoacylation with crown-ether ligated lanthanum

18-crown-6 and dibenzo-18-crown-6 were added to coordinate to lanthanum prior to aminoacylation reactions. When crown-ether ligated lanthanum (100 eq.) was used in the aminoacylation of tRNA (50 ug) with BocPheEP (100 – 500 eq.) or PheEP (100 – 500 eq.) no aminoacylation was observed. (Figures 53, 54)
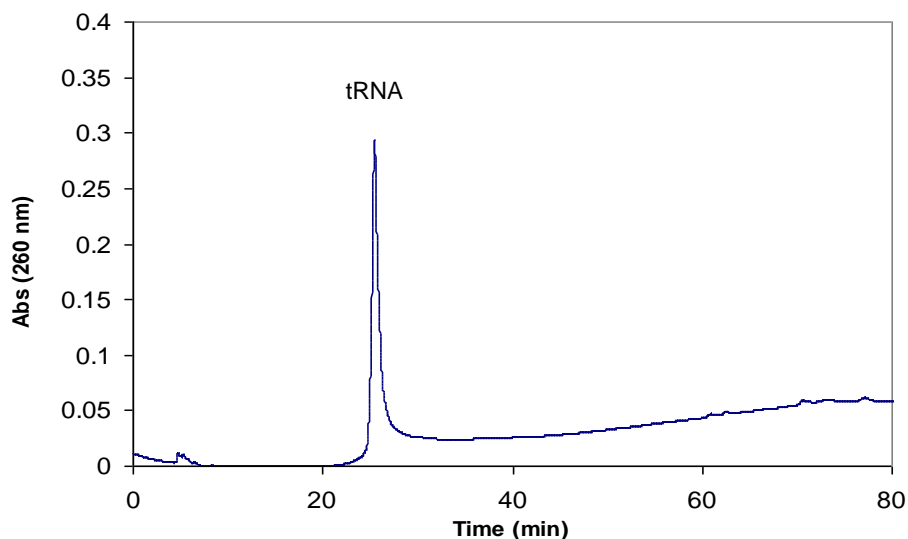
Figure 53. HPLC chromatogram for the reaction of tRNA (50 ug) with BocPheEP (100 eq.), 18-crown-6 (120 eq.), La(OTf)$_3$ (100 eq.) in EPPS (pH 8.0). Crude sample before purification.
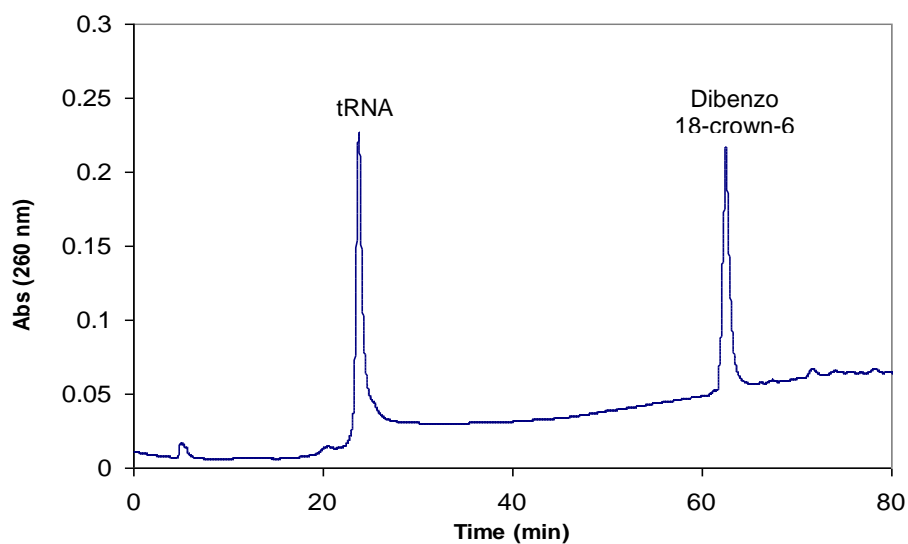
Figure 54. HPLC chromatogram for the reaction of tRNA (50 ug) with BocPheEP (100 eq.), dibenzo-18-crown-6 (120 eq.), La(OTf)$_3$ (100 eq.) in EPPS (pH 8.0). Crude sample before purification.

6.3.7 Efforts toward aminoacylation of tRNA with BocPheEP, FmocPheEP, TyrEP and FPheEP

Five different *N*-protected and unprotected aminoacyl ethyl phosphates were utilized in the lanthanum-mediated aminoacylation of tRNA to test their effect. In reactions with BocPheEP, FmocPheEP, TyrEP, FPheEP and PheEP under various reaction conditions ([tRNA] = 0.05 – 0.5 ug/uL, [buffer] = 0 – 500 mM, pH 6.0 – 8.0, [La(OTf)$_3$], [aaEP], [MgCl$_2$] = 0 – 5000 eq., reaction time = 0 – 240 min), no aminoacylation was observed.

6.3.8 Effect of poly-lysine in aminoacylation of tRNA

The effect of poly-lysine in neutralizing the negative charges of the phosphate backbone was studied. Poly-lysine (1 – 5 eq. relative to tRNA) was added to the tRNA (10 ug) solution to form a complex prior to subjecting to lanthanum-mediated reactions with PheEP and BocPheEP (1000 eq. of lanthanum and aaEP) in HEPES (pH 7.0, 50 mM) for 30 minutes. Crude and purified samples were analyzed by HPLC, and showed no aminoacylation. (Figure 55, 56)
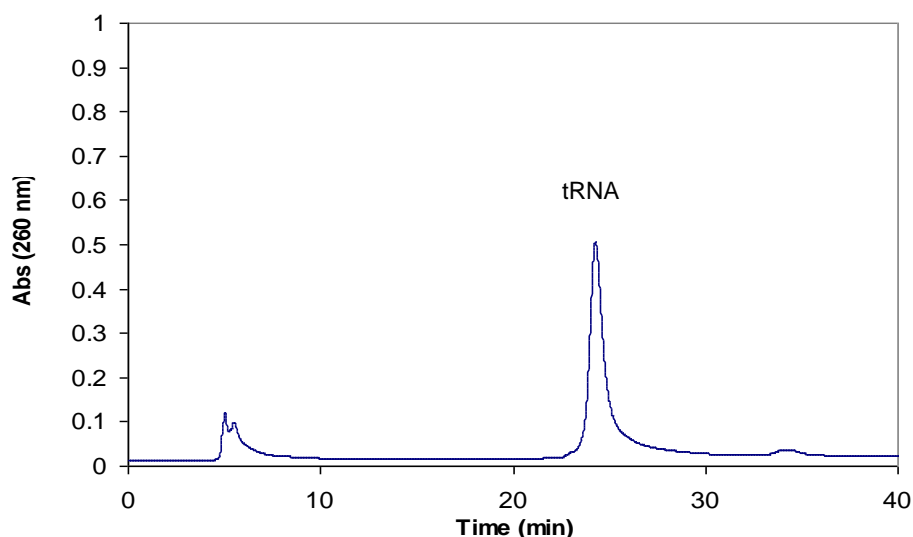
Figure 55. HPLC chromatogram for the reaction of tRNA (10 ug) with PheEP (1000 eq.) and La(OTf)$_3$ (1000 eq.) in the presence of polylysine (1 eq.) in pH 7.0 HEPES buffer.
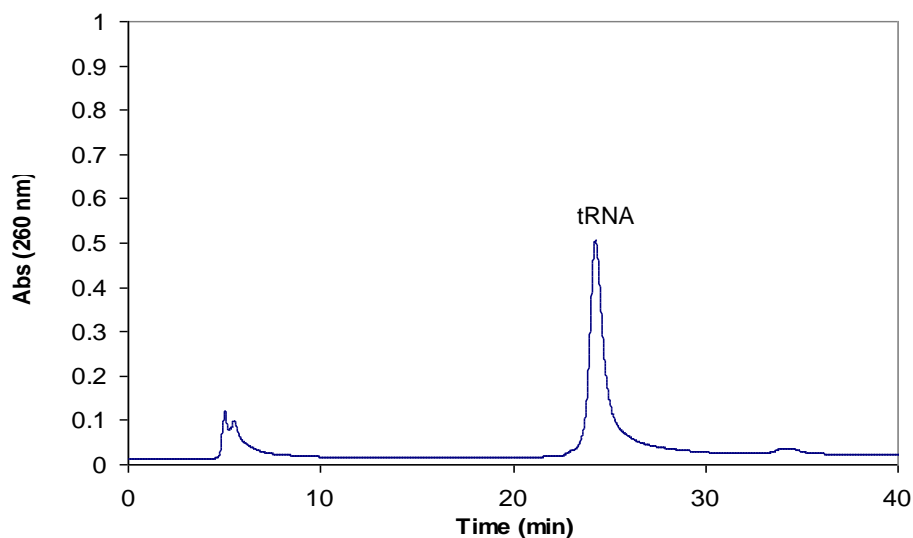
Figure 56. HPLC chromatogram for the reaction of tRNA (10 ug) with PheEP (1000 eq.) and La(OTf)$_3$ (1000 eq.) in the presence of polylysine (5 eq.) in pH 7.0 HEPES buffer.

6.3.9   Testing the stability of ester bond in aminoacyl-tRNA

The stability of ester bond in aminoacyl-tRNA was studied by incubating the enzymatically charged Phe-tRNA^{Phe} in the lanthanum-mediated aminoacylation reactions solutions. When Phe-tRNA^{Phe} (10 ug) was incubated in a solution containing 1000 eq. MgCl$_2$, 500 eq. La(OTf)$_3$ in HEPES (pH 7.0, 50 mM) buffer, deacylation is observed after 30 minutes. The peak corresponding to the free tRNA increases after 60 minutes of reaction.
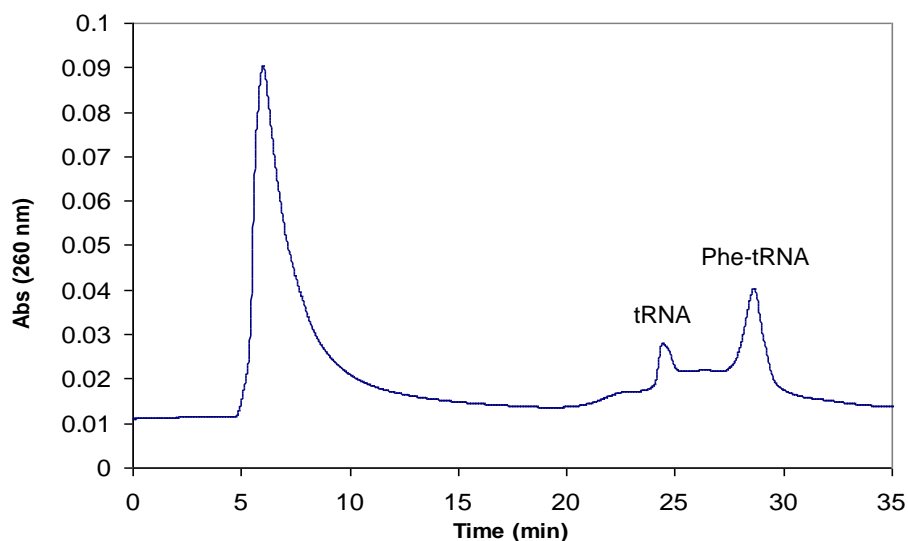
Figure 57. HPLC chromatogram illustrating deacylation of Phe-tRNA (10 ug) after 30 min incubation in a solution containing MgCl$_2$ (1000 eq.), La(OTf)$_3$ (500 eq.) and HEPES (pH 7.0).
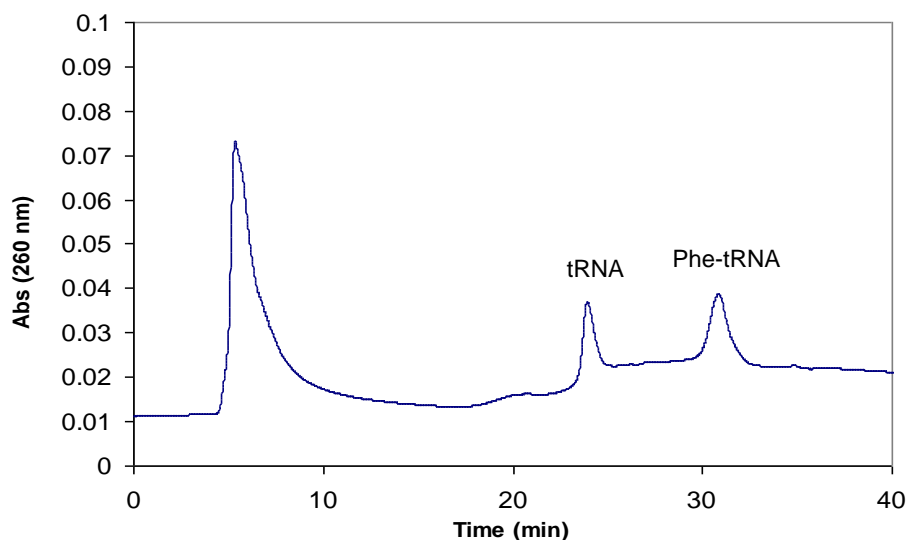
Figure 58. HPLC chromatogram illustrating deacylation of Phe-tRNA (10 ug) after 60 min incubation in a solution containing $MgCl_2$ (1000 eq.), $La(OTf)_3$ (500 eq.) and HEPES (pH 7.0).

6.4   Discussion

6.4.1   HPLC detection of lanthanum-mediated aminoacylation of tRNA

An HPLC method utilizing a C8 reversed-phased column has been developed to provide a rapid analysis of the aminoacylation of tRNA. This method uses eluents that are high in ionic strength (20 mM NH_4OAc, 500 mM NaCl, 10 mM MgOAc) and low in pH (pH 5.5). The high ionic strength assists in the separation by increasing the hydrophobic interaction between the polyanionic tRNA and the stationary phase. The low pH ensures that the labile ester bond of the aminoacyl-tRNA is maintained during analysis.

Direct aminoacylation of tRNA was tested under a variety of reaction conditions and analyzed by RP-HPLC. After 1 minute of reaction, a new peak appeared at approximately 29 minutes. However, when co-injected with the enzymatically acylated sample of Phe-tRNA[Phe], the two peaks do not co-elute. This peak is present in reactions in the absence of lanthanum, but disappears upon purification through a size-exclusion column. Based on these observations, the additional peak near 29 minutes appears to be a side product arising from the large excess of La^{3+} and PheEP. Aminoacylation of tRNA was studied under various conditions that differ in the buffer concentration, reagent concentrations, reaction time, solvent composition and temperature.

However, aminoacylation was not detected by RP-HPLC. Based on the lack of aminoacylation observed from the preliminary studies, efforts were made to determine the factors that affect successful aminoacylation.

6.4.2   Increasing La^{3+} coordination to 3'-terminal diol of tRNA

It is possible that there is a lack of coordination between La^{3+} and the 3'-terminal diol of tRNA. The three-dimensional secondary structure of tRNA may be blocking the terminal diol from forming an efficient coordination with La^{3+}. Therefore, in order to make the terminal diol more accessible for coordination, tRNA was chemically and thermally denatured. The addition of organic co-solvents, such as ethanol, methanol and DMSO, perturbs the three-dimensional structure of tRNA, and also reduces the concentration of water reducing hydrolysis. Thermal unfolding of tRNA was achieved by conducting the reaction at 60 ℃ with constant addition of reagents or heating the tRNA to 95 ℃. Both methods of unfolding tRNA did not result in successful aminoacylation.

Another possibility for the lack of coordination between La^{3+} and the 3'-terminal diol of tRNA is the formation of unproductive binding lanthanum to phosphates. Lanthanides are known to coordinate strongly to phosphates. Considering that there are multiple phosphate groups in the backbone of tRNA and the byproduct of aminoacyl ethyl phosphate, lanthanum may not be available for catalysis if unproductive coordination is formed with the phosphate groups. In order to overcome this challenge, La^{3+} ions were added in combination with a smaller lanthanide. By "lanthanide contraction" which describes the decrease in the atomic radius of lanthanides with the increase in atomic number, smaller lanthanides coordinate to phosphates tighter than larger lanthanides.[55] The addition of smaller lanthanides would preferentially bind to the phosphate groups freeing the larger lanthanum ions to coordinate to the terminal diol. Various equivalents of lanthanum and smaller lanthanides (Pr^{3+}, Nd^{3+}, Tb^{3+}, Yb^{3+}) were tested to determine their effects.

Ligand-coordinated La^{3+} complexes were investigated as an alternative method of selectively coordinating to the terminal diol over phosphate groups. Kobayashi et al. have developed chiral crown ether-ligated lanthanides for asymmetric aldol reactions.[86] In this reaction, the steric bulk introduced by the ligand directs preferential reaction at one face of the starting material. In utilizing the ligand-coordinated lanthanide complexes, the choice of ligand is

critical. Ligands with a strong coordinating ability would reduce the Lewis acidity of the lanthanide ion, and those with a weak coordinating ability would generate free metal cations due to the lack of coordination to the ligand.[86] For the preliminary studies involving crown-ethers, 18-crown-6 and dibenzo-18-crown-6 were selected for coordination to lanthanum and subjected to aminoacylation of tRNA. It was proposed that the crown-ether ligand would favour coordination to the terminal diol over the sterically hindered phosphates of the tRNA backbone. However, no aminoacylation was detected by HPLC.

Poly-lysine and magnesium ions were also added to restrict the unproductive binding of lanthanum to phosphate backbone. Blocking the phosphate backbone would enhance the availability of lanthanum for coordination to the terminal diol and successful aminoacylation. The lack of influence of magnesium suggests that the trivalent lanthanum may be binding to the phosphate group stronger than the divalent magnesium ions. Moreover, multiple positive charges introduced by the addition of poly-lysine may interfere with the efficient formation of bis-bidentate complex. The phosphate end of the aminoacylating reagents are also negatively charged, which may have electrostatic interactions with poly-lysine.

6.4.3 Subsequent addition of reagents

During the course of lanthanum-mediated aminoacylation of tRNA, the reagents (La^{3+} and PheEP) may undergo side reactions, such as polymerization or hydrolysis, which reduces the amount of reagent that is available for aminoacylation. To ensure that sufficient reagents are available throughout the reaction, a fresh solution of La^{3+} and PheEP were added to the reaction every 5 minutes to a total of 30 minutes. In comparison to the reaction where the same amount of reagents was added only at the beginning of the reaction, subsequent addition throughout the reaction had no effect on the aminoacylation. Following the purification through a size-exclusion column, no aminoacylation was observed.

6.4.4 Determining stability of aminoacyl-tRNA during lanthanum-mediated aminoacylation

Given the lack of observed aminoacylation by HPLC, we considered the possibility that the reaction conditions may be promoting the deacylation of aminoacyl-tRNA. Since aminoacyl-tRNAs without the *N*-protecting group are less stable in aqueous solution than the protected aminoacyl-tRNAs, the stability of enzymatically acylated Phe-tRNA[Phe] was tested in the reaction

conditions. After 30 minutes of incubation in the reaction solution containing lanthanum and buffer, we observe the peak corresponding to the free tRNA on the HPLC. After 60 minutes of incubation, the tRNA peak increases further. This suggests that deacylation may occur during the course of the reaction, and this could be a cause for the lack of observed aminoacylation.

Aminoacylation reactions were also studied with amino acids that contain bulky protecting groups. Aminoacyl-tRNAs that contain bulky amino acid side chains or large hydrophobic *N*-protecting groups have been reported to have greater stability than those without. However, when reactions were performed with BocPheEP and FmocPheEP, acylation was not detected by HPLC.

6.4.5   Limit for detection of aminoacyl-tRNA

Analysis by HPLC provides a simple quantitative method of detecting aminoacylation. In comparison to the previous methods that utilize specialized detection probes, such as radio- or fluorescence labeling,[64] HPLC separation followed by UV detection can be achieved with any natural or unnatural amino acids. However, one drawback of HPLC-UV detection technique is its lower sensitivity. The UV detection limit for the analysis of tRNA was approximated to be 0.2 – 1 ug, which corresponds to 10^{-12} – 10^{-11} mol of tRNA.[87] The practical detection limit may be even higher than the reported value due to the unstable baseline. In comparison, the detection limit for Uhlenbeck's method utilizing α-^{32}P-tRNA is estimated to be 3 x 10^{-22} mol/mm^2, and the fluorescence labeling technique using dansylated amino acid has a limit of 10^{-14} mol.[88-90]

In these studies, 10 – 50 ug of tRNA was used to test lanthanum-mediated aminoacylation. Based on the reported detection limit for HPLC-UV analysis, a minimum of 0.4 – 2 % yield is required to detect aminoacylation by HPLC. Sensitivity may be further reduced by minor contaminants and the raised baseline, which would make detection by HPLC-UV difficult. Since the detection limit for HPLC-UV depends largely on the hardware, a range of concentrations of tRNA can be used to determine the limit for this elution method. Furthermore, detection by a more sensitive technique utilizing radioactive tRNA or amino acid may be required to observe aminoacylation.

6.5   Conclusions

The aminoacylation of tRNA can be monitored using RP-HPLC, which provides a simple and rapid separation of aminoacyl-tRNA from the free tRNA. The lack of observed aminoacylation may be due to various factors, including the formation of unproductive coordination of lanthanum to the phosphate backbone of tRNA, reduced accessibility of the terminal diol of tRNA, possible deacylation during the course of reaction, and the high detection limit of the UV detector. Further investigation must be conducted with tRNA in order to provide an insight into the mechanistic details of the reaction.

Chapter 7
Detection of aminoacylation by MALDI-MS and efforts toward direct aminoacylation of tRNA

7.1   Introduction

Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) is widely used for the analysis of various biomolecules and large organic compounds. It uses a soft ionization technique which minimizes fragmentation and allows ionization of large fragile molecules. MALDI-MS has also been proven useful for the rapid analysis of tRNAs and their complexes. This technique has been utilized to distinguish between aaRS complexes of cognate and non-cognate tRNAs, and to analyze tRNA mutants.[91,92] Petersson et al. also applied MALDI-MS technique to monitor the ligation of aminoacyl dinucleotide to a truncated tRNA for the formation of aminoacyl-tRNA.[93] This allows a quick and simple method of assessing aminoacylation of tRNA, which is crucial for the investigation of efficient aminoacylation methods.

Duffy et al. used the MALDI-TOF-MS technique to analyze lanthanum-mediated direct aminoacylation of tRNA using a photolabile protecting group.[94] They obtained tRNA charged with NVOC-protected amino acids in yields up to $5 - 25$ %. Following photolytic cleavage of the protecting group aminoacyl-tRNA has been utilized in an expression system in *Xenopus laevis* oocytes to obtain protein that has properties identical to those of the native protein. Unnatural amino acids are successfully incorporated into various neuroreceptors in order to provide an insight into the binding mechanism of natural ligands and pharmaceuticals. These results provide an important demonstration of the feasibility of direct aminoacylation for incorporating unnatural amino acids into proteins. However, the required deprotection prior to ribosomal utilization adds a step where direct utilization is clearly preferable. Furthermore, the procedure as reported requires at least a 1000-fold excess of reagents to obtain the aminoacylated tRNA in any useful quantity. In an effort to improve the efficiency of this method, aminoacyl phosphate monoester free of the protecting group has been used in the lanthanum-mediated aminoacylation. The

degree of aminoacylation was analyzed by MALDI-TOF-MS and its translational competency was attempted by protein expression in *Xenopus* oocytes (Figure 59).
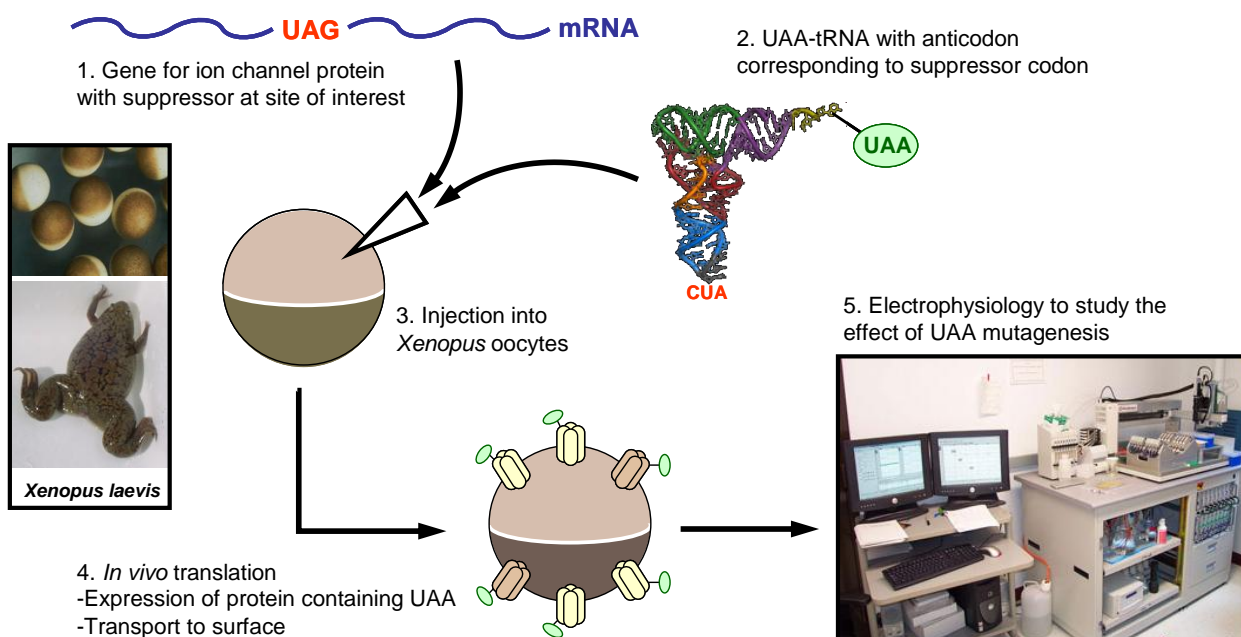
Figure 59. *In vitro* incorporation of unnatural amino acid into membrane protein by microinjection into *Xenopus* oocytes.

7.2   Experimental

7.2.1   Materials and Methods

Chemicals of reagent grade were used directly as received without further purification. Nuclease-free water was used for all reactions involving tRNA. CHROMA SPIN-30 size-elusion columns were purchased from Clontech. The transcription of 74 mer and 76 mer THG73 tRNAs were performed using T7 MEGAshortscript Transcription kit (Ambion) as previously reported by Saks et al.[95] Ligation of NVOC-Phe-dCA to 74 mer tRNA was performed as previously reported.[28,93] NVOC protecting group was removed by photolysis for 5 minutes prior to reaction to obtain Phe-tRNA. MALDI-MS analysis was performed on a Voyager DE PRO LAMDLI-TOF mass spectrometer operating in linear and positive ion modes.

7.2.2   MALDI-MS analysis

MALDI-MS analysis was performed as previously reported by Petersson et al.[93] NH_4^+ ion exchange beads were prepared by eluting Dowex 50WX8-200 hydrogen form, strongly

acidic (100 – 200 mesh size) ion exchange resin with NH_4OH and dried in air. NH_4^+ ion exchange beads (5 uL) were placed into an Eppendorf tube, and were added 3 uL 3-HPA matrix solution (42 mg 3-hydroxypicolinic acid, 2 mg picolinic acid, 2 mg diammonium citrate in 500 uL 9:1 water/acetonitrile) and 1 uL tRNA solution. The mixture was incubated for approximately 10 minutes. Calibration was performed with BSA standards in α-cyanohydroxycinnamic acid matrix solution. 0.5 – 1 uL tRNA matrix solution was spotted onto the target plate, and dried under airflow. Spectra were generated by accumulating 1000 – 2000 shots for each sample.

7.2.3   Lanthanum-mediated aminoacylation of tRNA

Stock solutions of lanthanum triflate, buffer, PheEP and tRNA were prepared fresh in nuclease-free water prior to reaction and kept on ice. Aliquots of each reagent were added in the order of tRNA, buffer, nuclease-free water, PheEP and lanthanum triflate to a total volume of 100 uL. Reactions were stirred vigorously at varying temperatures for varying times. Reactions were quenched with 100 mM DTPA (in 0.9M, pH 4.5 NaOAc). 2.2 M NH_4OAc (pH 5.2; 0.1 vol.) was added, and the solution was precipitated with EtOH (3 vol.) at -20'C overnight. tRNA was pelleted by centrifugation at 4'C (30 min, 14000 rpm). Supernatant was removed, and the isolated pellet was dried under vacuum for 10 min. The dry pellet was then dissolved in 25 uL nuclease-free H_2O, and purified through CHROMA SPIN-30 size-exclusion column to remove co-precipitated salt and amino acids. The purified solution was used directly for MALDI-MS analysis.

The reaction solution was subjected to deacylation conditions to confirm the ester bond formation. To the reaction solution was added 3.0 M Tris-HCl (pH 9.6) to a final concentration of 1.25 M Tris-HCl. This solution was incubated at 37'C for 4 hrs, and then purified through CHROMA SPIN-30 size-exclusion column to remove salts and amino acids.

7.2.4   Determining stability of ester linkage in aminoacyl-tRNA during lanthanum-mediated aminoacylation

Stock solutions of lanthanum triflate, $MgCl_2$, and HEPES (pH 7.0) buffer were prepared fresh in nuclease-free water prior to reaction and kept on ice. Phe-tRNA prepared by NVOC-Phe-dCA ligation to 74 mer tRNA was added to the buffered solution. To this was added $MgCl_2$ (5000 eq.) and lanthanum triflate (1000 eq.). Reactions were stirred vigorously at room temperature for 30 and 90 minutes, and then quenched with 100 mM DTPA (in 0.9M, pH 4.5

NaOAc). 2.2 M NH$_4$OAc (pH 5.2; 0.1 vol.) was added, and the solution was precipitated with EtOH (3 vol.) at -20'C overnight. tRNA was pelleted by centrifugation at 4℃ (30 min, 14000 rpm). Supernatant was removed, and the isolated pellet was dried under vacuum for 10 min. The dry pellet was then dissolved in 25 uL nuclease-free H$_2$O, and purified through CHROMA SPIN-30 size-exclusion column to remove co-precipitated salt and amino acids. The purified solution was used directly for MALDI-MS analysis.

7.2.5   Determining stability of THG73 tRNA during lanthanum-mediated aminoacylation

Stock solutions of lanthanum triflate, MgCl$_2$, and HEPES (pH 7.0) buffer were prepared fresh in nuclease-free water prior to reaction and kept on ice. To a buffered solution was added 76 mer tRNA, MgCl$_2$ (5000 eq.) and lanthanum triflate (1000 eq.). Reactions were stirred vigorously at room temperature for 30 minutes, and then quenched with 100 mM DTPA (in 0.9M, pH 4.5 NaOAc). To the quenched reaction, acidified PCI (1 vol., phenol/chloroform/isoamyl alcohol = 25:24:1) was added, vortexed for 1 minute, and then micro-centrifuged for 2 minutes to separate the layers. The top aqueous layer was removed. To the organic layer was added 1 vol. of nuc-free H$_2$O, vortexed for 1 minute and micro-centrifuged for 2 minutes. The top aqueous layer was removed. To the combined aqueous layers was added 1 vol. of CI (chloroform/isoamyl alcohol = 24:1), vortexed for 1 minute and micro-centrifuged for 2 minutes. The aqueous layer that contains tRNA was separated, and tRNA was isolated by ethanol precipitation. tRNA was pelleted by centrifugation at 4℃ (30 min, 14000 rpm). Supernatant was removed, and the isolated pellet was dried under vacuum for 10 min. The dry pellet was then dissolved in 25 uL nuclease-free H$_2$O, and purified through CHROMA SPIN-30 size-exclusion column to remove co-precipitated salt and amino acids. The purified solution was used directly for MALDI-MS analysis.

7.2.6   Injection of Phe-tRNA into *Xenopus* oocyte

mRNA for mouse serotonin receptor (5HT$_{3A}$) containing a TAG mutation at Y234 site was prepared by *in vitro* transcription. Each cell was injected with 50 nL of a mixture of mRNA (32 ng) and 21.4 ng of Phe-tRNA prepared using dCA ligation method or 16.5 ng of tRNA mixture from lanthanum-mediated aminoacylation. Uncharged 76 mer tRNA was injected as a

negative control. The cells were incubated for 24 hours, and the electrophysiological properties were analyzed by OpusXpress 6000A instrument in two-electrode voltage clamp mode.

7.3 Results

7.3.1 Lanthanum-mediated aminoacylation of tRNA

Full-length tRNA was subjected to lanthanum-mediated aminoacylation with PheEP. In the presence of large excess of reagents (> 1000 eq.), a new peak (24 430 m/z) corresponding to the mass of Phe-tRNA was observed (Figure 60). When the aminoacylation product was subjected to hydrolysis conditions in Tris-HCl (pH 9.6) buffer for 4 hours at 37 °C, the peak at 24 430 m/z disappeared. Additional peaks that may arise from multiple acylation were not observed. The peak at 24 488 m/z corresponds to an unidentified product from transcription which is present before and after the aminoacylation.
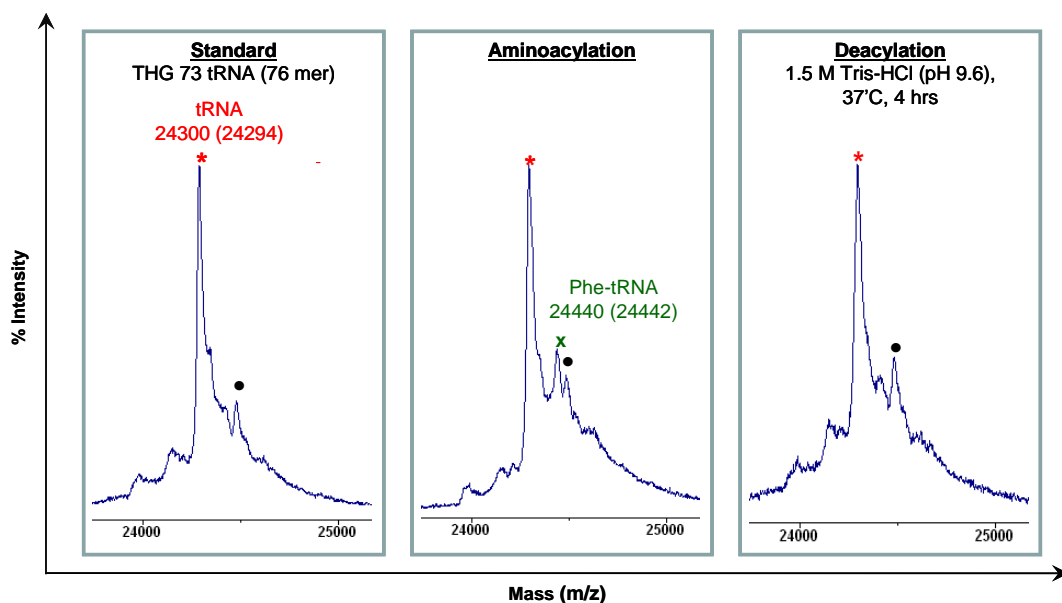
Figure 60. MALDI mass spectra of THG73 tRNA standard (left), after La^{3+}-mediated aminoacylation with PheEP (middle) and deacylation in mild alkaline solution (right). <u>Reaction conditions:</u> 31 ug tRNA, 25.4 mM PheEP (2000 eq.), 12.7 mM La(OTf)$_3$ (1000 eq.), 63.5 mM MgCl$_2$ (5000 eq.), 0.127 M HEPES (pH 7.0), 30 min.

Aminoacylation reactions were tested under a variety of conditions in order to optimize the yield of aminoacyl-tRNA product. In the presence of less than 1000 equivalents of lanthanum, aminoacylation was not detected by MALDI-MS. Varying the buffer concentration (5.14 – 100.3 mM), pH (6.5 – 8.0), temperature (4, 23, 37 °C), reagent concentration (lanthanum triflate,

PheEP, magnesium chloride, 0 – 5000 eq. relative to tRNA), reaction time (10 – 240 min) or solvent composition (10 – 25 % methanol or ethanol) did not have an effect on the extent of aminoacylation.

7.3.2 Determining stability of ester linkage in aminoacyl-tRNA during lanthanum-mediated aminoacylation
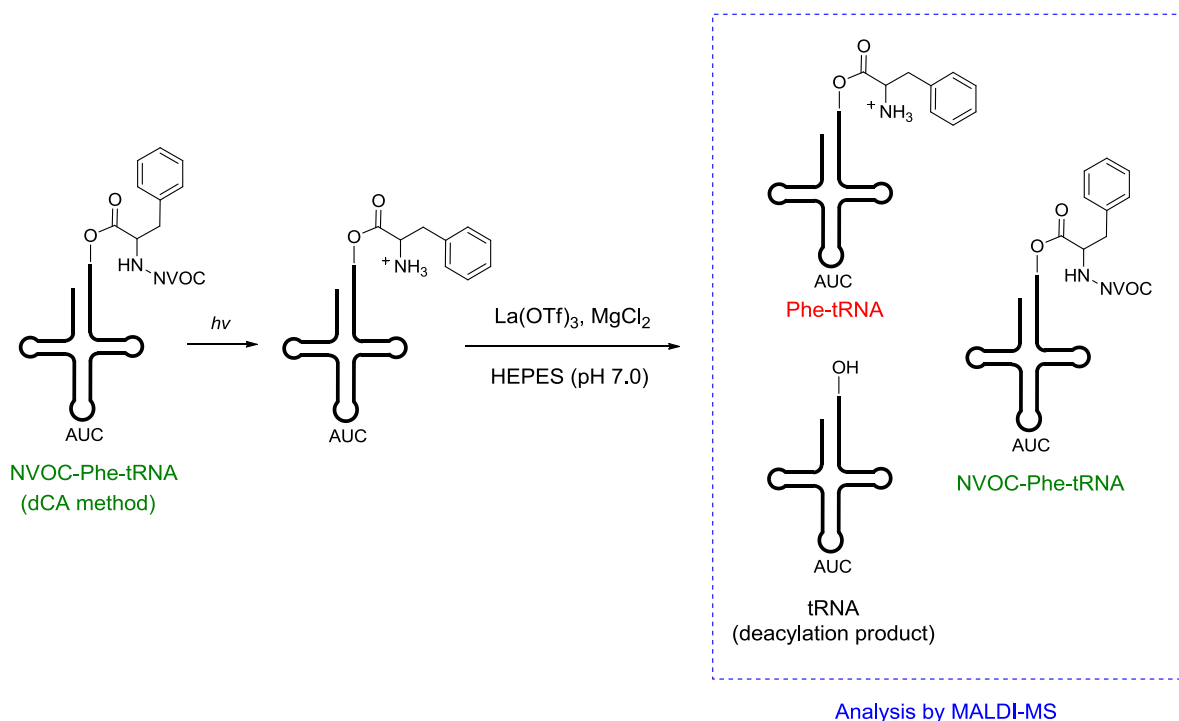
Figure 61. Reaction scheme showing the possible products from deacylation of aminoacyl-tRNA for detection by MALDI-MS.

Phe-tRNA that was prepared by enzymatic ligation of Phe-dCA to 74-mer tRNA was incubated in a solution containing the components of lanthanum-mediated aminoacylation. The degree of deacylation was estimated by MALDI-MS after 30 and 90 minutes of incubation at room temperature. With the increase in incubation time, an increase in the amount of free tRNA relative to Phe-tRNA and NVOC-Phe-tRNA was observed (Figure 62). The exact percentage change cannot be determined.
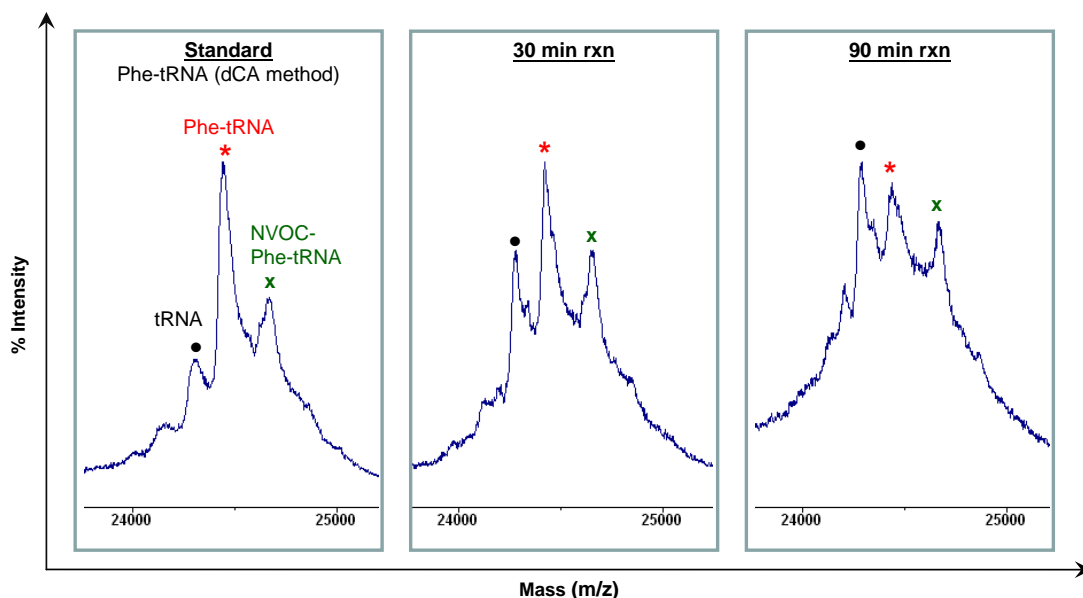
Figure 62. MALDI-MS spectra showing the change in the relative amounts of NVOC-Phe-tRNA, Phe-tRNA and free tRNA before (left) and after incubation in lanthanum-mediated acylation conditions for 30 minutes (middle) and 90 minutes (right). Reaction conditions: 10 ug tRNA, 4.17 mM La(OTf)$_3$, 20.8 mM MgCl$_2$, 41.7mM HEPES (pH 7.0).

7.3.3 Determining stability of THG73 tRNA during lanthanum-mediated aminoacylation

To test the possibility of metal-catalyzed degradation of tRNA during lanthanum-mediated aminoacylation, full-length tRNA was subjected to reaction conditions in the presence or absence of metal. The resulting reaction mixture was eluted through a size-exclusion column, which would remove small degradation products and reduce the recovery of tRNA. Degradation products that are larger in size and thus, not retained by size-exclusion column would appear in MALDI-MS analysis assuming that they are readily ionized under the same analytical conditions.

Based on the quantitative analysis by UV-spectroscopy, 99 % of tRNA was recovered in the absence of metal ions. When the metal ions (La^{3+} and Mg^{2+}) were added, 94 % of tRNA was recovered upon completion of reaction. From the MALDI-MS spectra, a new peak corresponding to the mass of (M − 87) m/z appeared when tRNA was incubated in a buffered solution containing lanthanum and magnesium metal. Small peaks in the lower mass range can also be seen in addition to the singly and doubly charged tRNA peaks (Figures 63, 64). From the relative magnitudes of singly charged tRNA peak to the newly appeared peaks after lanthanum-mediated acylation, it can be estimated that there is not a significant amount of degradation catalyzed by

lanthanum. Minimal degradation products suggest that the stability of tRNA is not significantly affected under the reaction conditions.
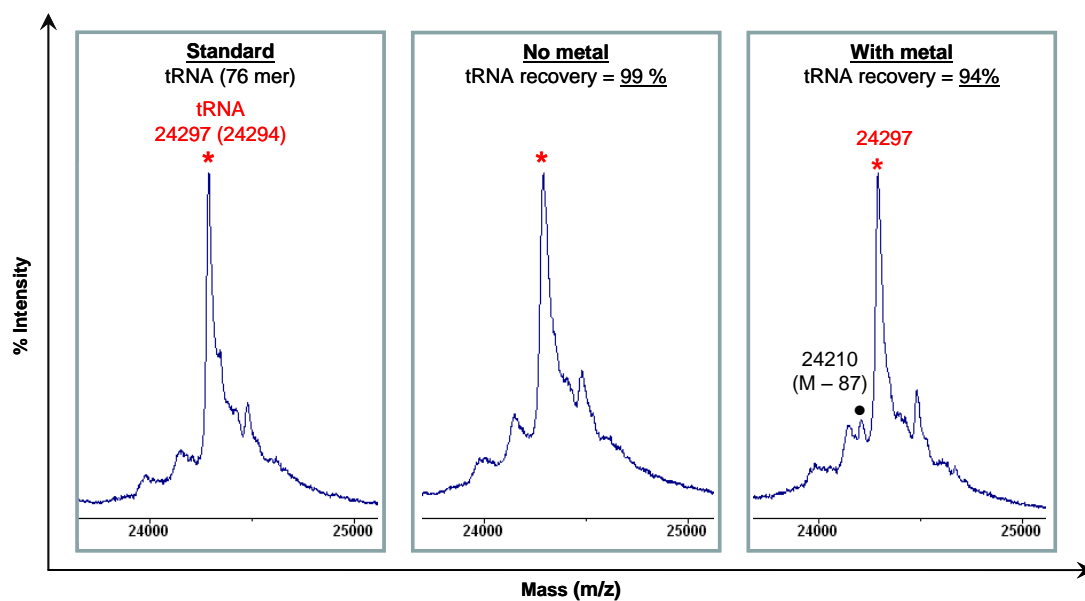
Figure 63. MALDI-MS spectra of full-length tRNA before (left) and after incubation in lanthanum-mediated acylation conditions in the absence (middle) and presence of metal (right). Reaction conditions: No metal: 10.7 ug tRNA (76 mer), 45.7 mM HEPES (pH 7.0), t=30 min; With metal: 10.7 ug tRNA, 1000 eq. La(OTf)$_3$, 5000 eq. MgCl$_2$, 45.7mM HEPES (pH 7.0), t=30 min.
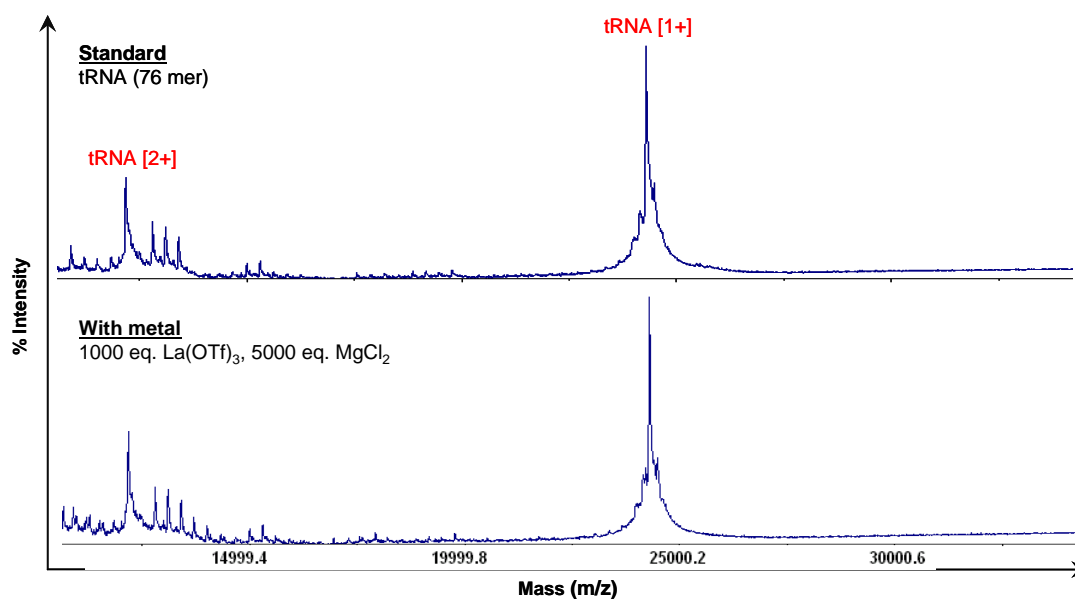
Figure 64. Wide-view of MALDI-MS spectra of full-length tRNA before (top) and after incubation in lanthanum-mediated acylation conditions in the presence of metal (bottom).

7.3.4   Injection of Phe-tRNA into *Xenopus* oocyte

A mixture of tRNA and aminoacyl-tRNA that was isolated from lanthanum-mediated aminoacylation was injected into *Xenopus* oocyte. Protein expression in the oocytes can be used as a functional test to determine the translational competency of the chemically acylated tRNA. The reaction solution was co-injected with mRNA that encodes mouse serotonin receptor (5HT3A) and contains TAG suppression at Y234. The receptor proteins are expressed and transported to the surface of the cell. Electrophysiology studies of the resulting oocytes were used to determine the effect of amber suppression on the receptor activity.

Phe-tRNA that was generated from the enzymatic ligation of Phe-dCA to 74 mer tRNA was utilized as a positive control. Based on the dose-response data obtained at nine different concentrations, a Hill plot was generated and an EC_{50} value of 11.00 (Figure 65, 66). As a negative control, unacylated 76 mer tRNA was injected, and no response was observed at all drug concentrations from 0 to 500 mM (Figure 67). When the reaction mixture was injected, no response was observed at serotonin concentrations of 250 mM or lower. Only at 500 mM of serotonin, the highest drug concentration tested, minimal response was obtained (Figure 68). The response was too low to generate an accurate Hill plot for full analysis.
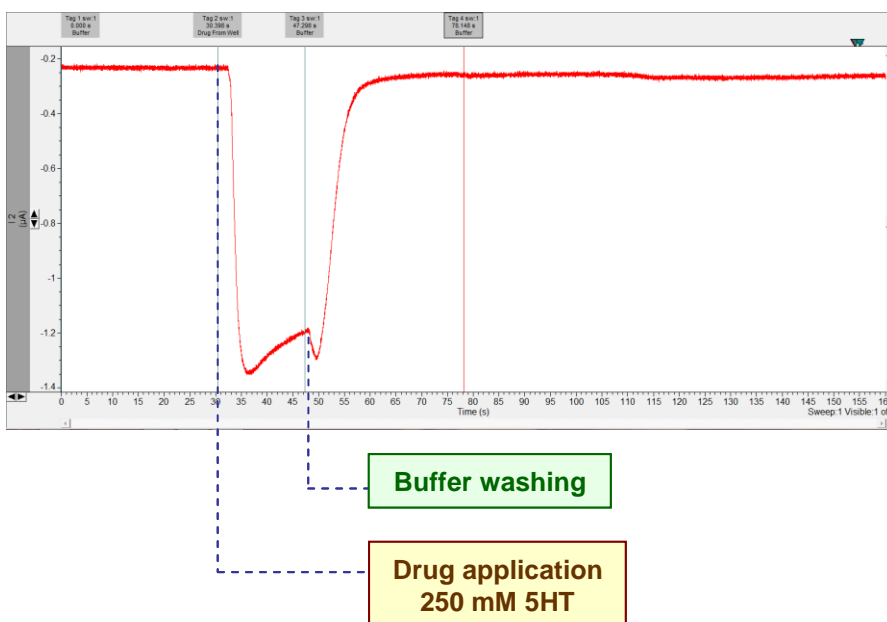
Figure 65. Electrophysiological response of *Xenopus* oocytes injected with Phe-tRNA generated by dCA-ligation method.
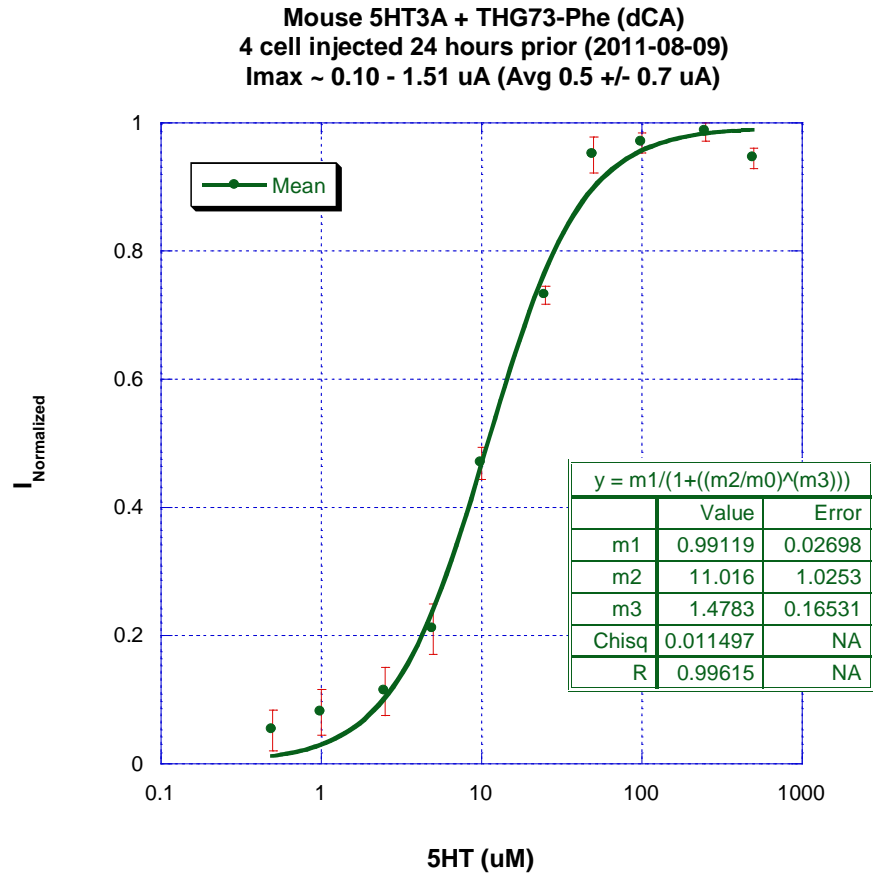
Figure 66. Hill plot generated for *Xenopus* oocytes injected with Phe-tRNA prepared by dCA-ligation method.

Negative control: unacylated tRNA
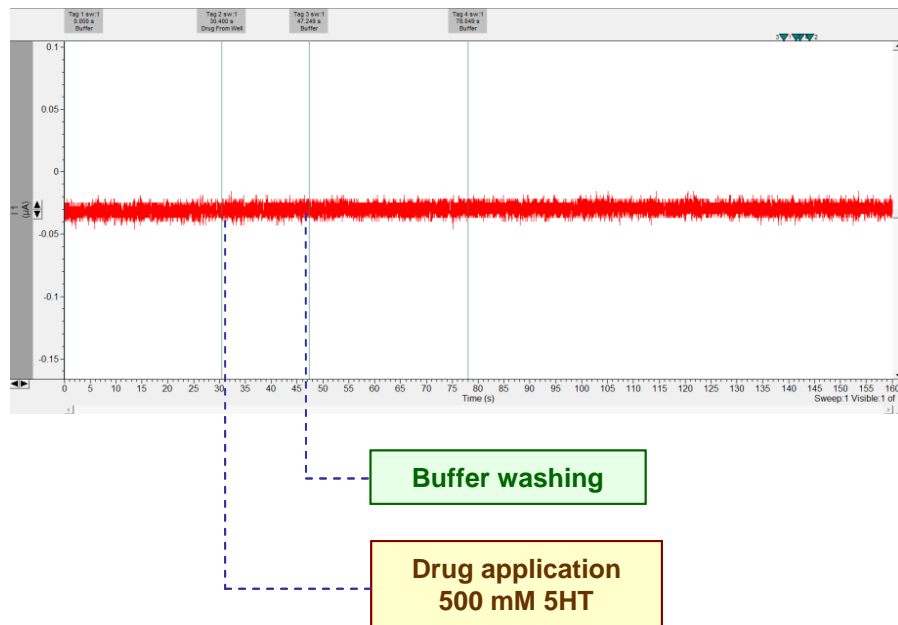
Figure 67. Electrophysiological response of *Xenopus* oocytes injected with unacylated tRNA.

Phe-tRNA (La/EP method)
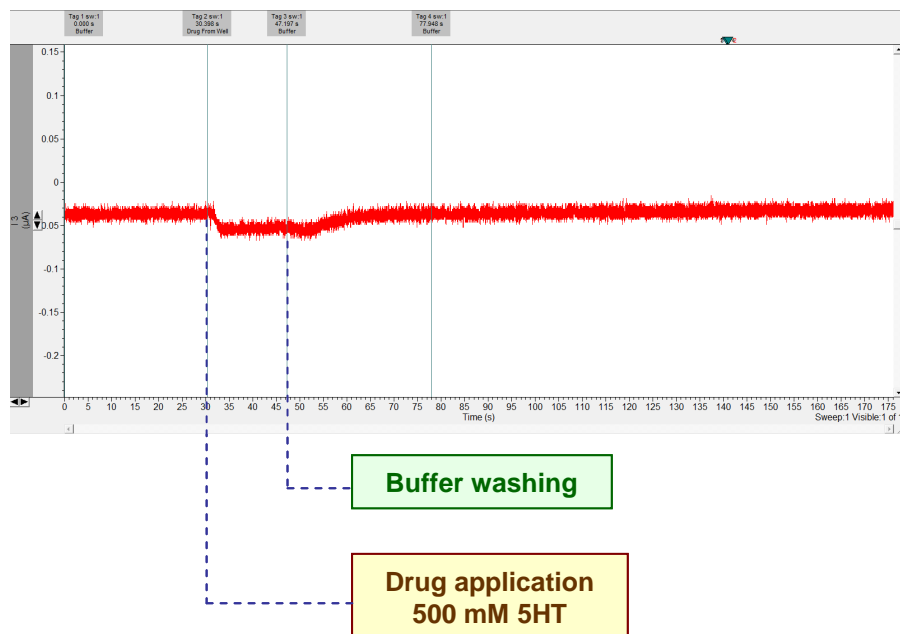
Figure 68. Electrophysiological response of *Xenopus* oocytes injected with tRNA/Phe-tRNA mixture generated by lanthanum-mediated aminoacylation.

7.4   Discussion

7.4.1   Lanthanum-mediated aminoacylation of tRNA

Lanthanum-mediate aminoacylation of tRNA was tested under a variety of reaction conditions and analyzed by MALDI-MS. In the presence of a large excess of La^{3+} and PheEP (> 1000 eq. relative to tRNA), a new peak was observed at 24 430 m/z. This mass is consistent with the expected mass of Phe-tRNA. To test whether this peak is due to an ester bond formation, it was incubated in a mild hydrolysis condition. Upon treatment with pH 9.6 Tris-HCl buffer for 4 hours at 37 °C, the peak at 24 430 m/z disappeared, which suggested a successful ester formation. The yield of aminoacylation cannot be determined due to the poor resolution of MALDI-MS technique and the differences in ionization of the free-tRNA and the aminoacyl-tRNA. Based on the qualitative analysis of the reactions, the yield of aminoacyl-tRNA appeared to be low since a significant amount of unacylated tRNA remained in all reactions. The peak at 24 488 m/z appears in all spectra. Although this peak could not be identified, it is proposed to be a transcription product that is unaffected by the lanthanum-mediated aminoacylation.

Several factors were considered in order to optimize the reaction conditions to maximize the yield of aminoacylation. In the presence of less than 1000 equivalents of PheEP and La^{3+}, aminoacylation could not be detected by MALDI-MS. Large excess of reagents may be required in order to overcome the unproductive binding of La^{3+} ions to the phosphate backbone of tRNA. Reactions were further investigated in terms of the reaction time, temperature and buffer concentrations; however, no significant different was observed. Co-solvent systems involving 10 − 25 % methanol, ethanol and DMSO were also studied to unfold the tRNA which could potentially make the 3'-terminal diols more accessible. Increasing the composition of organic solvent did not affect the yield of aminoacylation. Based on the qualitative analysis of MALDI-MS, the most optimized reaction conditions were 31 ug tRNA, 25.4 mM PheEP (2000 eq.), 12.7 mM $La(OTf)_3$ (1000 eq.), 63.5 mM $MgCl_2$ (5000 eq.) in 0.127 M, pH 7.0 HEPES buffer for 30 minutes at room temperature.

7.4.2   Determining stability of ester linkage in aminoacyl-tRNA during lanthanum-mediated aminoacylation

Aminoacyl-tRNAs that are unprotected at the α-amino group are known to have low stability in aqueous solutions, especially at high pH and temperature. According to the published

report by Hentzen et al. and Gilbert, the half life for hydrolysis of Phe-tRNA is 93 minutes in a phosphate buffer at pH 7.0 and 14 °C, and 16 minutes in 0.1 M, pH 8.6 Tris-HCl at 37 °C.[69,70] Stability of the ester linkage in aminoacyl-tRNA during the reaction and/or work-up was investigated. Phe-tRNA was prepared via ligation of NVOC-Phe-dCA to a truncated tRNA (74 mer) followed by photolytic cleavage to deprotect the NVOC protecting group. MALDI-MS analysis of the reaction after work-up and purification showed three peaks corresponding to the NVOC-Phe-tRNA from incomplete deprotection, Phe-tRNA and the free tRNA. Qualitative analysis of MALDI-MS revealed that deacylation occurs after 30 and 90 minutes of reaction followed by work-up. This observation suggests that a significant amount of aminoacyl-tRNA may be deacylated under the reaction conditions.

One possible method of protecting aminoacyl-tRNA from deacylation is to utilize elongation factor Tu (EF-Tu). EF-Tu is one of the most abundant proteins and is involved in the translation of proteins. EF-Tu binds to aminoacyl-tRNA to stabilize the ester linkage and is coupled to GTP-hydrolysis to load the correct aminoacyl-tRNA into the A-site of ribosome.[1] Further investigation will be required to test the ability of tRNA · EF-Tu complex to undergo lanthanum-mediated aminoacylation of tRNA while minimizing deacylation.

7.4.3 Determining stability of THG73 tRNA during lanthanum-mediated aminoacylation

Lanthanides are known to form monomeric and dimeric complexes in water. The dimeric complexes are reported to be the catalytically active species in the hydrolysis phosphodiester bonds. Lanthanide-catalyzed hydrolysis of phosphodiester bonds have been reported by various groups including Komiyama and co-workers, Morrow et al. and Chin et al.[61-63,96-98] The rate of hydrolysis by lanthanide ions depend on the pH and the nature of lanthanide. Smaller lanthanides which appear later in the lanthanide series catalyze RNA hydrolysis better than the earlier lanthanides. Also, the rate of hydrolysis is faster in a more alkaline solution than in lower pH.

Based on the extent of recovery of tRNA and the MALDI-MS analysis, it was shown that minimal degradation occurs during the reaction. This is consistent with the expected results since lanthanum is the largest atom in the lanthanide series, and the reactions were conducted at a neutral pH, where lanthanide-catalyzed hydrolysis is reported to be slow. However, caution must be taken in using MALDI-MS to analyze the stability of tRNA. Detection by MALDI-MS is

dependent on the ionization of the molecule. If the degradation product is not efficiently ionized under the same analytical conditions as the tRNA, it may not be detected by MALDI-MS. Analysis by gel electrophoresis or HPLC would provide a more accurate representation of the possible degradation of tRNA under lanthanum-mediated aminoacylation conditions.

7.4.4   Protein expression in *Xenopus* oocytes

The translational competency of Phe-tRNA produced by the lanthanum-mediated aminoacylation was tested by injection into *Xenopus* oocytes to express a serotonin receptor. Dose-response data were obtained at 9 different concentrations to generate a Hill plot and compared with literature value to determine successful incorporation of amino acid by amber suppression. Positive control using the Phe-tRNA generated from the enzymatic ligation of Phe-dCA to 74 mer tRNA showed an EC_{50} value of 11.00. This suggests that the chemically acylated tRNA is effective in incorporating an amino acid at the site specified by the amber suppressor codon. When a unacylated 76 mer tRNA was used as the negative control, no response to serotonin was observed. This is consistent with expected result as the insertion of unacylated tRNA into the A-site of ribosome would result in an early termination of protein translation. When the reaction mixture was injected, response was only observed for the highest concentration of serotonin at 500 mM. In comparison to the negative control, the signal is significant suggesting that chemically acylated Phe-tRNA is successful in inserting phenylalanine in response to the amber suppressor. However, the lack of response at lower drug concentrations suggests that the yield of protein expression was low. This could be due to the fact the Phe-tRNA was only present in very low concentrations in the reaction mixture injected into the cell. In order to improve the yield of protein expression, Phe-tRNA should be separated from the unacylated tRNA before injection into the cell.

7.5   Conclusions

Utilizing MALDI-MS technique, low levels of tRNA aminoacylation was detected with PheEP. It has been found that the full-length tRNA is stable against degradation under the lanthanum-mediated aminoacylation conditions. However, deacylation of aminoacyl-tRNA was observed. Protein expression in *Xenopus* oocytes revealed successful incorporation of phenylalanine using chemically acylated tRNA. Nevertheless, improvements are needed to increase the yield or protein expression for a more accurate analysis. Further studies need to be

performed utilizing a variety of *N*-protected and unprotected aminoacyl phosphate monoesters in order to improve the yield of aminoacylation. Ultimately, the results of these studies would contribute to the development of highly efficient lanthanum-mediated aminoayclation of tRNA and its applications in *in vitro* protein synthesis.

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