

Biomimetic Aminoacylation: Optimization of Reaction Conditions

by

Shannon E. Bunn

A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Chemistry
University of Toronto

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Abstract

“Biomimetic Aminoacylation: Optimization of Reaction Conditions”

Shannon E. Bunn

Master of Science, 2009

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Synthesizing proteins containing unnatural amino acids inserted at specific positions within the protein sequence has been a longstanding goal of biological chemists. This poses unique challenges, as aminoacyl tRNA synthetases, the enzymes responsible for protein synthesis, are highly specific. To overcome this, an *in vitro* lanthanum-catalyzed, biomimetic tRNA aminoacylation method has been developed¹. However, due to unproductive lanthanum coordination of ethyl phosphate, a reaction byproduct, a full equivalent of lanthanum must be added to each reaction. This may threaten the integrity of tRNA, as lanthanides are known to catalyze the hydrolysis of RNA^{2,3}. Using uridine as a simplified tRNA mimic, magnesium, which is known to coordinate strongly with phosphate ions, has been utilized to optimize this reaction and increase the selectivity of lanthanum towards esterification. In the presence of magnesium, ester yield is substantially increased. In addition to this, optimal pH and buffer reaction conditions were determined.

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List of Abbreviations

5'AMP	Adenosine-5'-monophosphate
ATP	Adenosine triphosphate
Boc (tBoc)	Tertbutyl oxycarbonyl
BocPhe	α -N-Boc-(L)-phenylalanine
BocPEP	α -N-Boc-(L)-phenylalanyl ethyl phosphate
DCC	N, N'-dicyclohexylcarbodiimide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EPSP	N-(2-hydroxyethyl)piperazine-N'-(3-propane) sulfonic acid
ESI MS	Electrospray ionization mass spectrometry
^1H NMR	Proton nuclear magnetic resonance
H₂O	Water
HPLC	High pressure liquid chromatography
La³⁺	Lanthanum
La(OTf)₃	Lanthanum triflate
L	Litre
Mg²⁺	Magnesium
Mg(OTf)₂	Magnesium triflate
M	Molar
μV	Microvolt
mRNA	Messenger ribonucleic acid
OH	Hydroxyl
^{31}P NMR	Phosphorous nuclear magnetic resonance
RNA	Ribonucleic acid
RP	Reverse phase
TFA	Trifluoroacetic acid
tRNA	Transfer ribonucleic acid

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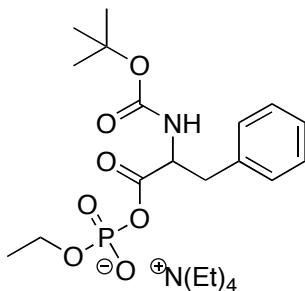
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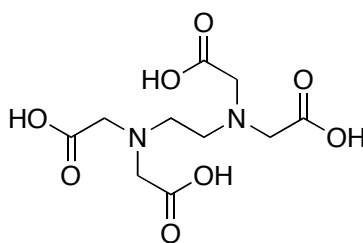
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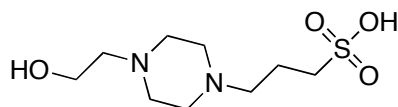
Structures of Relevant Compounds



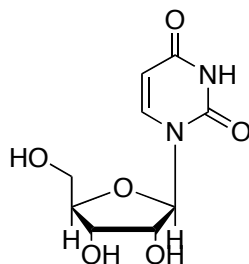
N-Boc-(L)-phenylalanyl ethyl phosphate (BocPEP)



Ethylenediaminetetraacetic acid



N-(2-hydroxyethyl)piperazine-N'-(3-propane) sulfonic acid (EPPS)



Uridine

CHAPTER 1: General Introduction

1.1 *Biochemical Protein Synthesis*

In the cell, protein synthesis begins with transcription of the deoxyribonucleic acid (DNA) that codes for a given protein, into messenger ribonucleic acid (mRNA)⁴. The mRNA is then read by the ribosome, which initiates a process called translation. To ensure translation specificity, each *L*-amino acid must be attached to its corresponding transfer RNA (tRNA), forming an aminoacyl-tRNA. Each aminoacyl-tRNA is then transported to the ribosome where the mRNA codon corresponding to the tRNA-anticodon is read. The location where each amino acid is inserted into the amino acid sequence is determined by the pairing of a codon in mRNA with a particular aminoacyl-tRNA anticodon⁶. As a result, if a tRNA for a given amino acid can be misacylated with an unnatural amino acid or an incorrect amino acid, that amino acid will likely be incorporated into the protein sequence.

1.1.1 *In vivo* tRNA Aminoacylation Mechanism

tRNA aminoacylation is catalyzed by a family of twenty aminoacyl tRNA synthetases, which are responsible for charging the correct tRNA with its cognate amino acid. Each aminoacyl tRNA synthetase is specific for one amino acid and one or more isoaccepting tRNAs. The aminoacyl tRNA synthetase family can be divided into two distinct classes, Class I and Class II, based on active site topology and the position of

acylation of tRNA⁵. Class I enzymes charge tRNAs at the 2'-OH while Class II enzymes catalyze acylation at the 3'-OH⁵.

The acylation reaction proceeds in two steps and both are catalyzed by an aminoacyl tRNA synthetase. First, the amino acid is activated as an aminoacyl-adenylate in a reaction with adenosine triphosphate (ATP). This reaction intermediate forms a complex with the aminoacyl tRNA synthetase. In the second step, the aminoacyl transfer, the amino acyl moiety is transferred from the phosphate of 5'AMP (adenosine 5'- monophosphate) to the 2'- or 3'- hydroxyl group of the tRNA molecule. Mechanistic analysis of this second step suggests that formation of the aminoacyl tRNA is achieved simply by bringing the reagents into reactive proximity, while ensuring proper alignment¹.

1.2 Biomimetic *in vitro* Synthesis of Aminoacyl tRNA

Proteins that contain unnatural amino acids inserted at specific locations within the protein sequence can be engineered to function pharmaceutically as enzyme inhibitors or to carry spin or fluorescent labels for study of protein structure, kinetics, folding, and other applications^{6,7,8}. The accuracy of aminoacyl-tRNA synthesis depends on the specificity of the individual aminoacyl tRNA synthetase and the average error rate is about 1 in 10 000⁽⁶⁾. This specificity makes it difficult to manipulate aminoacyl tRNA synthetase *in vivo* to produce proteins that contain unnatural amino acids. As a result, most research into the synthesis of unnatural amino acid-containing proteins has been directed towards the development of *in vitro* methods. Several methods for preparing misacylated aminoacyl-tRNA have been developed and successfully used to incorporate non-natural amino acids into proteins^{7,8,9,10}. The lanthanum-catalyzed tRNA aminoacylation method recently developed by Tzvetkova and Kluger is novel in that it does make it possible to aminoacylate any tRNA molecule at the 3' terminus with any natural or unnatural amino acid in water¹. They accomplished this by eliminating the use of aminoacyl tRNA synthetases and using an aminoacyl alkyl phosphate as an analogue for the aminoacyl adenylate and lanthanum (La^{3+}) as a catalyst.

1.2.1 Aminoacyl Alkyl Phosphates

The first step of *in vivo* protein synthesis is the formation of an aminoacyl adenylate, a mixed anhydride. The adenylate portion of the enzymatic intermediate is not involved in the reaction with tRNA, presumably serving to bind to the enzyme. Since the complexity of the adenosine is not needed for the chemical reaction, Kluger *et al.*

developed a simplified aminoacyl donor: an aminoacyl alkyl phosphate (Figure 1)⁹.

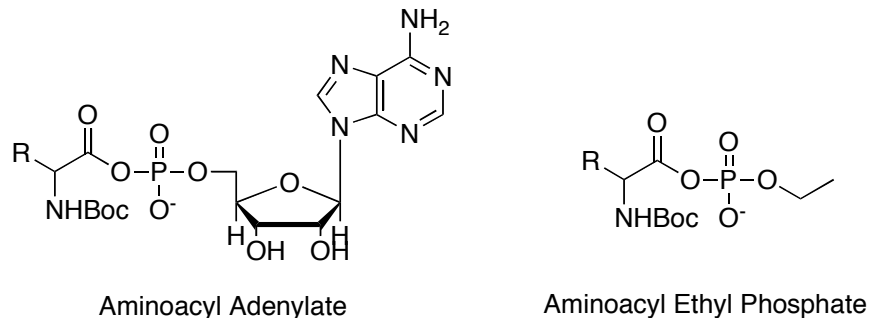


Figure 1: Aminoacyl adenylate and aminoacyl alkyl phosphate. (R=amino acid side chain; R1=alkyl group)

Kluger and co-workers determined that the rate of hydrolysis of aminoacyl phosphates was enhanced by acid, base and metal ions¹⁰. They subsequently developed a method that employs lanthanide ions, which coordinate and activate aminoacyl phosphates and demonstrated that this can be used to acylate of alcohols (mono- and diols), nucleosides, nucleotides, and RNA^{1,10,11,12}.

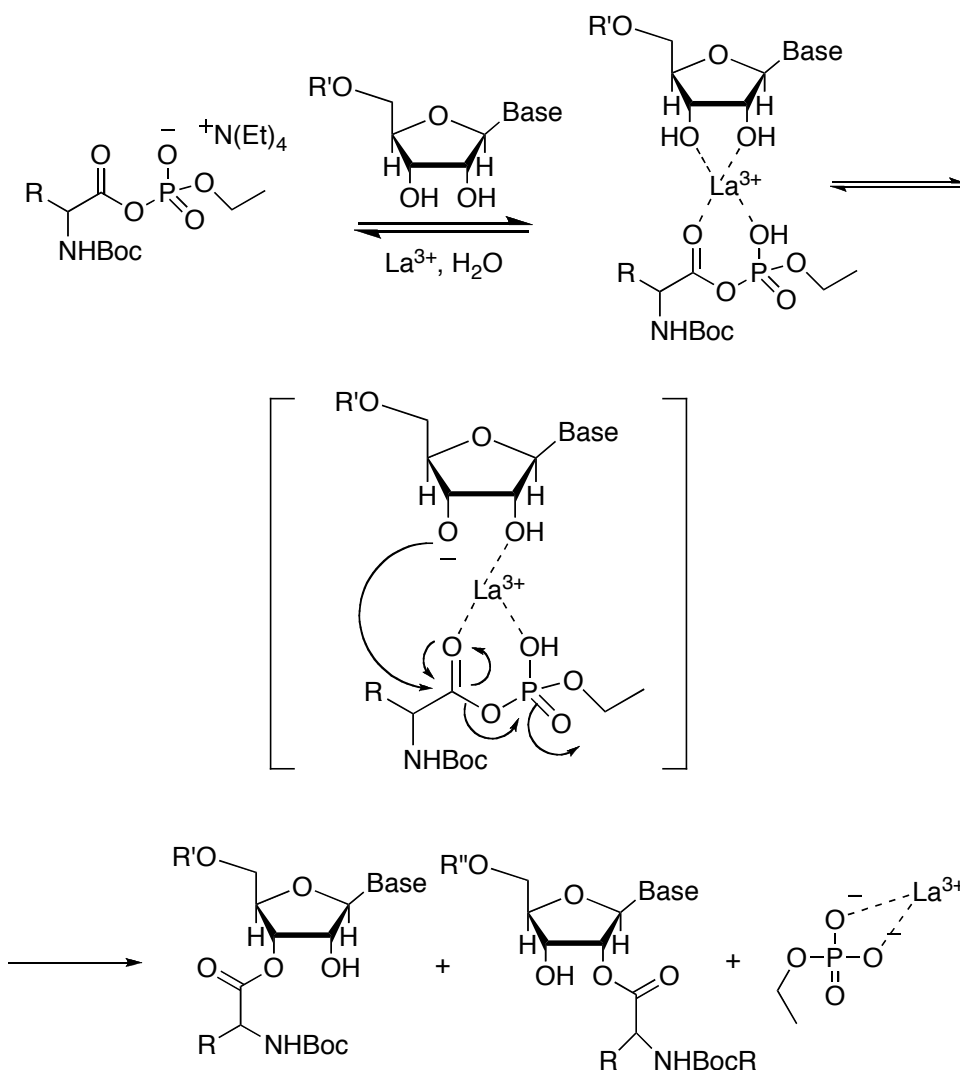
1.2.2 Lanthanum

In order to circumvent the stringent specificity requirements imposed by aminoacyl tRNA synthetases, our method employs lanthanum, which promotes aminoacylation reactions by coordinating to the reagents to bring them into reactive proximity. Lanthanum is a non-toxic element with no known biological role¹³. It has a high level of coordination, coordinating up to eight or nine ligands¹³, which is catalytically important as it allows for simultaneous coordination and activation of both the nucleophile and electrophile. In addition to creating a low enthalpic barrier, this simultaneous coordination also results in the lowering of the entropy barrier as a reaction that is bimolecular becomes pseudo-unimolecular upon coordination. In addition to this,

lanthanum is a highly electropositive, hard Lewis acid and is known to form flexible complexes predominantly with oxygen-containing ligands, which make it ideal to coordinate aminoacyl phosphates and tRNA, as less specific substitute for an aminoacyl tRNA synthetase¹³.

1.2.3 Lanthanum-Catalyzed Aminoacylation

Lanthanum (La^{3+}) promotes aminoacylation reactions by forming a bis-bidentate complex with a diol and the aminoacyl phosphate¹. In the proposed mechanism shown in Scheme 1, the two reacting species are brought close together, which facilitates a nucleophilic attack on the aminoacyl phosphate in a similar manner as occurs *in vivo* by aminoacyl tRNA synthetases.



Scheme 1: A general lanthanum-catalyzed aminoacylation (R' corresponds to the amino acid side chain, R is variable (H for nucleoside, phosphate for nucleotide, or refers the remainder of a tRNA molecule))

The pK_a values of the 2' and 3' hydroxyl groups are very similar (between 12 and 13 in linear RNA)¹⁴ and it is thought that upon coordination to the Lewis acidic lanthanum (La^{3+}), the 2' and 3' hydroxyl groups become more acidic, which results in the deprotonation of either the 2' or 3' hydroxyl group by bases in solution to form an oxyanion. When this occurs, the highly nucleophilic oxyanion becomes detached from the lanthanum ion and the other hydroxyl group, still coordinated, acts as a tether,

delivering the nucleophilic oxygen to attack the aminoacyl phosphate that is also coordinated to lanthanum. The preassociation of reactants in the coordination complex with lanthanum ensures proper geometry of attack and high selectivity for the nucleophilic attack.

Once aminoacylation has occurred, the product esters equilibrate, at with the 3'-ester present at a slightly higher concentration as it is thermodynamically more stable¹. This is similar to what is observed in *in vivo* protein synthesis. It has been shown previously that lanthanides promote the aminoacylation of *cis*-1,2-cyclopentanediol, nucleotides, nucleosides, and bulk RNA with aminoacyl phosphates such as α -N-Boc-(L)phenylalanyl ethyl phosphate (BocPEP)¹. For each of the aforementioned molecules, aminoacylation was selective for the 2' or 3' hydroxyl group on the diol functionality. In the case of the nucleosides, no aminoacylation was observed at the 5' hydroxyl group¹. It has been shown that if the diol functionality is removed or protected, no aminoacylation occurs¹.

1.3 Involvement of Magnesium to Increase Reaction Efficiency

The lanthanum-catalyzed aminoacylation (Scheme 1) is not catalytic and requires that a full equivalent of lanthanum be added to each reaction. It is thought that lanthanum remains coordinated to the alkyl phosphate at the end of each reaction, which is observed as a white precipitate. In order to optimize the reaction and increase the selectivity of lanthanum towards esterification, as opposed to hydrolysis of the aminoacyl phosphate¹, magnesium (Mg^{2+}) has been utilized.

Magnesium is commonly found in biological systems as a cofactor in metalloenzymes, and is known to coordinate strongly with phosphate ions. Magnesium is also known to be essential for maintaining the activity and structure of tRNA. In a study of interactions of magnesium with inorganic and nucleoside phosphates, Stuehr and co-workers showed that it coordinates exclusively to the phosphate oxygen atoms¹⁵. For the lanthanum-catalyzed aminoacylation reaction in question, it is thought that an excess of magnesium will coordinate to the ethyl phosphate byproduct, while allowing the lanthanum to be added in a catalytic amount. Magnesium has no catalytic activity and thus is ideal to displace lanthanum from its coordination to the alkyl phosphate reaction byproduct.

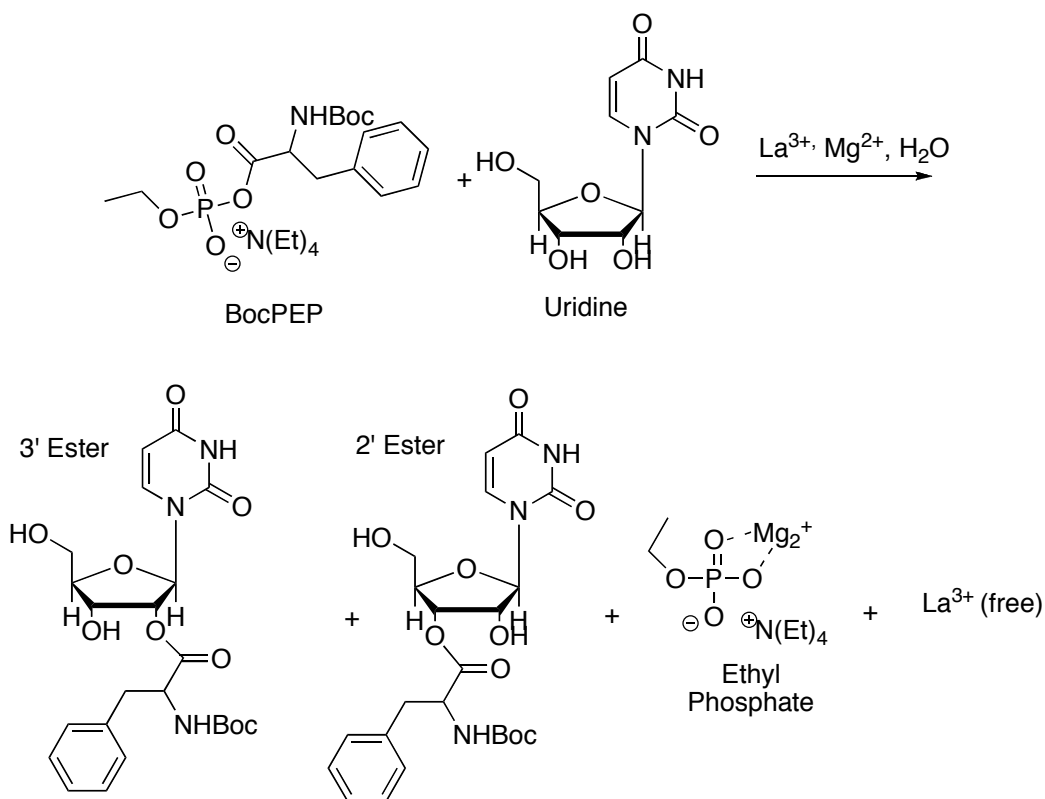
A further motivation for decreasing the concentration of lanthanum relative to substrate is that Morrow *et al.* and Komiyama *et al.* have observed that lanthanides catalyze the decomposition of RNA^{2,3}. The half time for the decomposition of an adenosine dinucleotide (ApA) by free lanthanum ions reported by Komiyama *et al.* is 23 000s or 6.38 hours with 1×10^{-3} M lanthanum present at pH 7.2 and 50°C ⁽³⁾. The

aminoacylation reaction involving lanthanum and uridine is complete in roughly 30 seconds and the lanthanum is quenched after a period of five minutes so it is anticipated that the tRNA decomposition by lanthanum to insignificant. Still, optimizing the reaction with respect to lanthanum concentration will preserve the structural integrity of tRNA, allowing aminoacylated tRNA molecules to participate in translation processes on the ribosome.

CHAPTER 2: Magnesium-Mediated, Lanthanum-Catalyzed Aminoacylation

2.1 *Introduction*

During the process of translation in the cell an aminoacyl tRNA synthetase aminoacylates a specific tRNA molecule with its cognate amino acid, activated as an aminoacyl adenylate. In the biomimetic, lanthanum-catalyzed system developed by Tzvetkova and Kluger, lanthanum acts as a “less specific aminoacyl tRNA synthetase” and aminoacyl ethyl phosphates act as simplified versions of aminoacyl adenylates. However, as is shown in Scheme 1, this process requires a full equivalent of the lanthanum salt to be added to each reaction, as lanthanum remains coordinated to the ethyl phosphate leaving group at the end of each aminoacylation. It is predicted that the addition of an excess of magnesium ions will result in the magnesium displacing the lanthanum from the negatively charged alkyl phosphate, allowing lanthanum to catalyze multiple aminoacylations (Scheme 2).



Scheme 2: Magnesium-mediated, lanthanum-catalyzed BocPEP aminoacylation of uridine

The addition of excess magnesium should permit lanthanum to be added in catalytic amounts, which will help preserve the structural integrity of tRNA, and reduce the competing hydrolysis of the aminoacyl phosphate reagent.

I investigated the effect of magnesium utilizing a model reaction between uridine and BocPEP. Uridine serves as a tRNA mimic as it contains the 2', 3' diol functionality found at the 3'-terminus of tRNA that has been shown to be essential for lanthanum-catalyzed aminoacylation to occur¹. It is also highly soluble in buffered aqueous solution at pH 8, significantly more cost-effective than tRNA, and can be more easily analyzed. BocPEP was chosen as it is easily synthesized and its aromatic functionality allows for UV-absorbance detection of both starting material and esters, which greatly enhances

high performance liquid chromatography (HPLC) analysis with a UV-visible detector. Optimizing this reaction will yield useful information that can be applied to systems utilizing tRNA.

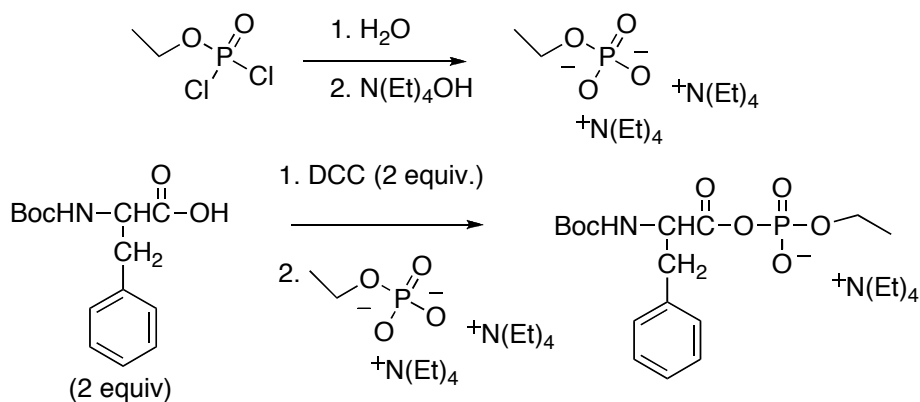
2.2 Experimental

2.2.1 Materials and Methods

All commercial reagents were purchased from Sigma Aldrich Canada and were used as received without further purification. All water was doubly distilled and deionized before use. All proton and phosphorus nuclear magnetic resonance (NMR) spectra were recorded at 300MHz. High-resolution mass spectrometry was performed at the QStar Chemistry Mass Spectral Facility, University of Toronto. High performance liquid chromatography (HPLC) analysis was performed on C18-reverse phase column and the products were detected at 263nm. The mobile phase consisted of 40% acetonitrile and 0.1% TFA in water.

2.2.2 BocPEP Synthesis

The synthesis of BocPEP requires two steps, as is shown in Scheme 3.



Scheme 3: General synthesis of BocPEP

The synthesis of tetraethyl ammonium ethyl phosphate was performed as outlined by Kluger *et al.*⁹ A brief synthetic description follows. Ethyl dichlorophosphate was added to a ten-fold excess of water over ten minutes in an ice-cooled round-bottom flask and stirred for one hour. Hydrochloric acid is produced as a byproduct, and was removed by rotary evaporating for at least two hours. The resulting ethyl phosphoric acid was neutralized with two equivalents of tetraethylammonium hydroxide. The neutral solution was freeze-dried and used in the next step without further purification.

In the second step, α -N-Boc-(L)-phenylalanine (BocPhe) (two equivalents) was activated with dicyclohexylcarbodiimide (DCC) (two equivalents) in dichloromethane for three minutes. Tetraethylammonium ethyl phosphate (one equivalent), dissolved in dichloromethane, was added to the mixture, which was left to stir at room temperature for 2.5 hours. By adding the DCC-activated amino acid in two-fold molar excess, it was ensured that no tetraethylammonium ethyl phosphate remained unreacted. Tetraethylammonium ethyl phosphate acts as an inhibitor in aminoacylation reactions by coordinating to the lanthanum ions and preventing the formation of bis-bidentate coordination complexes. The final products were extracted with water to remove excess DCC-activated BocPhe and other impurities. The solution was freeze-dried and used in the aminoacylation experiments without further purification. BocPEP was obtained as a clear, glassy solid in an 80-85% yield. Analytical data for BocPEP are given in Figure 2 and are consistent with other reports⁹.

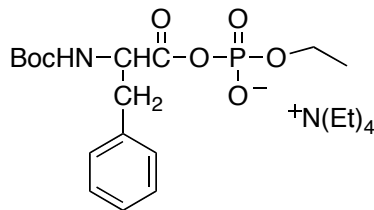


Figure 2: α -N-Boc-(L)-phenylalanyl ethyl phosphate structure

^1H NMR (300MHz, D_2O): δ 7.2 (5H, m, Ar), 4.3 (m, 1H, CHCO), 3.8 (2H, quintet, POCH_2CH_3), 3.1 (10H, quartet, $\text{N}^+(\text{CH}_2\text{CH}_3)_4$, POCH_2), 2.1 (1H, s, NH), 1.1 (15H, m, POCH_2CH_3 , $\text{N}^+(\text{CH}_2\text{CH}_3)_4$)

^{31}P NMR (121MHz, D_2O): δ -6.141

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

2.2.3 Aminoacylation

Aminoacylation reactions were performed with uridine and BocPEP in the presence and absence of magnesium. All reactions were performed at room temperature (23°C) with constant stirring. The molar ratios of magnesium and lanthanum were optimized with respect to uridine, the tRNA mimic, with the aim of determining reactant ratios that produced the highest ester yields. The concentrations of uridine and BocPEP were held constant at 1.5×10^{-2} M for all reactions performed. A reaction volume of 1.0×10^{-3} L was used in all cases. All reactions were conducted in quadruplicate and the mean amount of ester products, as determined by HPLC peak integration and standard deviations were computed. The HPLC output gives peak areas in units of $\mu\text{Volt} \cdot \text{second}$ ($\mu\text{V} \cdot \text{s}$), which can simply be considered as area since the units and actual magnitude of

the peak areas are relative. It is only the ratios of the ester peak areas that are meaningful as they permit comparison of the efforts of different reaction conditions on esterification.

The ester peak areas were examined as opposed to the percentage of total peak area on the chromatogram. The high molar absorptivity coefficient of the unreacted uridine peak consistently saturates the detector. Therefore, using the integrated peak areas as opposed to peak area percentages for the uridine-Boc-phenylalanyl esters is more accurate when comparing the extent of esterification under different reaction conditions. The HPLC sample injection volume used was 9.5×10^{-5} L for reactions outlined in section 2.3.1. The injection volume was increased to 1.2×10^{-4} L for reactions outlined in section 2.3.2 and 2.3.3 to ensure ester peak areas were large enough for integration, as the reactions were catalyzed by lower molar ratios of lanthanum (relative to uridine).

All stock solutions (lanthanum triflate ($\text{La}(\text{OTf})_3$), magnesium triflate ($\text{Mg}(\text{OTf})_2$), BocPEP, and uridine) were freshly prepared in pH 8 *N*-(2-hydroxyethyl)piperazine-*N'*-(3-propane) sulfonic acid (EPPS) solution. The triflate salts of lanthanum ($\text{La}(\text{OTf})_3$) and magnesium ($\text{Mg}(\text{OTf})_2$) were utilized in all aminoacylations and are referred to simply as magnesium and lanthanum in the following text. Reagents were added in the following order: uridine, BocPEP, magnesium. The reaction was initiated upon addition of lanthanum. Tzvetkova and Kluger determined that the aminoacylation of uridine by α -N-Boc-*p*-fluorophenylalanyl ethyl phosphate (BocFPEP), is complete in 30 seconds. All aminoacylation reactions were quenched after five minutes with 2.0×10^{-4} L of saturated ethylenediaminetetraacetic acid (EDTA), which coordinates the lanthanum catalyst.

2.3 Results

2.3.1 Effect of Magnesium on Aminoacylation

The effect of the presence of excess magnesium ions on the aminoacylation of uridine by BocPEP was investigated. The molar ratio of lanthanum to uridine was varied. Reactions were conducted in the presence and absence of magnesium at a molar ratio 10: 3 (relative to uridine). A sample chromatogram that is overlaid for reactions with and without magnesium is shown below in Figure 3. All molar ratios discussed are relative to uridine.

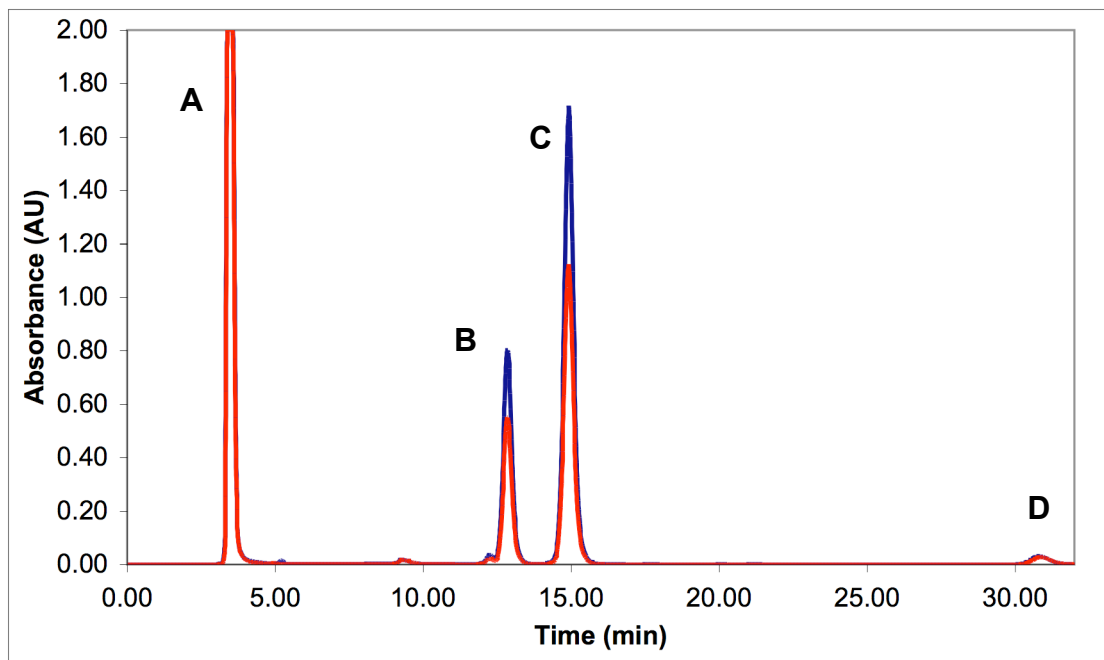


Figure 3: Chromatograms from reactions containing lanthanum at a molar ratio of 1: 15 (relative to uridine). The blue chromatogram represents a reaction also containing excess magnesium at a molar ratio of 10: 3. The red chromatogram represents a reaction without magnesium. Peaks labelled A, B, C, and D correspond the unreacted uridine, 2'-Boc-phenylalanyl-uridine ester, 3'-Boc-phenylalanyl-uridine ester, and BocPhe (hydrolysis product), respectively.

Peak identities were confirmed by comparing the chromatogram with data by Tzvetkova and Kluger¹. It should be noted that when reactions contained magnesium and no lanthanum, esterification was not observed. Table 1 shows the average total ester peak area for reactions where the concentration lanthanum was varied in the presence and absence of excess magnesium.

[Lanthanum]: [uridine]	Average total ester peak area with magnesium	Average total ester peak area without magnesium
1:15	$5.5 \pm 0.1 \times 10^7$	$3.9 \pm 0.9 \times 10^7$
1:3	$5.9 \pm 0.4 \times 10^7$	$4.9 \pm 0.4 \times 10^7$
2:3	$6.7 \pm 0.2 \times 10^7$	$6.2 \pm 0.1 \times 10^7$
1:1	$7.0 \pm 0.3 \times 10^7$	$6.1 \pm 0.5 \times 10^7$

Table 1: Average total ester peak area (2' ester + 3' ester) for the aminoacylation of uridine with BocPEP at various lanthanum to uridine molar ratios. Reactions were performed in the presence and absence of excess magnesium at a molar ratio of 10: 3 (relative to uridine). The standard deviation is indicated as error.

The impact of magnesium on the competing lanthanum-catalyzed hydrolysis of BocPEP was investigated by comparing the peak areas corresponding to the hydrolysis product, BocPhe, for reactions on various scales of lanthanum. Reactions were performed with and without excess magnesium. Results are shown in Figure 4.

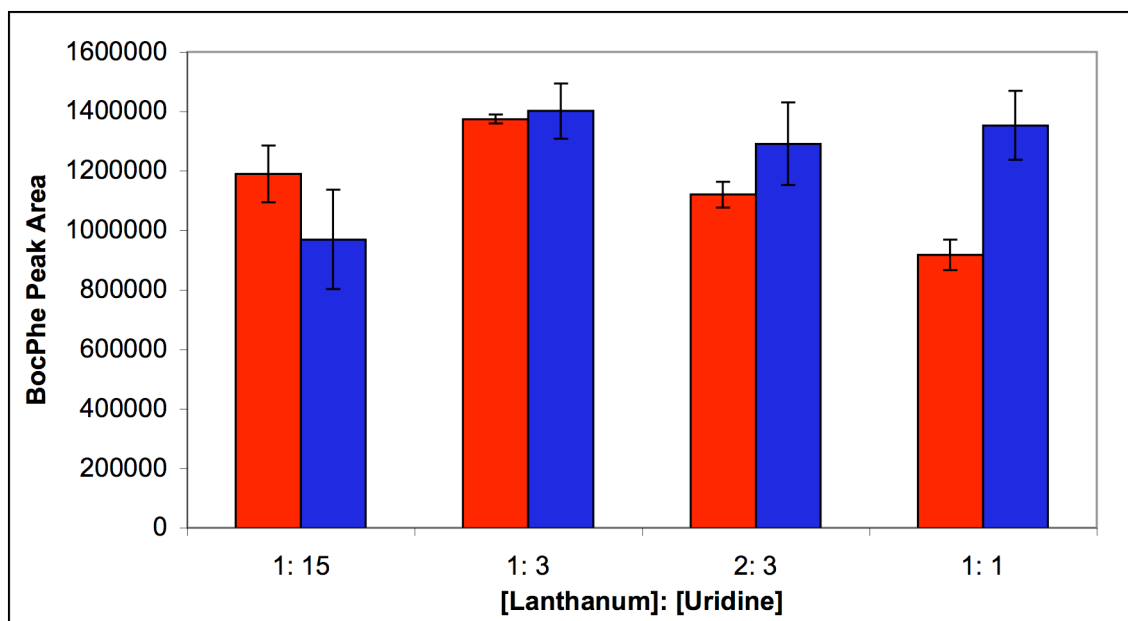


Figure 4: Average BocPhe peak area at various lanthanum to uridine molar ratios. Data points shown in blue correspond to reactions without magnesium, while those in red correspond to reactions with excess magnesium at a 10: 3 molar ratio (relative to uridine). Error bars indicate the standard deviation.

2.3.2 Determining Optimal Magnesium Concentration

The molar ratio of magnesium relative to uridine that results in maximum esterification was determined. BocPEP and uridine were present in 15-fold excess to lanthanum. These conditions were chosen so that in the future when tRNA is utilized, lanthanum can be added on a low scale, which will help to protect the tRNA from lanthanum-catalyzed hydrolysis.

Data obtained from HPLC analysis are summarized in Figure 5 and corresponding values and respective standard deviations are listed in Table 2.

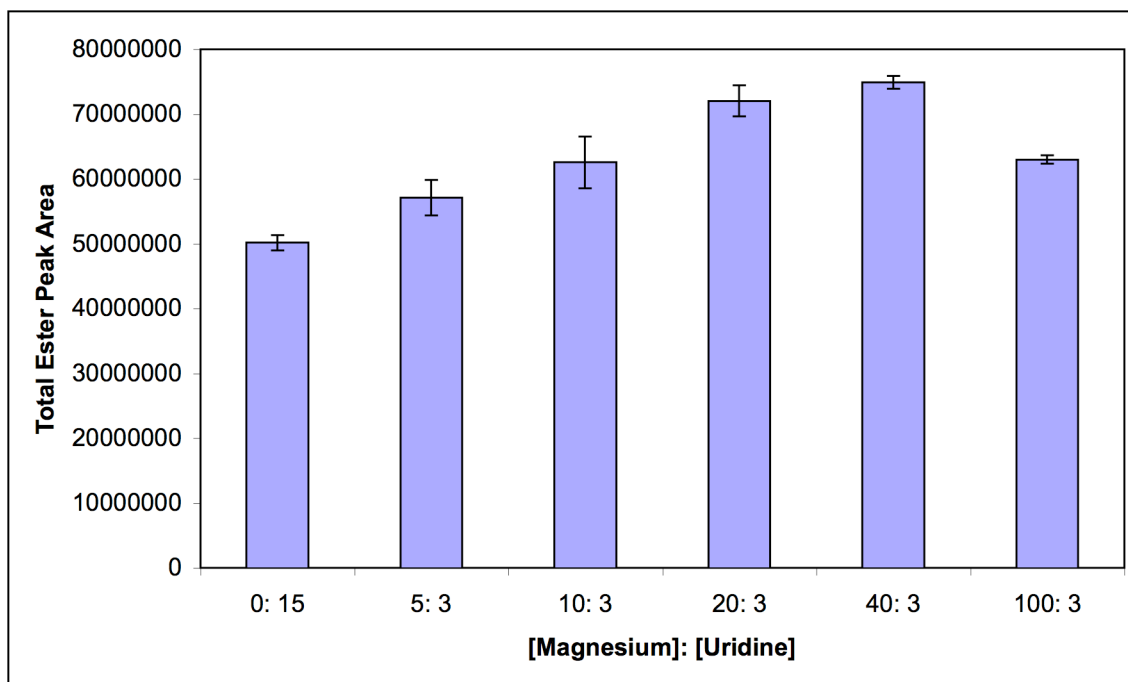


Figure 5: Average total ester peak area (2' ester + 3' ester) for the aminoacylation of uridine with BocPEP at various magnesium to uridine molar ratios. All reactions contained lanthanum at a molar ratio of 1: 15 (relative to uridine). Error bars indicate the standard deviation.

[Magnesium]: [Uridine]	Average total ester peak area (HPLC Area Units)
0: 15	$5.0 \pm 0.1 \times 10^7$
5: 3	$5.7 \pm 0.3 \times 10^7$
10: 3	$6.3 \pm 0.4 \times 10^7$
20: 3	$7.2 \pm 0.2 \times 10^7$
40: 3	$7.5 \pm 0.1 \times 10^7$
100: 3	$6.30 \pm 0.07 \times 10^7$

Table 2: Average total ester peak area (2' ester + 3' ester) for the aminoacylation of uridine with BocPEP at various magnesium to uridine molar ratios. All reactions contained lanthanum at a molar ratio of 1: 15 (relative to uridine). The standard deviation is indicated as error.

2.3.3 Further Reducing Lanthanum Concentration

The optimal magnesium to uridine molar ratio is 40: 3, as determined in section 2.3.2. Using this scale of magnesium, the relative amount of lanthanum was further reduced to determine if the increase in ester yield is enhanced on lower scales. All reactions were performed in quadruplicate and all samples were analyzed by HPLC analysis.

[Lanthanum]: [Uridine]	Average total ester peak area with magnesium (HPLC Area Units)	Average total ester peak area without magnesium (HPLC Area Units)
1: 30	$3.5 \pm 0.3 \times 10^7$	$3.3 \pm 0.3 \times 10^7$
1: 100	$1.48 \pm 0.08 \times 10^7$	$1.3 \pm 0.3 \times 10^7$

Table 3: Average total ester peak area (2' ester + 3' ester) for the aminoacylation of uridine with BocPEP at various lanthanum to uridine molar ratios. Reactions were performed in the presence and absence of excess magnesium at a molar ratio of 40: 3 (relative to uridine). The standard deviation is indicated as error.

2.4 Discussion

2.4.1 Effect of Excess Magnesium on Aminoacylation

Even without any added magnesium, it is possible to reduce the concentration of lanthanum to as low as one percent of the concentrations of uridine and BocPEP and still observe significant esterification. This makes it possible to use lanthanum on a low scale when tRNA is utilized in order to preserve its structural integrity. This also shows that lanthanum shows great selectivity towards the diol functionality on the uridine molecules.

The addition of magnesium at a molar ratio of 10: 3 (relative to uridine) causes an average increase in the yield of boc-phenylalanyl-uridine esters of 17% at all lanthanum conditions studied (Table 1). It should be noted that similar ester peak areas are observed in reactions containing equimolar concentrations of lanthanum and uridine in the absence of magnesium (average total ester peak area = $6.1 \pm 0.5 \times 10^7$) and those containing 33% lanthanum relative to uridine in the presence of excess magnesium at molar ratio of 10: 3 (average total ester peak area = $5.9 \pm 0.4 \times 10^7$) (Table 1). This suggests that magnesium ions are able to displace lanthanum from the ethyl phosphate byproduct, freeing up lanthanum ions to catalyze further reactions.

Since lanthanum is known to catalyze the hydrolysis of aminoacyl phosphates¹⁰, the effect of magnesium on BocPEP hydrolysis was also investigated (Figure 4). Magnesium effectively increases the lanthanum concentration available for catalysis and BocPEP hydrolysis. Figure 4 shows that for reactions containing lanthanum at molar ratios of 2: 3 and 1: 1 (relative to uridine), added magnesium suppresses the competing hydrolysis reaction. However, when lanthanum is present on a much lower scale (1: 15)

more hydrolysis product is observed in reactions containing magnesium compared than those without.

The hydrolysis product increases as lanthanum decreases for reactions with and without magnesium (Table 3). This can be explained by considering that in buffered aqueous solutions both lanthanum-bound water and buffer can catalyze aminoacyl phosphate hydrolysis¹⁰. This occurs when there is unreacted BocPEP, which is present when lanthanum is added on a lower scale. Also the presence of magnesium ensures more free lanthanum is available, which may explain the increase in BocPhe yield for magnesium-containing reactions where lanthanum present at a molar ratio of 1: 15 (relative to uridine) (Table 3).

2.4.2 Determining Optimal Magnesium Concentration

For reactions containing uridine at 15-fold excess to the lanthanum catalyst, the magnesium to uridine molar ratio that resulted in the greatest yield of esters was 40: 3 (Figure 5). In this case, an increase in ester yield of 33% was observed over reactions without added magnesium (Table 3). The yield of esters produced in the aforementioned reaction is within 5% of the yield produced in a reaction containing ten times more lanthanum (lanthanum to uridine molar ratio of 2: 3) with no added magnesium as seen in Table 2 (differences in HPLC injection volumes were accounted for). This is quite remarkable and a testament to the role that magnesium plays in displacing the lanthanum ions from the ethyl phosphate byproduct. In the future when this reaction is used to aminoacylate tRNA, it will be possible to reduce the amount of lanthanum added to each reaction and thus preserve the structural integrity of the tRNA.

As can be seen in Figure 5, when the magnesium to uridine molar ratio is further increased (100: 3), the total ester peak area is reduced, indicating that esterification is being depressed. This is likely because the excess magnesium is saturating BocPEP and competing with the lanthanum catalyst.

2.4.3 Further Reducing Lanthanum Concentration

Using the optimal magnesium to uridine molar ratio of 40: 3 (Section 2.4.2), the concentration of lanthanum was further reduced to determine if the increase in ester yield is observed at molar ratios less than 1: 15 (1: 30 and 1: 100, relative to uridine). Esters were observed with and without added magnesium for all scales of lanthanum studied (Table 3). A small increase in ester yield was observed in reactions containing magnesium, though much less substantial than the increase observed when the lanthanum to uridine ratios was 1: 15. This is likely to be due to the presence of extremely low amounts of lanthanum (3.33% and 1%) relative to uridine, which may indicate that the practical catalytic concentration limits of the lanthanum catalyst have been reached.

CHAPTER 3: Optimization of Reaction pH and Buffer Conditions

3.1 *Introduction*

The pK_a of water coordinated to lanthanum is seven units lower than uncoordinated water¹⁶. Since changes to the pH can affect the reaction rate of aminoacylation, it is important to buffer reactions so that upon adding lanthanum, the pH change is minimized. However, high concentrations of buffer can also catalyze the decomposition of aminoacyl phosphates. It is important to find a balance between buffering the reaction at a relatively constant pH while also minimizing BocPEP hydrolysis and most importantly ensuring maximum esterification.

I optimized the reaction conditions by varying the concentration of pH 8 EPPS buffer relative to the concentration of uridine. For each reaction, the pH before lanthanum addition and at various time points during the reaction is measured. Various molar ratios lanthanum and magnesium were considered in order to provide insight as to whether the increase in ester yields in the presence of magnesium is due to the magnesium salt altering the pH.

3.2 *Experimental*

All commercial reagents were purchased from Sigma Aldrich Canada and were used as received without further purification. All water was doubly distilled and deionized before use. High performance liquid chromatography (HPLC) analysis was performed on C18-reverse phase column and the products were detected at 263nm. The mobile phase consisted of 40% acetonitrile and 0.1% TFA in water.

Aminoacylation reactions were performed with 1.5×10^{-2} M uridine and BocPEP and all other reagents were added in relative amounts. Lanthanum was added at the following molar ratios relative to uridine: 1:1, 2: 3, 1: 3, and 1: 15. All reactions were performed both in the presence and absence of excess magnesium at a molar ratio of 10: 3 relative to uridine (5.0×10^{-2} M). The buffer utilized was pH 8 EPPS buffer at the following concentrations: 0 M, 1.0×10^{-2} M, 5.0×10^{-2} M, 1.0×10^{-1} M, and 2.0×10^{-1} M. These correspond to buffer to uridine molar ratios of 2: 3, 10: 3, 20: 3, and 40: 3, respectively.

The pH of each reaction was recorded before the addition of lanthanum ($t = 0$), 2.5 minutes after the addition of lanthanum ($t = 2.5$), and at 5 minutes after the addition of lanthanum ($t = 5$), which is immediately before the reaction was quenched. All aminoacylation reactions were quenched with 2.0×10^{-4} L of saturated ethylenediaminetetraacetic acid (EDTA). All reactions were performed in triplicate and the mean amount of ester products were determined by HPLC peak integration and standard deviations were computed.

All stock solutions (lanthanum triflate ($\text{La}(\text{OTf})_3$), magnesium triflate ($\text{Mg}(\text{OTf})_2$), BocPEP, and uridine) were freshly prepared in the concentration of pH 8

EPPS buffer in question. Reagents were added in the order of uridine, BocPEP, magnesium, and initiated upon addition of lanthanum. The triflate salts of lanthanum ($\text{La}(\text{OTf})_3$) and magnesium ($\text{Mg}(\text{OTf})_2$) were utilized in all aminoacylations and are referred to as magnesium and lanthanum where appropriate.

3.3 Results

The average measured pH at each time point and reaction condition is shown in Tables 4, 5, 6, and 7 for reactions containing EPPS to uridine molar ratios of 2: 3, 10: 3, 20: 3, and 40: 3, respectively. Reactions were also performed in water (unbuffered) and the average pH before the reaction was initially 3.80 and it did not fluctuate significantly over the five-minute reaction. No esterification was observed in any reaction where the solution was not buffered.

[Lanthanum]: [Uridine]	[Magnesium]: [Uridine]	Average pH at t = 0min	Average pH at t = 2.5min	Average pH at t = 5min
1: 1	10: 3	6.90	4.84	4.72
1: 1	0	7.13	4.67	4.64
2: 3	10: 3	6.91	4.92	4.81
2: 3	0	7.20	4.73	4.63
1: 15	10: 3	6.96	6.44	6.39
1: 15	0	7.26	6.75	6.67

Table 4: Average pH at t = 0, 2.5, and 5 for the aminoacylation of uridine with BocPEP in pH 8 EPPS buffer at a molar ratio of 2: 3 (relative to uridine). Molar ratios of lanthanum and magnesium are indicated. The average standard deviation is 0.04 for each data point.

[Lanthanum]: [Uridine]	[Magnesium]: [Uridine]	Average pH at t=0min	Average pH at t=2.5min	Average pH at t=5min
1: 1	10: 3	7.53	6.30	6.14
1: 1	0	7.61	6.22	6.05
2: 3	10: 3	7.56	6.52	6.50
2: 3	0	7.61	6.51	6.38
1: 15	10: 3	7.56	7.34	7.29
1: 15	0	7.58	7.38	7.34

Table 5: Average pH at t = 0, 2.5, and 5 for the aminoacylation of uridine with BocPEP in pH 8 EPPS buffer at a molar ratio of 10: 3 (relative to uridine). Molar ratios of lanthanum and magnesium are indicated. The average standard deviation is 0.04 for each data point.

[Lanthanum]: [Uridine]	[Magnesium]: [Uridine]	Average pH at t=0min	Average pH at t=2.5min	Average pH at t=5min
1: 1	10: 3	7.76	7.28	7.30
1: 1	0	7.82	7.25	7.23
2: 3	10: 3	7.74	7.30	7.30
2: 3	0	7.82	7.34	7.33
1: 15	10: 3	7.83	7.62	7.59
1: 15	0	7.83	7.65	7.63

Table 6: Average pH at t = 0, 2.5, and 5 for the aminoacylation of uridine with BocPEP in pH 8 EPPS buffer at a molar ratio of 20: 3 (relative to uridine). Molar ratios of lanthanum and magnesium are indicated. The average standard deviation is 0.02 for each data point.

[Lanthanum]: [Uridine]	[Magnesium]: [Uridine]	Average pH at t=0min	Average pH at t=2.5min	Average pH at t=5min
1: 1	10: 3	7.81	7.56	7.56
1: 1	0	7.82	7.66	7.64
2: 3	10: 3	7.81	7.60	7.59
2: 3	0	7.82	7.68	7.68
1: 15	10: 3	7.82	7.76	7.77
1: 15	0	7.847	7.82	7.85

Table 7: Average pH at t = 0, 2.5, and 5 for the aminoacylation of uridine with BocPEP in pH 8 EPPS buffer at a molar ratio of 40: 3 (relative to uridine). Molar ratios of lanthanum and magnesium are indicated. The average standard deviation is 0.01 for each data point.

HPLC analysis was performed for each reaction and the average total ester peak area was determined. The results are presented in Figures 6 and 7. Figure 6 shows data from reactions at various molar ratios of lanthanum and pH 8 EPPS buffer in the absence of magnesium and Figure 7 shows complementary data in the presence of magnesium at a molar ratio of 10: 3 (relative to uridine).

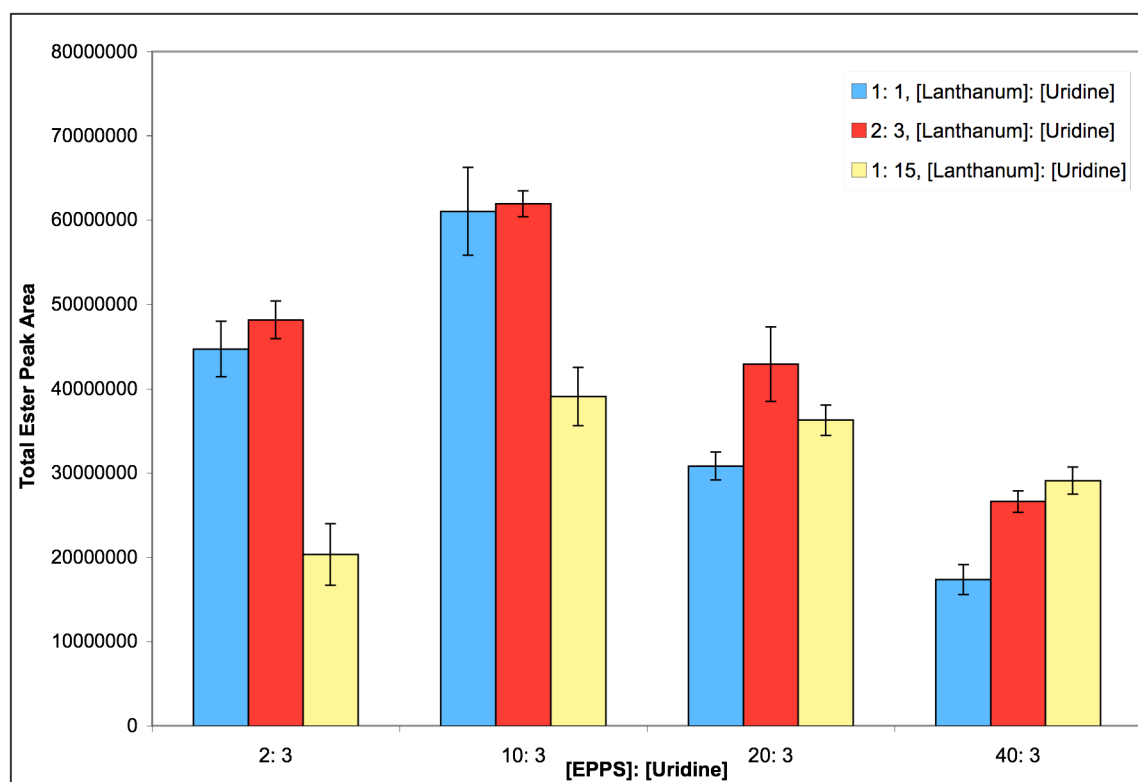


Figure 6: Average total ester peak area (2' ester + 3' ester) for the aminoacylation of uridine with BocPEP at various molar ratios of pH 8 EPPS buffer and lanthanum (relative to uridine). Error bars indicate the standard deviation. No magnesium was added.

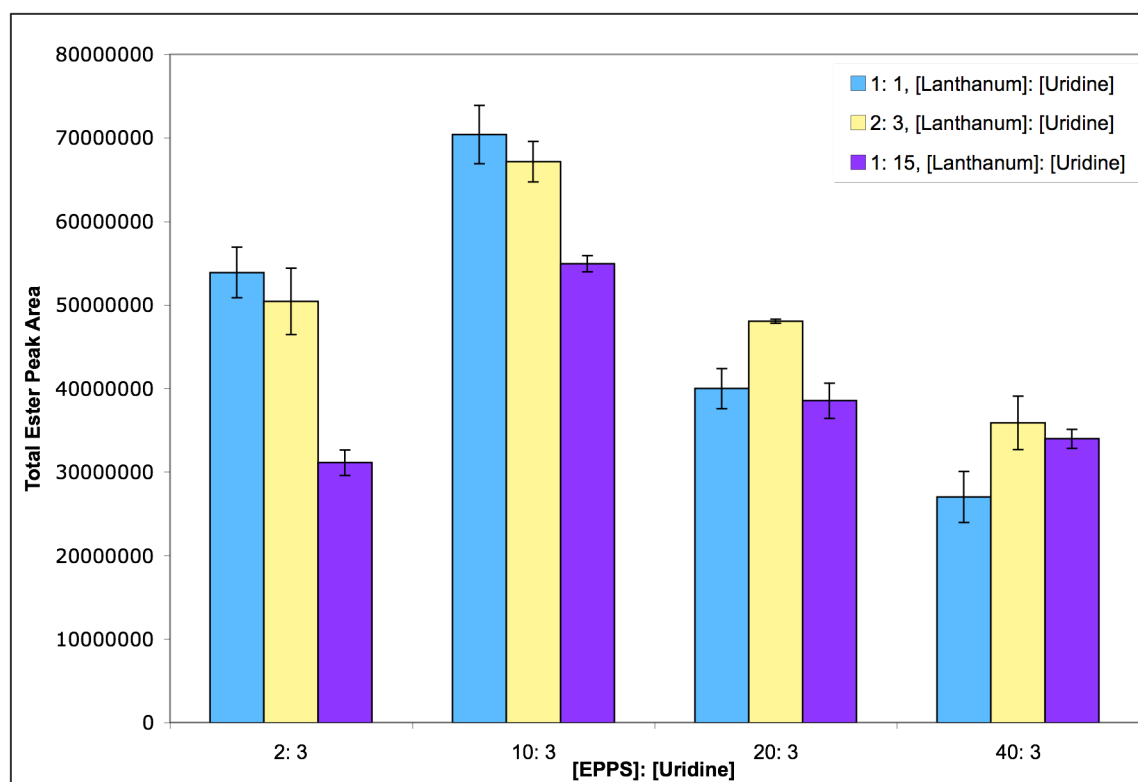


Figure 7: Average total ester peak area (2' ester + 3' ester) for the aminoacylation of uridine with BocPEP at various molar ratios of pH 8 EPPS buffer and lanthanum (relative to uridine). Error bars indicate the standard deviation. Excess magnesium was added at a molar ratio of 10: 3 (relative to uridine).

The effect of EPPS concentration on the competing BocPEP hydrolysis reaction was also investigated. Reactions containing uridine in 15-fold excess to lanthanum were examined, as these were found to contain the largest amount of hydrolysis product (Section 2.3.1). Figure 7 shows the average BocPhe peak area for reactions performed with and without excess magnesium and at various molar ratios of pH 8 EPPS buffer (relative to uridine).

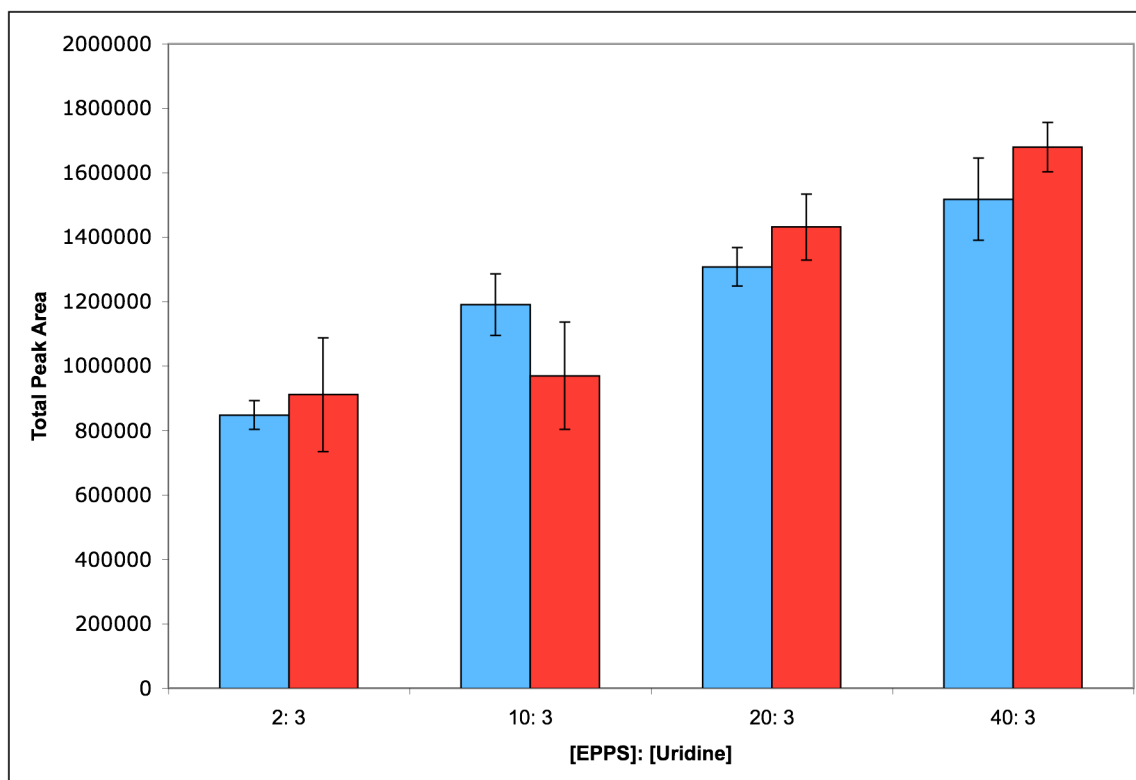


Figure 8: Average BocPhe peak area for the aminoacylation of uridine with BocPEP at various pH 8 EPPS buffer to uridine molar ratios. All reactions contained lanthanum at a molar ratio of 1: 15 (relative to uridine). Excess magnesium was added at a molar ratio of 10: 3 to reactions shown in blue. Reactions shown in red did not contain magnesium. Error bars indicate the standard deviation.

3.4 Discussion

All buffers were prepared initially at pH 8 and upon addition of uridine, BocPEP, and in some cases magnesium, the pH was observed to drop even before the lanthanum was added. This is likely to be due to a small amount of hydrolysis of BocPEP, which breaks down into ethyl phosphate and BocPhe, an amino acid (which has a pK_a of approximately two). As expected, a pH decrease was observed in all buffered reactions upon the addition of lanthanum, though its magnitude was reduced in reactions performed in greater concentrations of pH 8 EPPS buffer. The pH decrease was minimized when less lanthanum was added across all buffer conditions. There is little difference in magnitude of the observed decrease in pH between reactions containing magnesium and those not containing magnesium, although the final reaction pH is slightly lower in reactions that do not contain magnesium. This indicates that the increase in esterification in reactions containing magnesium is not likely to be a result of buffering caused by the magnesium or triflate ions.

The highest ester yields were observed for reactions performed in a pH 8 EPPS buffer at a buffer to uridine ratio of 10: 3. This was the case for every scale of lanthanum studied (Figures 6 and 7). The yield of esters was depressed at higher buffer to uridine molar ratios (40: 3 and 100: 3). This observation suggests that EPPS may be inhibiting lanthanum when it is present at high concentration. Although lanthanides do not coordinate well to organic sulfates, EPPS has hydroxyl groups, which can weakly coordinate to the metal¹³. As was the case in section 2.3.1, reactions containing the high molar ratios of lanthanum (relative to uridine) produced the highest ester yields. Again, the yield was greatest in reactions containing excess magnesium (Figure 7).

Interestingly, reactions that experience a significant decrease in pH over the course of the reaction produce the highest ester yields (Tables 4 and 5). This occurs for reactions performed in pH 8 EPPS at a buffer to uridine molar ratios of 2: 3 and 10: 3, with 10: 3 producing the highest ester yields. Though the pH decrease is minimized when the scale of lanthanum is reduced, it is clear that reactions, where a significant decrease in pH occurs, produce the highest yield of esters. This decrease in pH likely stabilizes the esters, preventing them from hydrolyzing. In more basic solutions, esters are more susceptible to hydrolysis, which may account for the decrease in ester yield at higher buffer to uridine molar ratios (40: 3 and 100: 3), where the pH was maintained at 7.5. As expected, as the buffer to uridine molar ratio increased not only did yield decrease, but BocPEP hydrolysis also increased (Figure 8). This is probably due to buffer catalyzing to the decomposition of BocPEP.

3.5 Conclusions and Future Considerations

The addition of excess of magnesium at a 40: 3 molar ratio (relative to uridine) increases the yield of esters for a lanthanum-catalyzed aminoacylation of uridine by up to 33% relative to reactions without magnesium. An increase in yield in the presence of magnesium was observed at all scales of lanthanum studied. Further reagent optimization can be achieved by using pH 8 EPPS buffer at a molar ratio of 10: 3 (relative to uridine). On this scale, the competing BocPEP hydrolysis reaction is minimized and ester yield is maximized. These conditions should be kept in mind when optimizing a lanthanum-catalyzed system using tRNA.

In the future, the addition of excess magnesium may allow the amount of lanthanum to be reduced in cases where tRNA is aminoacylated to ensure its structural integrity is maintained. Of equal importance, the results presented demonstrate that the lanthanum catalyst shows great selectivity towards the diol functionality present both on uridine and the 3' terminus of tRNA.

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