Biomimetic Aminoacylation

by

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Graduate Department of Chemistry
University of Toronto

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Abstract

“Biomimetic Aminoacylation”

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The accuracy of ribosomal protein synthesis depends on the fidelity of highly specific enzymes, aminoacyl tRNA synthetases, towards amino acid – tRNA pairs. These biological catalysts are responsible for activating the amino acids as aminoacyl adenylates and for their subsequent attachment to the 2’- or 3’-OH at the 3’-terminal of the correct tRNA to give aminoacyl-tRNA.

Extended diversity in protein structure and function could be achieved if non-natural side chains can be introduced in protein synthesis. This requires that the acceptor stem of a tRNA molecule be synthetically aminoacylated. The most widely used methods for charging tRNA with non-natural amino acids involve multi-step synthesis of an aminoacyl-pCpA and its consequent enzymatic ligation to truncated tRNA. No direct route to these species has been reported.

We have developed a method for direct biomimetic aminoacylation of the 3’-terminal hydroxyls of tRNA. Our approach shows to be promising in reactions leading to direct 2’- or 3’-O-aminoacylation of not only nucleosides and nucleotides but also RNA in general and tRNA in particular.

The system we have developed provides: 1) efficient activation of the amino acids as aminoacyl phosphates, analogues of the enzymatic intermediates, and 2) specific recognition of the 3’-terminal of tRNA by lanthanide ions present in the reaction. The aminoacylating reagents used in our studies were carefully selected to provide handles to
follow the reaction: UV absorbance, fluorescence spectroscopy and $^{19}$F NMR. Lanthanide (III) ions can play a role similar to a key part of the aminoacyl tRNA synthetases – they bring the aminoacyl close to the 3’-terminal of tRNA, in this case by forming a bis-bidentate complex with the aminoacyl phosphate and the 2’,3’-diol functionality of the 3’-terminal adenosine. This process relies on the specificity towards the unique 3’-terminal diol on tRNA, provided by the metal ion and the simultaneous complexation of the aminoacyl phosphate.
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BocTEP

TEP

BocFPEP

Isopropyl ester of BocFPhe

\(\varepsilon\)-DNS-\(\alpha\)-BocLysEP

\(\alpha\)-DNSGlyEP

\(\alpha\)-DNSPheEP

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<th>Full Form</th>
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<tbody>
<tr>
<td>aaRS</td>
<td>aminoacyl tRNA synthetase</td>
</tr>
<tr>
<td>5’AMP</td>
<td>Adenosine-5’-monophosphate</td>
</tr>
<tr>
<td>anti-DNS-Ab</td>
<td>anti-dansyl antibody</td>
</tr>
<tr>
<td>ApC</td>
<td>Adenyl (3’→5’) cytidine</td>
</tr>
<tr>
<td>ApCpC</td>
<td>Adenyl (3’→5’) cytidyl (3’→5’) cytidine</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>tBoc (Boc)</td>
<td>tertbutyl oxycarbonyl</td>
</tr>
<tr>
<td>BocFPEP</td>
<td>α-N-Boc-(L)p-fluorophenylalanyl ethyl phosphate</td>
</tr>
<tr>
<td>BocFPhe</td>
<td>α-N-Boc-(L)p-fluorophenylalanine</td>
</tr>
<tr>
<td>BocTEP</td>
<td>α-N-Boc-(L)tyrosyl ethyl phosphate</td>
</tr>
<tr>
<td>BocTyr</td>
<td>α-N-Boc-(L)tyrosine</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>2’CMP</td>
<td>Cytidine-2’-monophosphate</td>
</tr>
<tr>
<td>3’CMP</td>
<td>Cytidine-3’-monophosphate</td>
</tr>
<tr>
<td>5’CMP</td>
<td>Cytidine-5’-monophosphate</td>
</tr>
<tr>
<td>DCC</td>
<td>N, N’-dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNS</td>
<td>dansyl</td>
</tr>
<tr>
<td>DNS-Boc-Lys</td>
<td>ε-N-dansyl-α-N-Boc-(L)lysine</td>
</tr>
<tr>
<td>DNSGly</td>
<td>α-N-dansyl-glycyl</td>
</tr>
<tr>
<td>DNSGlyEP</td>
<td>α-N-dansyl-glycyl ethyl phosphate</td>
</tr>
<tr>
<td>DNSPhe</td>
<td>α-N-dansyl-(L)phenylalanine</td>
</tr>
<tr>
<td>DNSPheEP</td>
<td>α-N-dansyl-(L)phenylalanyl ethyl phosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EPPS</td>
<td>N-(2-hydroxyethyl)piperazine-N’-(3-propane) sulfonic acid</td>
</tr>
<tr>
<td>ESI MS</td>
<td>electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>5’GMP</td>
<td>Guanosine-5’-monophosphate</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>IF</td>
<td>initiation factor</td>
</tr>
<tr>
<td>La&lt;sup&gt;3+&lt;/sup&gt;</td>
<td>lanthanum</td>
</tr>
<tr>
<td>Ln&lt;sup&gt;3+&lt;/sup&gt;</td>
<td>lanthanide</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>oxRNA</td>
<td>oxidized RNA</td>
</tr>
<tr>
<td>oxtRNA</td>
<td>oxidized tRNA</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RP</td>
<td>reverse phase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecylsulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate EDTA</td>
</tr>
<tr>
<td>TEP</td>
<td>tyrosyl ethyl phosphate</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>5’TMP</td>
<td>Thymidine-5’-monophosphate</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>5’UMP</td>
<td>Uridine-5’-monophosphate</td>
</tr>
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Structures of Relevant Compounds

Buffers

\[
\text{N-(2-hydroxyethyl)piperazine-}\text{-N'-(3-propane) sulfonic acid (EPPS)}
\]

\[
\text{Ethylenediaminetetraacetic acid (EDTA)}
\]

RNA bases

\[
\text{Adenine}
\]

\[
\text{Guanine}
\]

\[
\text{Cytosine}
\]

\[
\text{Uracil}
\]
Ribonucleosides and derivatives

- Adenosine
- 2'-Deoxyadenosine
- 2',3'-O-isopropylideneadenosine
- Uridine
- 2'-Deoxyuridine
- Cytidine
Nucleotides and derivatives

Adenosine-5′-monophosphate

Guanosine-5′-monophosphate

Uridine-5′-monophosphate

Thymidine-5′-monophosphate

Cytidine-5′-monophosphate

Cytidine-3′-monophosphate

Cytidine-2′-monophosphate
Structure of tRNA
CHAPTER 1: General introduction

1.1. Biochemical protein synthesis

Protein synthesis starts with transcription of the DNA coding for a given protein into messenger RNA (mRNA). This new form of genetic information is then read by the ribosomes in a process called translation.

Proteins in living organisms are synthesized from twenty naturally occurring L-amino acids. Each of them must be attached to its corresponding transfer RNA (tRNA) to form an aminoacyl-tRNA. Each of these aminoacylated tRNAs is brought to the ribosome by specific proteins (factors) when the codon on the mRNA, corresponding to the tRNA-anticodon is read. Where the amino acid will be inserted into the amino acid sequence is determined by the pairing of a codon in mRNA with a particular aminoacyl-tRNA.¹

Figure 1: From DNA to proteins (http://www.scq.ubc.ca/wp-content/translation)
1.1.1. **Aminoacylation of tRNA**

The aminoacylation process is catalyzed by a family of twenty enzymes, the aminoacyl tRNA synthetases (aaRS). They play an essential role in protein synthesis because they are responsible for charging the correct tRNA with its cognate amino acid. Each aaRS is specific for one amino acid and one or more isoaccepting tRNAs.

The aaRS family is divided in two classes: Class I and Class II. There are several differences, including active site topologies, binding of tRNA and position of acylation of tRNA. Class I enzymes charge tRNAs at the 2’-OH while Class II enzymes catalyze acylation at the 3’-OH.¹

The acylation reaction proceeds in two steps.²⁻³ First, the amino acid is activated as an aminoacyl-adenylate in a reaction with ATP (adenosine triphosphate). This reaction intermediate forms a complex with the enzyme.⁴ In the second step, the acyl transfer, the amino acyl moiety is transferred from the phosphate of 5’AMP (adenosine 5’-monophosphate) to the the 2’- or 3’- hydroxyl group of the tRNA molecule. Both steps are catalyzed by the same enzyme.

The activation step requires the presence of ATP and Mg²⁺ (Figure 2). The metal ion stabilizes the conformation of ATP and withdraws electrons from the β-phosphate to facilitate the formation of aminoacyl adenylate. The enzyme orients the two substrates in optimal positions for transition state formation by in-line nucleophilic displacement, which lowers the activation barrier for the reaction.¹, ³ The resulting acyl phosphate is retained by the aaRS for the second step, nucleophilic attack of the 2’- or 3’- hydroxyl group at the 3’-terminal of tRNA.
Figure 2: Formation of aminoacyl adenylates

The accuracy of aminoacyl-tRNA synthesis depends on the specificity of the aaRS. The overall error rate is about 1 in 10,000. The discrimination of amino acids by aaRS is based mostly on recognition of the side chain. Some amino acids such as cysteine have unique functional groups which makes their recognition easy. Additional discrimination, however, is necessary for amino acids that are very close in structure, such as valine and isoleucine. In those cases, the enzyme has two distinct catalytic editing sites, which act as a “double sieve”. The first site excludes amino acids larger than the specific substrate. Hydrolysis of the incorrect aminoacyl adenylate occurs in the second site. In the selection of the cognate tRNA, the accuracy of the process depends on the recognition and the stabilization of the transition state for tRNA charging. The recognition is based mainly on interactions with one or more discriminator bases (N73), the acceptor stem and the anticodon.
1.1.2. Ribosomal protein synthesis

Proteins are synthesized in the ribosomes in an N- to C-terminal direction. The ribosome reads mRNA which codes the amino acid sequence for the synthesizing protein.

![Ribosomal peptide-bond formation](image)

**Figure 3**: Ribosomal peptide-bond formation

The tRNA charged with the correct amino acid is brought to the ribosome by specific proteins, called factors (initiation and elongation factors). There are three sites on the ribosome as it reads the mRNA: A (aminoacyl-tRNA binding), P (peptidyl-tRNA binding) and E (exit) sites. The aminoacyl-tRNA is delivered first to site A by the elongation factors. Upon nucleophilic attack of the amino group of the aminoacyl-tRNA to the peptidyl-tRNA at the P site, the elongated with peptide is moved from peptidyl-tRNA to the aminoacyl-tRNA (Figure 3). tRNA is released through site E as a free molecule and is available for another aminocyclation cycle. The completion of the protein synthesis is marked by a stop codon on the mRNA. There is no endogenous tRNA with an anticodon corresponding to a stop codon. Once the ribosome reaches the stop codon which is recognized by release factors, the protein is released in the cytoplasm.
1.2. **In Vitro incorporation of non-natural amino acids into proteins**

Synthesis of peptides and proteins with novel properties require incorporation of amino acids with non-natural side chains. Such peptides can be developed to function as enzyme inhibitors or to carry spin labels or fluorescent labels for study of protein structure, kinetics, folding, and other applications.\(^8\)-\(^{10}\)

Due to the high specificity of aaRSs, as mentioned above, non-natural amino acids can not be incorporated biosynthetically, unless they differ very little from the natural amino acids. For example, fluorinated aromatic amino acids can be incorporated into a bacterial expression system by including them in the growth medium and eliminating the ability of the cell to synthesize that amino acid endogeneously.\(^{11}\) However, this results in nonspecific incorporation. In order to incorporate non-natural amino acids into a protein using the biological translational apparatus, new methods must be developed.

*In vitro* methods usually use a combination of misacylated tRNA and a unique codon for the non-natural amino acid. Thus, the important interaction in this case is codon/anticodon and it does not depend on the nature of the amino acid. These methods include amber suppression, frameshift suppression and N-terminal labeling.

**1.2.1. Amber suppression**

In nature, there are three degenerate stop codons (UAA – ochre, UAG – amber, and UGA – opal) whose role is to stop translation and cause protein release.\(^{12}\) Out of the three stop codons, only one, UGA, occurs frequently. Thus the remaining two, UAA and UAG, can be used to code for a non-natural amino acid. The amber stop codon, UAG, is the primary choice in the stop codon suppression method.

Incorporation of such a stop codon into a DNA sequence is done by site-directed mutagenesis. The codon is complemented by a misacylated tRNA bearing the anticodon complementary to the stop codon. This allows for the insertion of a non-natural amino acid at a specific position in the protein sequence.

Hecht and co-workers developed a method for synthesizing “misacylated” tRNAs.\(^{13}\) Essentially, this method relies on T4 RNA ligase-mediated coupling of 2'(3')-O-acetylated pCpA derivative with a truncated tRNA molecule (tRNA-\(\text{C}_{\text{OH}}\)). The dinucleotide is
acylated through a multiple step process involving protection of the amino groups on cytidine and the amino acid. However, the conditions for the removal of standard protecting groups (tBoc, Fmoc, Cbz, etc.) destroy the integrity of the macromolecule. The protection of the amino acid here is required due to the conditions of the ligation reaction. The acylated tRNA prepared by this method, however, can not be directly used for ribosome-based translation unless the protecting group is removed. The original method was improved by using protecting groups such as pyroglutamyl\textsuperscript{14}, and N-pentenoyl\textsuperscript{15}, which can be removed from the “misacylated”-tRNA enzymatically (pyroglutamate aminopeptidase) or by mild treatment with aqueous iodine.

**Figure 4:** Strategy of biosynthetic incorporation of non-natural amino acids via Amber suppression method
As mentioned above, the amber suppression approach involves the replacement of the codon for the amino acid of interest with a stop codon, TAG, by site-directed mutagenesis (Figure 4).\textsuperscript{16, 17} There are several requirements that must be met before the amino acid of interest is incorporated into the protein. The suppressor tRNA that delivers the amino acid must efficiently insert the amino acid in response to the UAG codon and must not be recognized by the endogeneous aaRS. If a tRNA is used from the same organism as the one from which the translational system is derived, it will be deacylated and acylated with the correct amino acid by the enzyme. The suppressor tRNA is prepared via anticodon-loop replacement procedure developed by Bruce and Uhlenbeck\textsuperscript{18} and then chemically acylated. Schultz and co-workers used tRNA\textsuperscript{Phe} from yeast with an altered anticodon in \textit{E. coli} derived translational system.\textsuperscript{17} Their approach to the aminoacylation of tRNA is very similar to Hecht’s method, with some improvements. First, a dinucleotide, pCpA\textsuperscript{17} or pdCpA\textsuperscript{16} is acylated with the amino acid of interest. The amino groups of cytidine and amino acid are protected with the same protecting group (\textit{o}-nitrophenyl sulfenyl group = \textit{o}-NPS) which allows for easy one-step deprotection with aqueous thiosulfate at the end of the process. The yield of this reaction is 14 percent. In the second, the fully deprotected aminoacylated dinucleotide is ligated to the truncated suppressor tRNA\textsuperscript{Phe}. Chamberlin and co-workers developed a similar method which relies on a combination of chemical synthesis and run-off transcription to prepare the misacylated nonsense suppressor tRNA.\textsuperscript{19}

Despite the promise of this technique, it also has many drawbacks. The yield of protein synthesized by stop codon suppression is low. The synthesis of the suppressor tRNA and its acylation require multiple steps. Also, the suppressor tRNA is in competition with the release factors which can result in synthesis of truncated proteins. The suppressor tRNA must also be orthogonal to the organism the \textit{in vitro} system is derived. Another reason for low protein production is that once deacylated the tRNA cannot be reused. Using only one stop codon limits this method to incorporation of only one non-natural amino acid into a given protein.

Later, Schultz\textsuperscript{20} and RajBhandary\textsuperscript{21} improved this methodology by generating orthogonal pairs of aaRS and suppressor tRNAs which increased the yields and solved the problem of deacylation by endogeneous aaRS. The approach involves import of the
aaRS and the suppressor derived from a cognate tRNA from heterologous or the same organism. The tRNA is an amber suppressor and the enzyme is mutated in order to recognize the non-natural amino acid and the tRNA\textsubscript{CUA} and not the endogenous tRNAs.

### 1.2.2. Frameshift suppression

Amber suppression is a widely used method for incorporation of non-natural amino acids but it does not allow for incorporation of multiple amino acids into a single protein. This is possible through frameshift suppression. Another advantage of using frameshift over amber suppression is that the competition with the endogenous release factors for the stop codon is avoided.

The reading frame of a gene can be changed by inserting or deleting a nucleotide. Despite such a mutation in mRNA, proteins can still be synthesized if a suppressor tRNA is present. The suppressor tRNA (GCC) recognizes a 4 nucleotide codon (CGGG) (Figure 5A). In this case, after the peptidyl transfer, the new codon (CGU) at the A-site is shifted with +1 nucleotide, and full length protein is synthesized. If the endogenous tRNA recognizes the 3 nucleotide codon on the mRNA (CGG) (Figure 5B), truncated protein is synthesized as the ribosome reaches the in-frame stop codon. After the formation of the new peptide bond, the peptidyl-tRNA is moved to the ribosomal P-site and a new codon (GCG) is positioned at the A-site.

Sisido et al. found that suppressions of AGGU and CGGG in E. coli in vitro system effectively insert nonnatural amino acids with p-nitrophenyl, 1- and 2- naphtyl, 2-antranyl, and p-phenylazophenyl groups, among others. Later, they successfully introduced two nonnatural amino acids, 2-naphtylalanine and nitrophylalanine, into Streptavidin by using a combination of two four-base codons, CGGG and AGGU. Enzymatically synthesized frameshift suppressor tRNAs (tRNA\textsubscript{ACCU(-CA)} and tRNA\textsubscript{CGGG(-CA)}) were aminoacylated via ligation to aminoacyl-pdCpA by T4 RNA ligase.
Figure 5: Frameshift suppressor tRNA recognizes the four base codon CGGG and incorporates the non-natural amino acid into a full length protein (A); In the absence of the frameshift suppressor tRNA, truncated protein is synthesized (B)

1.2.3. N-terminal labeling

In prokaryotes, the initiator tRNA (tRNA\(^{Met}\)) delivers formylmethionine to the ribosome with the help of the initiation factors (IF). Three initiation factors (IF1, IF2 and IF3) are responsible for this process. IF1 prevents entering of the aminoacyl-tRNA at the A ribosomal site. IF3 plays an important role in stabilizing the free 30S subunit of the ribosome and its binding to the initiation site of mRNA. IF2 selects fMet-tRNA\(^{Met}\) from a pool of tRNAs upon recognition of the protected \(\alpha\)-amino group on formylmethionine.\(^{25,26}\) Studies have shown that IF2 binds strongly to tRNAs with attached N-protected methionine (Acetyl-Met-tRNA\(^{Met}\) and AcetylMet-tRNA\(^{Met}\)).\(^{27}\) Thus, IF2 shows poor discrimination between tRNAs carrying N-protected amino acids.
The elongation factors, responsible for delivery of subsequent amino acids along the peptide chain, however, show much greater specificity.

This fact allows for an easy N-terminal labeling of proteins. First, tRNA$_{f\text{Met}}$ is enzymatically charged with methionine.$^28$ Then the amino acid is modified to carry a specific label (fluorophore, radioactive tag, etc.). The aminoacylated tRNA is then used directly in an in vitro translational system. However, the elongation process is sometimes affected by the size of the fluorophore.$^29$ Bulkier fluorophores result in less efficient protein synthesis. However, some improvements in this method are required as the N-terminal labeled Met-tRNA$_{f\text{Met}}$ is competing with endogeneous fMet-tRNA$_{f\text{Met}}$ for the start codon (AUG), resulting in only 1-2 mol% labeled protein.$^30$ This problem was overcome by Rothschild and co-workers who used mRNA with the amber codon (UAG) instead of AUG.$^31$ The suppressor tRNA$_{(\text{-CA})}$ was chemically aminoacylated. This new method is highly efficient and provides specific N-terminal labeling of cell-free translational products.

1.2.4. Other methods for generating aminoacyl-tRNA

Recently Sisido and co-workers reported a new method for aminoacylation of tRNA using a cationic micellar system.$^32$ They first used this approach to acylate pdCpA.$^33$ The aminoacyl-dinucleotide, however, still had to be ligated to the tRNA. The system consists of an N-protected (N-pentenoyl) amino acid activated as a cyanomethyl ester, a target suppressor tRNA$_{\text{CCCG}}$, and a cationic detergent at pH 7.5.$^32$ The cationic detergent/micelle solubilizes the hydrophobic amino acid into its core and concentrates the negatively charged tRNA on its positively charged surface. The amino acid and tRNA react only upon ultrasonic agitation. There are several drawbacks of this method. No acylation occurs without sonication. Only one detergent, hexadecyltrimethylammonium chloride (CTACl), efficiently promotes the reaction. Analysis of the products shows that, in addition to the correct aminoacylation products, minor acylation products were also formed with nucleotides of cytidine and guanine. After acylation, the aminoacyl-tRNA$_{\text{CCCG}}$ was used in an in vitro translational system from E. coli. The yield was determined to be 20% with respect to the wild type.
Suga and co-workers developed a method for the acylation of tRNA based on the recognition of tRNAs by ribozymes.\textsuperscript{34, 35} An artificial ribozyme transfers an aminoacyl group from the 3’ terminus of a short nucleotide (aminoacyl donor) to the 3’ terminus of tRNA (aminoacyl acceptor) via the formation of a 5’-aminoacyl-ribozyme intermediate (Figure 6).\textsuperscript{34, 36}

The recognition of the aminoacyl donor by the ribozyme is governed by Watson-Crick base-pairing. The specificity toward tRNA is controlled by interactions with both CCA at the 3’-terminus and the anticodon loop. Thus, the ribozyme can be programmed for acylation of a specific tRNA\textsuperscript{36}. However, extensive screening is necessary for this to be achieved.

Sisido and co-workers used a similar approach where a peptide nucleic acid (PNA) was used as the recognition element in a nonenzymatic aminoacylation of tRNAs.\textsuperscript{37} A thioester of the amino acid is attached to the N-terminus of a PNA. The amino acid and the PNA, which is complementary to the 3’-terminus of yeast tRNA\textsuperscript{Phe}, are linked through a specifically designed spacer. The spacer then brings the thioester close to the
adenosine at the 3’-terminus of tRNA (Figure 7)\textsuperscript{37} and aminoacyl-tRNA results from the ester exchange.

![Diagram of PNA-assisted aminoacylation of tRNA]

**Figure 7:** PNA-assisted aminoacylation of tRNA

In situ aminoacylation/\textit{in vitro} translation was carried out in the presence of an orthogonal tRNA with a 4-base anticodon (CCCG), PNA linked to a nonnatural amino acid (H-2napAla-S-sp-PNA) and mRNA encoding for Streptavidin containing a 4-base codon (CGGG), in an \textit{E. coli} S30 in vitro system. It yielded a mutant protein in which the nonnatural amino acid was incorporated at the position directed by the 4-base codon/anticodon pair. This system is applicable to a variety of amino acids, and the acylation is specific to a particular tRNA.\textsuperscript{37}

### 1.3. Metal ion catalysis in reactions of phosphate derivatives

Phosphate esters are relatively unreactive under standard laboratory conditions.\textsuperscript{38} However, they play an important role in living organisms. For example, the hydrolysis of ATP is the main source of energy in the cell. Several classes of enzymes are involved in phosphoryl transfer reactions including phosphatases, kinases, phosphodiesterases, nucleases, and nucleotidyl transferases. These enzymes are metalloenzymes or require
metal co-factors. Some of these enzymes require a loosely bound divalent metal ion (Mg$^{2+}$ for kinases, Ca$^{2+}$ for some nucleases), while others require tightly bound cation (Zn$^{2+}$ for alkaline phosphatase and DNA polymerase I).$^{39}$

Ribozymes, or RNA enzymes, are another class of molecules that catalyze the hydrolysis of phosphodiester bonds. They require Mg$^{2+}$ to maintain their catalytic activity, and their reactions are classified according to the leaving group.$^{40}$ Reactions catalyzed by group I and II introns and RNase P have the 3′-OH as a leaving group. 5′-OH is the leaving group for hydrolysis catalyzed by hammerhead, hairpin, and delta agent ribozymes.$^{40}$

The enhanced reactivity of phosphate derivatives provided by biological catalysts is therefore related to the presence of metal ions. These can accelerate the rate of hydrolysis of phosphate esters through three direct methods of activation: Lewis acid activation (coordination of the phosphate oxygens to the metal), nucleophile activation (coordination of the nucleophile to the metal), or leaving group activation (coordination of the leaving group oxygen to the metal) (Figure 8).$^{41}$ Also, a water molecule coordinated to the metal can act as an intramolecular acid catalyst, while a coordinated hydroxyl group can act as an intramolecular base catalyst.

![Figure 8](image-url)

**Figure 8:** Metal ion acceleration of phosphate ester hydrolysis by Lewis acid activation (A), metal hydroxide activation (B), leaving group activation (C), or general base catalysis (D) (adapted from reference 41).
Metal-ion catalysis depends on the kinetics of ligand exchange, the coordination number and oxidation state of the metal. The pKa of a ligand bound to an electropositive metal is lower than the pKa of the free ligand in solution. This is a result of a decrease in the electron density of the bound ligand. For example, a water molecule coordinated to a metal ion can have a pKa ranging from 6 to 12, depending on the metal and its coordination number.

1.3.1. Lanthanides

1.3.1.1. General properties

The Lanthanide series consists of lanthanum and the fourteen elements that follow it in the periodic table. They are non-toxic, but they have no known biological role. They are highly electropositive elements with prime oxidation number III and they form essentially ionic compounds. An important feature of the lanthanides is the “lanthanide contraction” – the progressive decrease in the ionic radius observed with increase of the atomic number. These elements are hard Lewis acids with high coordination numbers (6 to 12, 8 to 9 for biological molecules) and geometrical flexibility that form complexes predominantly with oxygen-containing ligands.

1.3.1.2. Spectroscopic applications

Some lanthanides have very high paramagnetic moments (with the exception of lanthanum and lutetium which are diamagnetic) and are used in NMR spectroscopy as shift reagents, and in luminescence spectroscopy. Based on their similarities to calcium, lanthanides (Ln³⁺) are capable of replacing Ca²⁺ in its binding sites in proteins. Thus, paramagnetic Ln³⁺ are used to determine the three-dimensional structure of protein–ligand complexes by NMR. Lanthanides were also found to displace magnesium from its binding sites on tRNA backbone, and so are also used to probe the environment of these sites by the cleavage reactions that they promote.
1.3.1.3. Lanthanides as catalysts in organic synthesis

Lanthanides find many applications as catalysts in organic synthesis\textsuperscript{47-51} as most Lewis acids are not stable in aqueous media.\textsuperscript{48} Kobayashi and co-workers extensively studied the catalytic properties of lanthanide triflates in reactions carried out in presence of water. The catalysts were efficient in both aqueous and organic meida and were found to catalyze several carbon-carbon bond forming reactions such as the Mannich reaction\textsuperscript{50}, Aldol and Michael reactions, Friedel-Crafts acylation\textsuperscript{48, 49}, Diels-Alder\textsuperscript{48} and Aza Diels-Alder reactions\textsuperscript{51}, and more.

1.3.1.4. General properties

Lanthanides and lanthanide complexes are also often used as catalysts of hydrolysis of phosphodiester bonds. Komiyama and co-workers reported fast hydrolysis of dinucleoside monophosphates by Eu\textsuperscript{3+}\textsuperscript{52}, RNA by La\textsuperscript{3+}-dinuclear complex\textsuperscript{53}, tRNA by La\textsuperscript{3+}, Eu\textsuperscript{3+}, and Ce\textsuperscript{3+} complexes\textsuperscript{54}, and esters and amides by Ln\textsuperscript{3+}\textsuperscript{55}. Morrow et al. designed a europium complex that promotes transesterification of RNA\textsuperscript{56} while Chin et al. used lanthanum dimers (La\textsubscript{2}(OH)\textsubscript{3}) to catalyze the hydrolysis of RNA.\textsuperscript{57} More selective cleavage of RNA/tRNA is achieved with Ln-complexes, as the specificity is dependent on the ligand structure.\textsuperscript{53, 54}

1.4. Aminoacyl phosphates

The activation of amino acids in nature involves formation of mixed anhydrides of phosphate esters and the amino acid. We have developed simplified chemical analogues to use in biomimetic processes.

1.4.1. Aminoacyl adenylates

Lipmann first suggested that the reactive intermediates of the biosynthesis of proteins are the aminoacyl adenylates.\textsuperscript{58} This was later proven by using chemically synthesized aminoacyl adenylates as substrates of the enzymatic reaction.\textsuperscript{4} Several syntheses of the enzymatic intermediate were developed. Berg attempted condensation of a N,N’ – dicyclohexylcarbodiimide (DCC) - activated free amino acid with adenylic acid in presence of aqueous pyridine which resulted in very low yields.\textsuperscript{59} Other methods relied
on the reaction of 5’-adenylic acid with N-protected amino acids activated by DCC\textsuperscript{60}, as mixed anhydrides\textsuperscript{61}, or as acyl chlorides\textsuperscript{4} followed by removal of the protecting group.

### 1.4.2. Aminoacyl phosphates as analogues of the enzymatic intermediates

Figure 9: Aminoacyl adenylate and aminoacyl alkyl phosphate. (R=amino acid side chain; R\textsubscript{1}=alkyl group)

The adenylate portion of the enzymatic intermediate is not involved in the reaction with tRNA, functioning only for binding to the enzyme. However, its complex functionality is a problem for the chemical synthesis of aminoacyl-adenylates (Figure 9).

Katchalsky and Paecht used the silver salt of N-Cbz protected amino acids in a coupling reaction with dibenzyl chlorophosphate\textsuperscript{62}. The protecting group was removed by treatment with anhydrous hydrogen bromide. These aminoacyl phosphates proved to be very reactive compounds at room temperature. At neutral pH and in aqueous media they not only hydrolyze but also rapidly form polypeptides. A major drawback of this method is the formation of benzyl bromide, which cannot be removed without destroying the acyl phosphate.

In 1996, Kluger and co-workers developed a convenient method for generating tetraalkylammonium aminoacyl alkyl phosphates\textsuperscript{63}. Later they studied the mechanism and catalysis of the hydrolysis of alanyl ethyl phosphate\textsuperscript{64}. The rate of hydrolysis was enhanced by acid, base and metal ions. However, the reaction with alcohols was slow and development of a new catalyst was required. A new system was proposed where
lanthanide ions enhance not only the hydrolysis of benzoyl methyl phosphate (BMP) but also the acylation of alcohols (mono- and diols).\textsuperscript{65,66}

1.4.3. Acyl phosphates in lanthanide-mediated acylation reactions

\begin{center}
\textbf{Scheme 1:} Reaction scheme of La\textsuperscript{3+}-promoted benzylation of \textit{cis}-1,2-cyclopentanediol
\end{center}

Cameron et al. reported successful monobenzoylation of ethylene glycol, 1,2-propanediol, \textit{cis}-1,2-cyclopentanediol, and adenosine using lanthanides in water.\textsuperscript{66} No ester formation was observed when \textit{trans}-1,2-cyclopentanediol was reacted with BMP in presence of lanthanum. The high efficiency of this system is attributed to the formation of a bis-bidentate complex of La\textsuperscript{3+} with the diol and BMP (Scheme 1). Clarke et al. previously reported this pattern of coordination in La-catalyzed reactions of diols with acetic anhydride in organic media.\textsuperscript{67,68}
1.5. **Our approach**

**Scheme 2:** Formation of aminoacyl-tRNA. Comparison of the enzymatic and the La$^{3+}$-promoted reaction.

Several methods for preparing aminoacyl-tRNA have been developed and have been successfully used to incorporate non-natural amino acids into proteins.$^{13, 32, 37, 69}$ However, no direct method for charging tRNA at the 3’-terminal adenosine exists.

The objective of our studies is to generate a system that allows the 3’-aminoacylation of any tRNA molecule with any amino acid (natural or non-natural) in water. To accomplish this, a catalyst that lacks the high specificity of aaRSs is required. This catalyst, however, must be able to recognize the specific functionality of the 3’-terminus
of tRNA and charge only one of the two hydroxyl groups of the terminal adenosine. There are over 70 other hydroxyls in a typical tRNA.

The key components of our system are aminoacyl alkyl phosphates and lanthanide salts. The aminoacyl phosphates are donors of the aminoacyl moiety and are analogues of the enzymatic intermediates, aminoacyl adenylates. Lanthanides promote the aminoacylation reactions by forming a bis-bidentate complex with the adenosine diol and the aminoacyl phosphate. The two reacting species are brought close together, thus facilitating nucleophilic attack on the aminoacyl phosphate. In biological systems, the enzyme, aaRS, catalyzes the reaction in a similar manner (Scheme 2).

To study the applicability of this system to acylation of tRNA, several aminoacyl phosphates were synthesized and have been tested in reactions with nucleosides, nucleotides and RNA. Of great interest was also the capability of the generated species to donate the amino acid to the growing peptide chain in an in vitro protein synthesis.
CHAPTER 2: Synthesis and analysis of the aminoacylating reagents

2.1. Introduction

The aminoacylating reagents used in these studies (α-N-Boc-tyrosyl ethyl phosphate (BocTEP), α-N-Boc-p-fluorophenylalanyl ethyl phosphate (BocFPEP), ε-N-dansyl-α-N-Boc-lysyl ethyl phosphate (DNS-BocLysEP), α-N-Dansyl-glycyl ethyl phosphate (DNSGlyEP), and α-N-dansyl-(L)-phenylalanyl ethyl phosphate (DNSPheEP)) were synthesized using the method developed by Kluger and Loo. This method utilizes tetraethylammonium salts, which make the aminoacyl ethyl phosphates soluble in both organic and aqueous solvents. This property is also used in the separation of the final products from the reaction mixtures.

Acyl phosphates are not stable in base, which requires the use of acid-labile protecting group. Thus, a Boc group was used to protect the α-amino group of the amino acids. The aminoacyl phosphate can be deprotected by treatment with trifluoroacetic acid (TFA). In the case of BocFPEP, 19F NMR was used to monitor ester-product formation. The hydrolysis product, or BocFPhe, is commercially available and its chemical shift is easy to determine. To be able to compare the chemical shifts of the newly synthesized esters with a standard, we synthesized an isopropyl ester of BocFPhe.

The introduction of a fluorophore (DNS) at the aminoacyl phosphate permitted the study of reactions that require very low concentrations of reagents by fluorescence spectroscopy.

2.2. Materials and Methods

Commercial reagents were used as received. High-resolution mass spectrometry was performed at the QStar Chemistry Mass Spectral Facility, University of Toronto. NMR spectra were recorded at 300 or 400 MHz (1H), 75 or 100 MHz (13C), 282 MHz (19F) and 121 MHz (31P).
2.2.1. Synthesis

Briefly, ethyl dichlorophosphate (25 mmoles) was added to a 10 fold excess of water over 10 minutes in an ice-cooled round-bottom flask and stirred for 1 hour. The hydrochloric acid, generated as a by-product, was removed by rotary evaporation. 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.

The amino acid (Boc(L)Tyr, Boc(L)FPhe, DNSGly, DNS(L)Phe, or ε-DNS-α-Boc-(L)Lys) (1.3 equivalents) was activated with DCC (1 equivalent) in dichloromethane for 3 minutes. Tetraethylammonium ethyl phosphate (1 equivalent), pre-dissolved in dichloromethane, was added and the mixture was stirred at room temperature for 1 hour. The final products were extracted with water, freeze-dried and used in the aminoacylation experiments without further purification. The aminoacyl ethyl phosphates (BocTEP and BocFPEP, white, and DNSGlyEP, DNSPheEP, or ε-DNS-α-Boc-LysEP, yellow hygroscopic solids) were prepared in 70 - 80 % yield.
**ε-Dansyl-α-Boc-(L)lysine (ε-DNS-α-Lys)**

While stirring at 4°C, 0.046 mmol of α-Boc-(L)lysine was dissolved in 130 μl distilled deionized (dd) water and 130 μl DMF. To this mixture was then added 0.043 mmol of 1, 5–dansyl chloride in 130 μl dimethylformamide and 5.42 μl triethylamine. The reaction mixture was stirred continued for 4 hours at 4°C. The mixture was then diluted with ethyl acetate and washed with saturated NaCl solution. The organic phase was dried over anhydrous MgSO₄, filtered and the solvent was removed under vacuum. The resulting yellow oil was used in the coupling step outlined above.

**Removal of the protecting group of BocTEP**

1.09 mmol of BocTEP were dissolved in 40 ml dichloromethane. 7.1 ml TFA (15 % of the total volume) was added, and the mixture was stirred for 1 hour at room temperature. About 2 ml toluene were added to facilitate removal of TFA by rotary evaporation at 37-40°C. After the formation of viscous oil, another portion of dichloromethane and toluene were added and the evaporation was repeated. The resulting tyrosyl ethyl phosphate (TEP) was precipitated in an ice-bath through the addition of cold acetone. The product, white fine crystals, was filtered and air dried. The yield was 30 % due to incomplete precipitation.

**Synthesis of isopropyl ester of BocFPhe**

The isopropyl ester of N-Boc-\(p\)-Fluorophenylalanine was synthesized according to the method published by Hassner and Alexanian. N-Boc-\(p\)-Fluorophenylalanine (1 mmole) was dissolved in 15 ml dichloromethane. DCC (1.1 mmoles), isopropanol (1.1 mmoles) and 4-pyrrolidonopyridine (0.1 mmoles) were added. The solution was stirred at room temperature for 2 hours. Dicyclohexyl urea was filtered out and the filtrate was washed with water, acetic acid and water again. The isopropyl ester was dried over MgCl₂ and the organic solvent was removed under vacuum.
2.2.2. Analysis

BocTEP

![Chemical Structure of BocTEP]

$^1$H NMR (300 MHz, D$_2$O): δ 7.0 (t, 2H, Ar), 6.7 ((t, 2H, Ar), 4.3 (t, 1H, CHCO), 4.0 (q, 2H, POCH$_2$CH$_3$), 3.2 (q, 8H, N$^+$ (CH$_2$CH$_3$)$_4$), 3.0 (m, 2H, ArCH$_2$), 1.2 (t, 3H, POCH$_2$CH$_3$), 1.1 (s, 9H, tBu), 1.0 (t, 12H, N$^+$ (CH$_2$CH$_3$)$_4$);

$^{13}$C NMR (75 MHz, D$_2$O): δ 158.9 (CO-O-PO), 155.4 (p-OH-CAr), 155.1 (CO-O-tBut), 131.5 (Ar), 129.0 (p-OH-ArC), 115.8 (Ar), 81.6 (C(CH$_3$)$_3$), 64.3 (P-O-CH$_2$CH$_3$), 52.5 (NH-CH-CO), 49.0 (N$^+$ (CH$_2$CH$_3$)$_4$), 37.1 (Ar-CH$_2$), 28.9 (C(CH$_3$)$_3$), 15.9 (P-O-CH$_2$CH$_3$), 6.4 (N$^+$ (CH$_2$CH$_3$)$_4$);

$^{31}$P NMR (121 MHz, D$_2$O): δ -6.19

MS ESI (-): found m/z 388.1173, calculated m/z 388.1166

TEP

![Chemical Structure of TEP]

MS ESI (-): found m/z 288.0653, calculated m/z 288.0642
BocFPEP

\[
\begin{align*}
\text{F} & \quad \text{NH} \\
& \quad \text{O} \\
& \quad \text{O} \\
& \quad \text{O} \\
& \quad \text{P} \\
& \quad \text{O} \\
\end{align*}
\]

\(^1H\) NMR (400 MHz, D\(_2\)O): \(\delta\) 7.1 (t, 2H, Ar), 6.9 (t, 2H, Ar), 4.3 (t, 1H, CHCO), 4.0 (q, 2H, POCH\(_2\)CH\(_3\)), 3.2 (q, 8H, N\(^+\)(CH\(_3\)CH\(_3\))\(_4\)), 3.0 (m, 2H, ArCH\(_2\)), 1.2 (t, 3H, POCH\(_2\)CH\(_3\)), 1.1 (s, 9H, tBu), 1.0 (t, 12H, N\(^+\)(CH\(_2\)CH\(_3\))\(_4\));

\(^{13}C\) NMR (100 MHz, D\(_2\)O): \(\delta\) 163.1 (CO-O-PO), 160.5 (p-F-\(\text{C}\)Ar), 157.3 (p-F-\(\text{C}\)Ar), 132.2 (CO-O-tBut), 130.9 (Ar), 115.0 (Ar), 81.3 (CH\(_3\))\(_3\), 63.6 (P-O-CH\(_2\)CH\(_3\)), 55.9 (NH-CH-CO), 52.1 (N\(^+\)(CH\(_2\)CH\(_3\))\(_4\)), 36.2 (Ar-CH\(_2\)), 27.6 (CH\(_3\))\(_3\), 15.6 (P-O-CH\(_2\)CH\(_3\)), 6.7 (N\(^+\)(CH\(_2\)CH\(_3\))\(_4\));

\(^{19}F\) NMR (282 MHz, D\(_2\)O): \(\delta\) -117.2;

\(^{31}P\) NMR (121 MHz, D\(_2\)O): \(\delta\) -6.18;

MS ESI (-): found m/z 391.1203, calculated m/z 391.1196
Isopropyl ester of BocFPhe

\[
\begin{align*}
\text{H NMR (300 MHz, CD}_{3}\text{OD): } & \delta 7.2 (t, 2H, Ar), 6.99 ((t, 2H, Ar), 4.97 (\text{quintet, } 1H, \\
& \text{CH(CH}_{3}\text{)}_{2}), 4.26 (t, 1H, \text{CHNH}), 2.92 (m, 2H, \text{ArCH}_{2}), 1.38 (s, 9H, tBu), 1.19 (dd, 6H, \\
& \text{CH(}}\text{CH}_{3} \text{)}_{2}); \\
\text{F NMR (282 MHz, CD}_{3}\text{OD): } & \delta -118.9; \\
\text{MS ESI (+): } & \text{found } m/z 348.2, \text{ calculated } m/z 348.16 (M+Na)}
\end{align*}
\]

\[\varepsilon\text{--DNS-}\alpha\text{-BocLysEP}\]

\[
\begin{align*}
\text{H NMR (300 MHz, D}_{2}\text{O): } & \delta 8.4-8.2 (m, 3H, Ar), 7.7-7.5 (m, 3H, Ar), 4.4 (t, 1H, \\
& \text{CHCO}), 4.2 (q, 2H, \text{POCH}_{2}\text{CH}_{3}), 3.2 (q, 8H, \text{N}^+(\text{CH}_{2}\text{CH}_{3})_{4}), 3.1 (t, 2H, \varepsilon\text{--NH-CH}_{2}), \\
& 2.9(s,\text{DNS- N(}}\text{CH}_{3}\text{)}_{2}), 1.8 (t, 2H, \varepsilon\text{--NH-CH}_{2}\text{-CH}_{2}\text{-CH}_{2}\text{-CH}-\text{CH}), 1.6 (m, 2H, \varepsilon\text{--NH-CH}_{2}\text{-CH}_{2}\text{-CH}_{2}\text{-CH}_{2}\text{-CH}), 1.5 (t, 3H, \text{POCH}_{2}\text{CH}_{3}), 1.2 (m, 2H, \varepsilon\text{--NH-CH}_{2}\text{-CH}_{2}\text{-CH}_{2}\text{-CH}_{2}\text{-CH}_{2}\text{CH}), 1.1 (s, 9H, tBu), 1.0 (t, 12H, \text{N}^+(\text{CH}_{2}\text{CH}_{3})_{4}); \\
\text{MS ESI (-): } & \text{found } m/z 586.2, \text{ calculated } m/z 586.2
\end{align*}
\]
\[\alpha\text{-DNSGlyEP}\]

\[
\begin{array}{c}
\text{HN} \\
\text{O}\end{array}
\begin{array}{c}
\text{O-S=O} \\
\text{O-P-O} \\
\text{O} \\
\text{-} \\
\text{O} \\
\text{N} \\
\text{O}\end{array}
\]

\[\begin{array}{c}
\text{HN} \\
\text{O}\end{array}
\begin{array}{c}
\text{O-S=O} \\
\text{O-P-O} \\
\text{O} \\
\text{-} \\
\text{O} \\
\text{N} \\
\text{O}\end{array}
\]

\[^{1}\text{H NMR} (300 \text{ MHz, D}_2\text{O}): \delta 8.4-8.1 (m, 3\text{H, Ar}), 7.7-7.4 (m, 3\text{H, Ar}), 4.2 (q, 2\text{H, POC}_{\text{H}_2}\text{CH}_3), 3.6 (s, 2\text{H, NH-CH}_2), 3.3 (q, 8\text{H, N}^+(\text{CH}_2\text{CH}_3)_4), 2.9 (s, \text{DNS- N(CH}_3)_2), 1.5 (t, 3\text{H, POC}_{\text{H}_2}\text{CH}_3), 1.3 (t, 12\text{H, N}^+(\text{CH}_2\text{CH}_3)_4), 1.1 (s, 9\text{H, tBut});\]

\text{MS ESI (-): found m/z 415.1, calculated m/z 415.07}\]

\[\alpha\text{-DNSPheEP}\]

\[
\begin{array}{c}
\text{HN} \\
\text{O}\end{array}
\begin{array}{c}
\text{O-S=O} \\
\text{O-P-O} \\
\text{O} \\
\text{-} \\
\text{O} \\
\text{N} \\
\text{O}\end{array}
\]

\[\begin{array}{c}
\text{HN} \\
\text{O}\end{array}
\begin{array}{c}
\text{O-S=O} \\
\text{O-P-O} \\
\text{O} \\
\text{-} \\
\text{O} \\
\text{N} \\
\text{O}\end{array}
\]

\[^{1}\text{H NMR} (300 \text{ MHz, D}_2\text{O}): \delta 8.4-8.3 (m, 3\text{H, DNS-Ar}), 7.7-7.5 (m, 3\text{H, DNS-Ar}), 7.2 (m, 5\text{H, Ar}), 4.4 (m, 1\text{H, NH-CH}_2), 4.2 (q, 2\text{H, POC}_{\text{H}_2}\text{CH}_3), 3.2 (d, 2\text{H, Ar-CH}_2), 3.3 (q, 8\text{H, N}^+(\text{CH}_2\text{CH}_3)_4), 2.9 (s, \text{DNS- N(CH}_3)_2), 1.5 (t, 3\text{H, POC}_{\text{H}_2}\text{CH}_3), 1.3 (t, 12\text{H, N}^+(\text{CH}_2\text{CH}_3)_4);\]

\text{MS ESI (-): found m/z 505.1, calculated m/z 505.1}\]
CHAPTER 3: Effect of pH on the hydrolysis of BocTEP

3.1. Introduction

Aminoacyl ethyl phosphates are stable at neutral pH and 25°C. Kluger and co-workers reported a half-life of 100 hours for N-Boc-phenylalanyl ethyl phosphate at these conditions. In more basic solutions (pH 10-11) the rate of hydrolysis increases with concentration of OH⁻ and at pH 4-5 buffer catalysis is observed. Kluger and Cameron reported acceleration of hydrolysis of BMP upon addition of lanthanide salts. The carbonyl group of the acyl phosphate has increased electrophilicity due to the coordination to the metal, while lanthanides activate the coordinated water molecules to produce OH⁻ nucleophiles, and hydrolysis proceeds via intramolecular base catalysis.

Because our aminoacylation approach involves acylation of nucleotides in aqueous solutions, hydrolysis of the aminoacylating reagent competes with the desired acylating reaction. Hydrolysis of BocTEP was studied at different buffer concentration and pH levels to determine reaction conditions which would eliminate or suppress the hydrolysis side reaction.

3.2. Experimental

3.2.1. Materials and Methods

Commercial reagents (lanthanum(III) triflate, EPPS, acetonitrile and TFA) were purchased from Sigma and used as received. Water was doubly distilled and deionized prior to use. BocTEP was used without further purification. All solutions were freshly prepared before starting the reaction. The buffer stock solutions were adjusted to pH 8. The amount of hydrolysis product was measured as the integrated area of the HPLC peaks. HPLC analysis was performed on a C18 reverse phase (RP) column and the products were detected at 280 nm. The mobile phase consisted of 30% acetonitrile and 0.1% TFA in water.
3.3. Results

The concentration of buffer (EPPS) was varied and pH of the actual reaction mixture in presence of La$^{3+}$ was measured. BocTEP and La(OTf)$_3$ were used in equivalent amounts (20 mM). Reactions were quenched with EDTA at 20 sec, 20 and 60 minutes.

<table>
<thead>
<tr>
<th>[EPPS], mM</th>
<th>pH reaction media</th>
<th>Hydrolysis product (BocTyr), %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20 sec</td>
</tr>
<tr>
<td>0</td>
<td>3.5</td>
<td>0.00</td>
</tr>
<tr>
<td>10</td>
<td>4.5</td>
<td>0.00</td>
</tr>
<tr>
<td>100</td>
<td>7.5</td>
<td>66.01</td>
</tr>
<tr>
<td>167</td>
<td>8.0</td>
<td>70.13</td>
</tr>
</tbody>
</table>

Table 1: Dependence of BocTEP hydrolysis on the pH and [EPPS]

When no buffer is present in the solution, the pH measured is 3.5 (Table 1). At these conditions hydrolysis of BocTEP was very slow resulting in overall BocTyr formation of 11.29% in 60 minutes. With increase of [EPPS] to 10 mM, the pH increases to 4.5 and the amount of hydrolysis product also increased. At [EPPS] 100 and 167 mM more than 60% of the starting material is hydrolyzed immediately. The optimal [EPPS] for hydrolysis of BocTEP in presence of 20 mM La$^{3+}$ appears to be 100 mM, where at 60 minutes 96% of the starting material is converted to BocTyr.
3.4. Discussion

EPPS is an effective buffer from pH 7 to pH 9. Upon binding to La$^{3+}$, the pK$_a$ of coordinated water is lowered by 7 units.$^{72}$ When no buffer is present, the pH goes from 7 to 3.5. 10 mM buffer can slightly diminish the effect of metal ions: at half the concentration of La$^{3+}$ it increases the pH to 4.5. When the buffer’s concentration exceeds that of the metal ion, the solution is slightly basic.

Acyl phosphates are reported to be unstable in alkaline solutions.$^{64, 65}$ In the presence of buffer in higher concentration, not only the La$^{3+}$-bound water but also the buffer itself can catalyze the hydrolysis.$^{64}$ The observed hydrolysis product was formed faster in reaction media at pH 7.5 and 8. Maximum BocTyr was formed at pH 7.5, or 100 mM buffer (96.12% in 60 minutes). After increasing the concentration of EPPS, the hydrolysis product was 89.92%. This observation could be explained with inhibition of the catalyst. Lanthanides do not coordinate well to organic sulfates. However, EPPS has hydroxyl groups at one end of the molecule and they can weakly coordinate to the metal and thus inhibits it.$^{43}$ A similar effect is observed in reactions of hydrolysis of BMP.$^{73}$ At lower [EPPS] and lower pH, the buffer-catalyzed hydrolysis is diminished and the amount of BocTyr is four times (at 10 mM EPPS) and eight times (no EPPS) lower compared to the amount of hydrolysis product at pH 7.5 (100 mM buffer).

3.5. Conclusions

In the system for biomimetic aminoacylation that we developed all reactions are performed in aqueous buffered media. The acylation is necessarily accompanied by hydrolysis of the reagent due to the water coordinated to the metal ion. The results obtained from the studies of BocTEP hydrolysis suggest that lowering the pH of the solution or the concentration of the buffer can slow down the hydrolysis. This effect can be used to optimize the outcome of aminoacylation of nucleotides.
CHAPTER 4: Aminoacylation of nucleosides

4.1. Introduction

Selective monoacylation of ribonucleotides at either a 2’- or 3’-OH groups is a problem of great difficulty, due to the almost identical reactivity of these sites. Bulky acylating reagents that are used in a standard chemical esterifications react only at the less hindered 5’-OH group. Several methods have been developed for the monoacylation of nucleosides. Aminoacylation of ribonucleosides can be accomplished by acid-catalyzed reactions of an orthoester of an N-protected amino acid. The resulting ribonucleoside-2’,3’-cyclic orthoesters are then subjected to further acid hydrolysis to generate the 2’ (3’)-O-acylribonucleosides. Moffatt et al. reported acetylation of uridine via activation of the nucleoside as uracil 2’,3’-O-(dibutylstannylene). This activated nucleoside was reacted in an organic solvent with acetic anhydride to give a mixture of 2’-O-acetyl and 3’-O-acetyluridine.

Aminoacylation of the 3’-terminal adenosine of tRNA is an essential step of protein synthesis. This step is normally catalyzed by aaRSs, resulting in either formation of a 2’- or 3’-aminoacyl-tRNA, with the two esters in equilibrium. Thus, generation of either of them would fulfill the requirement for successful protein synthesis. In the system we have developed, La\textsuperscript{3+} must selectively bind to the 1,2-diol of the nucleoside and not charge the 5’-OH or react with any amino group of the purine or pyrimidine.

La\textsuperscript{3+}-catalyzed aminoacylation with D-ribose and D-2-deoxyribose resulted in ester formation. The opening and closing of the hemiacetal ring from ribose cyclization in solution, however, makes it difficult to determine the sites of reaction. Blocking the anomeric position by introducing a nucleic acid base limits the possible sites for coordination of La\textsuperscript{3+}. Thus, aminoacylation reactions of nucleosides will provide important information about the applicability of the proposed catalytic system to reactions with tRNAs.
4.2. Experimental

4.2.1. Materials and Methods

Commercial reagents (nucleosides, lanthanum (III) salts, EPPS, acetonitrile and TFA) were purchased from Sigma and used as received. Water was doubly distilled and deionized prior to use. NMR spectra were recorded at 300 MHz (\(^1\)H), 75 MHz (\(^{13}\)C), and 282.0 MHz (\(^{19}\)F). High-resolution mass spectrometry was performed at the QStar Chemistry Mass Spectral Facility, University of Toronto. HPLC analysis was performed on C18-RP column and the products were detected at 280 nm (for BocTEP) and at 263 nm (for BocFPEP). The mobile phase consisted of 30% acetonitrile and 0.1% TFA in water (for BocTEP) and 40% acetonitrile and 0.1% TFA in water (for BocFPEP).

The selectivity of La\(^{3+}\) towards aminoacylation versus hydrolysis is measured as the ratio between the equilibrium constants of acylation (\(K_{\text{acyl}}\)) and hydrolysis (\(K_{\text{hydr}}\)) (Equation 1).

\[
K = K_{\text{acyl}} / K_{\text{hydr}} = (\text{Esters Peak area} / [\text{Nucleoside}]) \times ([\text{H}_2\text{O}] / \text{Boc-amino acid Peak area})
\]

4.3. Results

Both BocTEP and BocFPEP were used as aminoacylating reagents in these studies and their reactivity was compared. All reactions were done at room temperature (23° C) with constant stirring. Each experiment was carried out in freshly prepared solutions.

4.3.1. Reaction of uridine with aminoacyl phosphates in the presence of La\(^{3+}\)

First, it was necessary to determine the sites of reaction. Acylation reactions were performed with uridine and 2’-deoxyuridine (10 mM) with La(OTf)_3 (1 mM) and BocTEP (6 mM) in 10 mM EPPS pH 8. Uridine was converted to two esters while its 2’-deoxy analogue did not react (Figure 10).
Figure 10: Comparison of HPLC chromatograms of La-catalyzed aminoacylation reactions of uridine (red) and 2’-deoxyuridine (blue).

BocFPEP was also studied as aminoacylating reagent in reactions with uridine and showed very high reactivity. After 35 sec, BocFPEP was completely consumed. The selectivity of the reaction is the same as the one observed for BocTEP with the ratio of 2’-: 3’-esters being 1:2.

4.3.2. $^1$H and $^{13}$C NMR analysis of Uridine-BocTyr esters

The HPLC peaks corresponding to the esters were collected, freeze-dried and characterized. ESI MS (-) confirmed that the products are monoesters of uridine (calculated m/z 507, found m/z 506). Additional analysis by $^1$H and $^{13}$C NMR was required to determine the site of reaction. The observed chemical shifts were compared to the ones of native uridine. The main differences in the $^1$H NMR spectra were in the chemical shifts of the 1’-proton. For non-modified uridine, the 1’-H had a chemical shift of 5.9 ppm. The ester that elutes first showed a chemical shift for 1’-H at 6.06 ppm. For the second ester, the chemical shift was at 5.94 ppm. $^{13}$C NMR spectra of the esters were
compared to that of uridine. Changes in the chemical shifts of C2 and C3 carbons indicated that 2’- and 3’-OH of uridine were aminoacylated (Table 2). The ester with the shorter retention time (12.9 minutes) was formed from the 2’-OH and the one with longer retention time (15.2 minutes) from the 3’ OH.

<table>
<thead>
<tr>
<th>Compound</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uridine</td>
<td>90.67</td>
<td>75.74</td>
<td>71.31</td>
<td>86.34</td>
<td>62.27</td>
</tr>
<tr>
<td>Ester 1</td>
<td>88.88</td>
<td>77.44</td>
<td>70.43</td>
<td>86.96</td>
<td>62.30</td>
</tr>
<tr>
<td>Ester 2</td>
<td>89.70</td>
<td>74.94</td>
<td>74.20</td>
<td>84.53</td>
<td>62.40</td>
</tr>
</tbody>
</table>

Table 2: $^{13}$C chemical shifts for uridine and BocTyr -uridine esters in CD$_3$OD.

Thus, ESI MS and NMR analysis of the products revealed that La$^{3+}$-mediated aminoacylation of uridine results in specific monoacylation of the nucleoside at the 2’- or 3’-OH group. The esters were obtained in regioisomERICally pure form ($^1$H NMR).

4.3.3. La$^{3+}$-mediated aminoacylation of adenosine

In the La$^{3+}$-catalyzed aminoacylation of adenosine, two ester products are formed (Figure 11). ESI MS (-) confirmed the formation of adenosine monoesters (calculated m/z 530.2, found m/z 529.1). When adenine, 2’-deoxyadenosine and 2’,3’-isopropylidene adenosine were subjected to the same reaction conditions, no esters were formed.
**Figure 11**: La$^{3+}$-mediated aminoacylation of adenosine with BocTEP ([Adenosine] = 10 mM, [BocPEP]=[La$^{3+}$]=3 mM, [EPPS]$_{pH8}$=10 mM)

### 4.3.4. La$^{3+}$-mediated acylation of cytidine with BocTyr and BocFPhe

The aminoacylation of cytidine with both BocTEP and BocFPEP in the presence of La$^{3+}$ as a mediator was successful (Figure 12). All reacting species (cytidine, aminoacylating reagent and La$^{3+}$) were present in equimolar concentrations, 10 mM. The buffer concentration was also 10 mM.

Two esters are formed in a very rapid reaction. They were separated by HPLC. ESI MS established that they are monoesters of cytidine (for BocTyr-cytidyl ester ESI MS (-) calculated m/z 506.2, found m/z 505.1; for BocFPhe-cytidyl ester ESI MS (+) calculated m/z 508, found m/z 509 (+H$^+$) and 531 (+Na$^+$).
Figure 12: La-mediated aminoacylation of cytidine with BocTEP (A) and BocFPEP (B) ([Cytidine] = [aminoacylating reagent] = [La$^{3+}$]=10 mM, [EPPS]_{pH8}=10 mM)

The reaction with BocFPEP proceeded faster than with BocTEP. The aminoacylating reagent reacted completely within 20 sec of mixing. Increasing the concentration of the buffer did not change the selectivity of ester formation, but it did accelerate the reaction/consumption of BocFPEP. At 100 mM EPPS pH 8 the reaction was completed in 18 sec. The esters accounted for 95.1 % of the total products (4.9 % was hydrolysis product BocFPhe). The ratio of 2’- to 3’-esters was the same as observed for the other studied ribonucleosides, 1:2.

The progress of the aminoacylation of cytidine with BocFPEP was also monitored with $^{19}$F NMR (Figure 13). In this case, LaCl$_3$ was used as a catalyst instead of La(OTf)$_3$, to avoid having additional fluorinated materials in the reaction mixture. When BocFPEP was subjected to hydrolysis, only two peaks were observed on the NMR spectrum, BocFPEP (-117.2 ppm) and BocFPhe (-117.8 ppm). The acylation reaction was stopped by the addition of EDTA immediately after mixing. The reaction mixture was then filtered and spiked with D$_2$O, such that the volume of D$_2$O, so that the volume was added to equal 1/3 of the final volume.
The signal corresponding to BocFPEP has a chemical shift $\delta$ -117.2 while the known peak of the free acid, BocFPhe, is at -117.8 ppm. After the reaction, a new peak at $\delta$ -116.9 appeared that corresponds to an ester being formed in the reaction. The isopropyl ester of BocFPhe was synthesized to compare its $^{19}$F NMR chemical shift to the chemical shifts of the products. In both cases, the chemical shifts of the esters are very close to those of BocFPEP and BocFPhe.

**4.3.5. Effect of uridine concentration on the reaction**

To determine the optimal conditions for this reaction, the concentrations of the reagents were varied one at a time in separate experiments. The studied range of uridine concentration was 1 to 10 mM (Table 3). In the reaction mixtures, La(OTf)$_3$ and BocTEP were in 3 mM concentration. Buffer (EPPS) at pH 8 was present in 10 mM concentration. The final pH of all reaction mixtures was 7. The maximum $K$, e.g. the highest selectivity for aminoacylation over hydrolysis, was observed with 2 mM uridine. The amount and distribution of the products were monitored for 24 hours at room temperature by HPLC.
In all cases, the aminoacylating agent was completely consumed in 20 minutes and the amount of produced esters was constant after 24 hours.

<table>
<thead>
<tr>
<th>[Uridine], mM</th>
<th>2’ester, %</th>
<th>3’ ester, %</th>
<th>( K_{2'} \times 10^4 )</th>
<th>( K_{3'} \times 10^4 )</th>
<th>( K \times 10^4 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>18</td>
<td>0.9</td>
<td>1.4</td>
<td>2.3</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>29</td>
<td>1.0</td>
<td>1.6</td>
<td>2.6</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>37</td>
<td>0.9</td>
<td>0.6</td>
<td>2.5</td>
</tr>
<tr>
<td>4</td>
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<td>0.5</td>
<td>1.2</td>
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</tr>
<tr>
<td>5</td>
<td>19</td>
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<td>0.5</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>6</td>
<td>21</td>
<td>43</td>
<td>0.5</td>
<td>1.1</td>
<td>1.6</td>
</tr>
<tr>
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</tr>
<tr>
<td>8</td>
<td>23</td>
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<td>0.6</td>
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</tr>
<tr>
<td>9</td>
<td>23</td>
<td>49</td>
<td>0.5</td>
<td>1.1</td>
<td>1.6</td>
</tr>
<tr>
<td>10</td>
<td>23</td>
<td>50</td>
<td>0.5</td>
<td>1.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Table 3: Aminoacylation of uridine with BocTEP in water. Effect of [Uridine] on ester formation and distribution.

Subsequent studies of this reaction showed that most of the ester formation occurs during mixing and in the first minute of reaction. After one minute, there was less than 1% change in the peak areas of the products.

4.3.6. Effect of La(OTf)₃ concentration on the reaction

The dependence of the aminoacylation of uridine with BocTEP on the concentration of La³⁺ was studied at 22 °C. The concentrations of uridine and BocTEP were 3 mM. La(OTf)₃ concentration was varied from 0.3 to 3 mM. Buffer was present in 10 mM concentration. Table 4 contains the data for ester formation and distribution, as well as \( K \) for the reaction at the specific concentration of the catalyst.

The reaction is most selective with 2 mM La(OTf)₃. In this case the ratio of reagents and catalyst is 3:3:2 uridine:BocTEP:La(OTf)₃.
Table 4: Aminoacylation of uridine with BocTEP in water. Effect of La$^{3+}$ on ester formation and distribution.

4.3.7. Effect of buffer concentration on the reaction

We investigated the effect of buffer concentration on the ester formation and distribution. All reacting species (adenosine, BocTEP and La$^{3+}$) were in constant, stoichiometric concentrations (5 mM). pH 8 buffer was used at 5, 10, 50, and 100 mM.

<table>
<thead>
<tr>
<th>[La$^{3+}$], mM</th>
<th>2' ester, %</th>
<th>3' ester, %</th>
<th>$K_2 \times 10^4$</th>
<th>$K_3 \times 10^4$</th>
<th>$K \times 10^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>0.0</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.5</td>
<td>9.3</td>
<td>18.1</td>
<td>0.5</td>
<td>0.9</td>
<td>1.4</td>
</tr>
<tr>
<td>0.8</td>
<td>20.6</td>
<td>32.8</td>
<td>1.2</td>
<td>2.0</td>
<td>3.2</td>
</tr>
<tr>
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<td>37.5</td>
<td>2.2</td>
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<td>5.9</td>
</tr>
<tr>
<td><strong>2.0</strong></td>
<td><strong>21.9</strong></td>
<td><strong>58.7</strong></td>
<td><strong>2.1</strong></td>
<td><strong>5.5</strong></td>
<td><strong>7.6</strong></td>
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<tr>
<td>3.0</td>
<td>26.8</td>
<td>40.3</td>
<td>1.5</td>
<td>2.2</td>
<td>3.7</td>
</tr>
</tbody>
</table>

Table 5: La$^{3+}$-mediated aminoacylation of adenosine with BocTEP. Dependence of products formation on buffer concentration (% ester products; BocTyr* - % BocTyr at 60 minutes reaction time)

<table>
<thead>
<tr>
<th>Reaction time, min.</th>
<th>5 mM</th>
<th>10 mM</th>
<th>50 mM</th>
<th>100 mM</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>0%</td>
<td>36%</td>
<td>13%</td>
<td>28%</td>
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<tr>
<td>5</td>
<td>36%</td>
<td>59%</td>
<td>21%</td>
<td>43%</td>
</tr>
<tr>
<td>10</td>
<td>40%</td>
<td>66%</td>
<td>26%</td>
<td>48%</td>
</tr>
<tr>
<td>15</td>
<td>45%</td>
<td>68%</td>
<td>24%</td>
<td>50%</td>
</tr>
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<td>70%</td>
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<td>50%</td>
</tr>
<tr>
<td>30</td>
<td>51%</td>
<td>73%</td>
<td>23%</td>
<td>51%</td>
</tr>
<tr>
<td>40</td>
<td>54%</td>
<td>74%</td>
<td>24%</td>
<td>51%</td>
</tr>
<tr>
<td>60</td>
<td>59%</td>
<td>76%</td>
<td>25%</td>
<td>55%</td>
</tr>
</tbody>
</table>

| BocTyr*             | 15%  | 15%   | 23%   | 35%    |
All reactions resulted in selective monoaminoacylation of the 2' and 3' OH-groups of the ribonucleoside. The ratio of the 2' to 3' esters was 1:2, similar to the ratio obtained with uridine. Buffer concentration did not affect the selectivity of the reaction but did affect the rate of ester formation (Table 5). The optimal buffer concentration was 10 mM, producing 76% esters and 15 % acid. Under these conditions, the resulting pH of the reaction mixture was 7.5. The lowest amount of product formation was observed with 50 mM buffer (25 %).

4.3.8. Inhibition of La$^{3+}$ by ethyl phosphate

Additional experiments were performed with this reaction to determine the availability of metal ion for further catalysis. Initially the reaction mixture consisted of uridine (8 mM), BocTEP (3 mM), La(OTf)$_3$ (3 mM) and EPPS at pH 8 (10 mM). The reaction was followed by HPLC. After 20 minutes reaction time BocTEP was completely consumed. A new portion of the aminoacylating reagent, in the same amount and concentration as the original, was added and the reaction was monitored for an additional 20 hours. A very small decrease in the peak area of the BocTEP and slight increase in the area of BocTyr was observed.

Phosphates are known to be very good ligands for lanthanides. We examined the possibility of inhibition of the metal ion by the byproduct of the reaction, ethyl phosphate. In a standard reaction uridine (8 mM) was combined with La(OTf)$_3$ (3 mM) and BocTEP (3 mM) in the presence of buffer. Tetraethylammonium ethyl phosphate was added (3 mM) and the formation of products was monitored. The reactions appeared to be very slow and very small amounts of esters were obtained.

4.4. Discussion

Ribonucleosides do not undergo hemiacetal ring opening, as ribose does. This eliminates some of the possibilities for coordination of La$^{3+}$. However, they also introduce functional groups through their purine or pyrimidine bases, which can lead to potential complications.
4.4.1. Coordination requirements

All nucleosides contain three hydroxyl groups. Two of them, the 2’-OH and 3’-OH, are vicinal and cis to each other, and according to the proposed scheme should be the only sites of coordination for La\(^{3+}\) to lead to successful ester formation. Purines and pyrimidines provide additional amino and carbonyl groups. As a hard Lewis acid, La\(^{3+}\) does not have high affinity for functional groups containing nitrogen.\(^{43}\) However, coordination to them is possible if there is a strong ligand close by that can form a chelate with the metal ion.

Acylation should proceed according to Scheme 4. La\(^{3+}\) forms a bis-bidentate complex with the ribonucleoside and the aminoacylating reagent. The pKa’s of both OH-groups are very close. For the 2’-OH group the pKa has been found to be between 12 and 13.\(^{79}\) Upon coordination to the metal, the pKa is lowered and the OH-group is activated for nucleophilic attack on the carbonyl group of the Boc-aminoacyl phosphate. Once formed, the esters reach equilibrium, at which point the 3’-ester is present at slightly higher concentration as it is thermodynamically more stable.

**Scheme 4:** Productive bis-bidentate coordination of La\(^{3+}\) to uridine and BocTEP which yields a mixture of 2’- and 3’-esters
4.4.2. **Selectivity of La\(^{3+}\)-mediated aminoacylation**

Several experiments were performed in order to determine the site of La\(^{3+}\)-coordination and the subsequent acylation reactions. All ribonucleosides studied (adenosine, uridine and cytidine) were successfully aminoacylated with either BocTEP or BocFPEP to give two esters. When one (2’-deoxyuridine and 2’-deoxyadenosine) or both of the OH groups involved in the reaction (2’,3’-isopropylidene adenosine) were eliminated, no esterification was observed. This observation proved that the 2’ and 3’ hydroxyl groups are both important for successful acylation. No 5’-ester was formed in any of the reactions. The resulting esters were formed at the same time and do not result from equilibration. When stored in aqueous solution, however, either ester will give an equilibrium mixture of both.

As outlined in the section “La\(^{3+}\)-mediated aminoacylation of adenosine”, additional studies with the adenine base confirmed that the metal ion does not coordinate to the base to promote acylation.

The sites of esterification were determined to be the 2’ and 3’ OH groups on the ribonucleoside by \(^1\)H and \(^{13}\)C NMR based on the comparison to non-modified uridine. The ratio of 2’- to 3’-esters in all cases was in the range 1:1.5 to 1:2.

These results are consistent with other studies of the aminoacylation reaction that suggested that acylation in aqueous solutions takes place on the ribose moiety and the nucleic acid base does not react.\(^{80-83}\)

4.4.3. **Optimization of the reaction conditions**

All aminoacylation reactions were performed in aqueous media and hydrolysis is always observed to a certain extent. In aqueous solutions, La\(^{3+}\) forms complex with water. This coordination results in the lowering of the pK\(_a\) of water, making it a stronger acid. The pH of the reaction mixture is always lower than the pH of the buffer, except in cases where the buffer concentration exceeds that of La\(^{3+}\).

Several studies were conducted to optimize the conditions for this reaction (Tables 3, 4 and 5; optimal conditions shown in bold). In general, the reaction is very rapid. The ester formation occurs in the first minute of mixing. The selectivity of the catalyst for acylation over hydrolysis was measured as \(K\). For acylation of uridine \(K\) was at a
maximum \((7.6 \times 10^4)\) when uridine:BocTEP:La\(^{3+}\) was 3:3:2. For concentrations of uridine from 1 to 10 mM, \(K\) decreases with an increase in the concentration. The optimal selectivity in this study was for 2 mM uridine (uridine:BocTEP:La\(^{3+}\) = 2:3:3). Thus, the catalyst shows great selectivity towards the diol, considering the large difference in concentrations (3 mM nucleoside vs. 55 M water).

The effect of buffer concentration on the aminoacylation of nucleosides was also studied in a reaction with adenosine. EPPS buffer was used at 5, 10, 50, and 100 mM concentration. The maximum amount of ester formation was in 10 mM buffer, where the pH of the reaction medium was 7-7.5. In 100 mM buffer, the consumption of the aminoacylating reagent is the fastest but it results in a large extent of hydrolysis (Table 5). This experiment suggests that the selectivity towards aminoacylation can by driven by changes in pH or buffer concentration.

Using a fluorinated aminoacylating reagent, BocFPEP, allows us to use an alternative method to analyze reaction products. By \(^{19}\text{F}\) NMR we were able to monitor the formation of esters. This analytical technique will be helpful in studies of larger molecules where HPLC or \(^1\text{H}\) NMR will not be applicable.

The aminoacylation of cytidine with BocFPEP was studied with \(^{19}\text{F}\) NMR. The observed chemical shift of the ester is very similar to those of BocFPEP and BocFPhe. This is also observed for the synthetically prepared isopropyl ester of BocFPhe and confirms that converting the carboxyl group into an ester does not lead to great change in the \(^{19}\text{F}\) NMR chemical shift. The F-atom shift is very sensitive to environment. Small changes in its surroundings could induce large chemical shifts. In our case, the environment directly around the atom is not changed and therefore the chemical shift is similar to the one of the parent compound. Small changes however are observed when the concentration of buffer or the amount of metal ions in the solution is higher.

4.4.4. Inhibition of the reaction by phosphates

An important feature of any catalyst is its ability to be regenerated after the completion of one reaction. La\(^{3+}\) was not active after complete consumption of BocTEP. When a fresh portion of BocTEP was added to the initial reaction mixture with unreacted
uridine present, no change was observed in the ester peaks. The hydrolysis product peak, however, increased slightly. If La\(^{3+}\) was available, both esters and BocTyr, would form.

When ethyl phosphate (byproduct of both acylation and hydrolysis) was added to a standard aminoacylation reaction, both reactions were greatly suppressed. Since anionic phosphates are much better ligands for lanthanides than neutral OH-groups\(^ {43}\), the equilibrium will shift to favor complex formation upon addition of phosphate. Free La\(^{3+}\) will be present in a very small amount, and no substantial change will be observed. Thus, La\(^{3+}\) is not available to promote further reaction due to inhibition by the generated phosphate. Similar observation was reported in Ln\(^{3+}\) -catalyzed hydrolysis of 4-nitrophenyl phosphate.\(^ {84}\) The inorganic phosphate, a product of the reaction, competes with the substrate, affects the successful complex formation, and thus reduces the activity of the catalyst.

### 4.5. Conclusions

BocTEP and BocFPEP are efficient acylating reagents in La\(^{3+}\)-mediated monoaminoacylation of ribonucleosides in aqueous media. The catalyst recognizes the diol moiety of the ribonucleoside and converts its 2’- or 3’-OH group to the aminoacyl esters. The reaction proceeds via formation of a bis-bidentate complex of La\(^{3+}\) with both reagents (Scheme 4). The reaction conditions were optimized by varying the concentrations of the reagents or the buffer. It was also noted that La\(^{3+}\) is inhibited by the generated ethyl phosphate.

\(^{19}\)F NMR is a valuable technique that allows studying the formation of esters of ribonucleosides with BocFPhe. This method could be used for the study of aminoacylation of larger molecules, such as polynucleotides or RNA.

Based on the results obtained from La\(^{3+}\)-mediated aminoacylation reactions of nucleosides, we can conclude that the approach provides selectivity towards acylation when performed in aqueous media. Moreover, esterification occurs only at the desired positions – the 2’ and 3’ hydroxyl groups and only monoesters on ribonucleosides are formed.
CHAPTER 5: Aminoacylation of nucleotides

5.1. Introduction

The chemical synthesis of 2’- or 3’-O-acyl nucleotides usually requires activation of the acid and presence of a catalyst in a multiple step process. Velikyan and co-workers designed a synthesis similar to the acylation of nucleosides in which nucleoside-5’-monophosphates are prepared via acid catalyzed reaction of carboxylic acid orthoesters with ribonucleotides. A ribonucleotide-2',3’-cyclic orthoester is formed and is then subjected to acid hydrolysis to give the 2’- and 3’-O-acyl nucleotides. This method, however, failed when protected amino acids were used. Later, Weber and co-workers proposed another system for the preparation of aminoacyl nucleotides. They reacted homoribonucleotides with N-(acetylaminoacyl)-imidazoles. The reaction was done in imidazole buffer at pH 5.7. They also reported an imidazole catalyzed transfer of N-acetylglycine from adenylate anhydride to the 2’-OH groups of homopolyribonucleotides. The 3’-O-ester of AMP could also be formed through an intramolecular transfer of the aminoacyl moiety from the 5’-phosphate of the aminoacyladénylate to the 3’-OH of the ribose.

Our studies involve the aminoacylation of mono-, di-, and trinucleotides in the presence of La$^{3+}$. As in the reaction with nucleosides, the role of the metal ion is to recognize the 2’,3’-diol moiety and to activate the bound ligands. Several complications, however, must be overcome first. Lanthanides are known to catalyze the hydrolysis of phosphodiester bonds of RNA and RNA-models. Phosphates are very good ligands for lanthanides. A complex with the nucleotide’s phosphate is much stronger than the one with the diol. Thus, we chose to investigate whether or not this particular metal ion is capable of aminoacylating the furanose ring in competition with forming an unreactive complex with the phosphate. Study of aminoacylation of di- and trinucleotides provide some information concerning the reaction of oligonucleotides under these conditions.
5.2. Experimental

5.2.1. Materials and Methods

Commercial reagents (nucleotides, lanthanide (III) salts, scandium(III) chloride, magnesium chloride, EPPS, acetonitrile and TFA) were purchased from Sigma and used as received. Water was doubly distilled and deionized prior to use. NMR spectra were recorded at 400 MHz (\(^1\)H) and 356 MHz (\(^{19}\)F). High-resolution mass spectrometry was performed at the QStar Chemistry Mass Spectral Facility, University of Toronto. HPLC analysis was performed on C18-RP column and the products were detected at 280 nm (for BocTEP) and at 263 nm (for BocFPEP). The mobile phase consisted of 30% acetonitrile and 0.1% TFA in water (for BocTEP) and 40% acetonitrile and 0.1% TFA in water (for BocFPEP). All reactions were performed at room temperature unless stated otherwise and with constant stirring.

5.3. Results

The reactions of mononucleotides with BocTEP and BocFPEP in the presence of La\(^{3+}\) were much slower than those of nucleosides. No ester formation was observed in the absence of La(OTf)_3. The acylation of nucleotides was performed with all ribonucleoside-5'-phosphates (5’AMP, 5’GMP, 5’CMP and 5’UMP). The possible ability to modify 2’-deoxy-5’-nucleosides (5’TMP) and ribonucleosides with phosphate groups at the 2’- and 3’- position was also addressed.

5.3.1. Aminoacylation of ribonucleoside-5’-monophosphates with BocTEP

5’AMP was acylated with BocTEP in a slow reaction resulting in the formation of two monoesters with elution times of 7.2 and 7.9 minutes (Figure 14) (ESI MS (-) calculated m/z 610, found m/z 609). The ester peaks were detectable after 60 minutes reaction time. 24 hours after mixing, a substantial amount of BocTEP was still present in the mixture. The area of the ester peaks in the HPLC increased by 23% from 60 to 360 minutes. The esters were stable over the course of the 24 hour reaction. When the temperature was increased to 35 °C and 45 °C, only one ester (retention time 7.2 minutes)
was formed, with the starting material still present; there was no significant improvement in the yield of formed esters.

![HPLC chromatogram of La\(^{3+}\)-mediated aminoacylation of 5’AMP with BocTEP at 24 hours reaction time (10 mM 5’AMP, 3 mM BocTEP, 3 mM La(OTf)\(_3\), 10 mM EPPS pH 8)](image)

**Figure 14:** HPLC chromatogram of La\(^{3+}\)-mediated aminoacylation of 5’AMP with BocTEP at 24 hours reaction time (10 mM 5’AMP, 3 mM BocTEP, 3 mM La(OTf)\(_3\), 10 mM EPPS pH 8)

In reactions of BocTEP with 5’UMP, 5’GMP and 5’CMP under the conditions mentioned above (10 mM nucleotide, 3mM BocTEP, 3 mM La(OTf)\(_3\), 10 mM EPPS; room temperature), only one ester was produced in each case (Figure 15). They were all identified as monoesters of these nucleotides with BocTyr. 5’UMP yielded an ester with elution time 7.1 minutes (ESI MS (-) calculated m/z 587, found m/z 586.1), 5’GMP – 7.2 minutes (ESI MS (-) calculated m/z 626, found m/z 625.2), and 5’-CMP – 7.5 minutes (ESI MS (-) calculated m/z 586, found m/z 585.2).
Figure 15: Aminoacylation of 5’UMP (A), 5’GMP (B), and 5’CMP (C) with BocTEP
Nucleotide | BocTEP conversion*, % | Esters, % area | BocTyr, % area
--- | --- | --- | ---
5’AMP | 66 | 55 | 11
5’ GMP | 66 | 58 | 8
5’CMP | 68 | 58 | 10
5’ UMP | 62 | 33 | 29

*BocTEP conversion to products from the starting amount in 2 hours.

Table 6: Relative yields of products from aminoacylation of 5’ribonucleotides catalyzed by 1.5 equivalents of La$^{3+}$ in aqueous media (3 mM nucleotide, 3mM BocTEP, 4.5 mM La(OTf)$_3$, 10 mM EPPS; room temperature).

In order to optimize the reaction conditions and to test if we could drive the reaction to completion, La$^{3+}$ was used in 1.5 equivalents to 1 equivalent of BocTEP and nucleotide. Still reaction completion was not observed, but formation of esters seemed to proceed faster than when 1 equivalent of the metal ion was used (Table 6).

The conversion of BocTEP into products after 30 minutes was greater than 30% in every case. The largest amount of hydrolysis product was observed in the aminoacylation of 5’UMP. No dependence, however, on the type of nucleotide (purine or pyrimidine) was observed. After 20 hours reaction time BocTEP was still present in the solution but ester hydrolysis had started to occur.

### 5.3.2. Aminoacylation of 5’CMP with NH$_2$-TEP

We studied the aminoacylation of 5’CMP with NH$_2$-TEP. The tBoc protecting group was removed from BocTEP as described in Chapter 1. The reaction mixture consisted of 15 mM 5’CMP, 11 mM TEP, 11 mM La(OTf)$_3$, and 13 mM EPPS pH 8. A single product, along with the hydrolysis product, was formed and identified as a monoester of 5’CMP (ESI MS (-) calculated m/z 486, found m/z 485.1). All three compounds, the ester, NH$_2$-TEP and Tyr, had very similar elution times. Further optimization of the
analytical method would provide more information as to whether one or two esters were formed.

5.3.3. The effect of Mg$^{2+}$ on the La$^{3+}$-mediated aminoacylation of nucleotides

In order to increase the selectivity of La$^{3+}$ towards esterification, an experiment was conducted with Mg$^{2+}$ added to the reaction mixture. Thus, a standard reaction mixture containing [5'UMP]=[BocTEP]=[La(OTf)$_3$]=15 mM at pH 8 ([EPPS] = 100 mM) gave 66% esters and 34% hydrolysis product. When MgCl$_2$ (25 mM) was present in the same reaction, the product distribution was 74% esters and 26% BocTyr. Mg$^{2+}$ alone did not catalyze ester formation under the reaction conditions.

5.3.4. Aminoacylation of thymidine-5'-monophosphate (5'TMP)

![Figure 16: Aminoacylation of 2-deoxy-5'-TMP with BocTEP](image-url)
Aminoacylation of 5’TMP with BocTEP was studied to determine whether modification of 2’-deoxy-5’-nucleotides is possible under our reaction conditions. 5’TMP was reacted under the same conditions as the ribonucleotides (10 mM 5’TMP, 3 mM BocTEP, 3 mM La(OTf)₃, 10 mM EPPS pH 8; at room temperature). One monoester of 5’TMP with retention time 7.4 minutes was produced (ESI MS (-) calculated m/z 585, found m/z 584.2) (Figure 16).

### 5.3.5. Aminoacylation of cytidine-2’ and 3’-monophosphates (2’CMP and 3’CMP) with BocTEP

Aminoacylation of 3’ and 2’CMP was attempted under the same reaction conditions as the aminoacylation of 5’TMP outlined above. 3’CMP formed one ester with retention time 8.1 minutes (ESI MS (-) calculated m/z 586, found m/z 585.2). 2’CMP did not produce esters in the interval that the reaction was monitored (24 hours) (Figure 17).

**Figure 17:** Aminoacylation of 3’CMP (A) and 2’CMP (B)
5.3.6. \(^{19}\)F NMR study of the aminoacylation reactions of nucleotides (5’, 3’, and 2’CMP and 5’TMP)

The acylation reactions were performed at 23°C with constant stirring. Lanthanide salts (LaCl\(_3\) or La(OTf)\(_3\)) were always added last to prevent hydrolysis of BocFPEP prior to possible acylation. Formation of products was monitored by RP-HPLC at 263 nm and \(^{19}\)F NMR at 367 MHz or 282 MHz, in D\(_2\)O, reference CFCl\(_3\). When \(^{19}\)F NMR was used to follow the reactions, LaCl\(_3\) was used as a catalyst. In cases where the ester products were separated by HPLC, the residual TFA from the mobile phase was removed by solid phase extraction (PL-HCO\(_3\) MP SPE Tubes, Polymer Labs) or acetic acid was used in the mobile phase instead. All reagents were used in equimolar amounts (nucleotide : BocFPEP : La\(^{3+}\) = 1:1:1). The nucleotides studied were 5’CMP, 3’CMP, 2’CMP and 5’TMP. Separate samples were studied by HPLC and \(^{19}\)F NMR.

The aminoacylation of 5’CMP was performed at two different concentrations of the buffer, 10 and 100 mM. At lower buffer concentration, the pH of the reaction mixture was 6.5, whereas at higher concentration, the pH was measured to be 8. In both cases monoesters of 5’CMP were formed (MS ESI (-) m/z calculated 588.15, found 587.2); however, the selectivity was very different. At pH 6.5, two esters were formed with elution times of 4.7 and 5.3 minutes. The ester that eluted first was the major product. The second ester was formed in a very low amount. After 60 minutes reaction time there was still some BocFPEP remaining. Increasing the buffer concentration to 100 mM led to faster consumption of BocFPEP, with only one ester formed (elution time 5.3 minutes). No starting material (BocFPEP) remained at 60 minutes. When the integrated areas of the peaks were compared, it was found that at higher pH the ester accounted for 48% of total products while at lower pH it accounted for 76% of the total products (Figure 18). After 18 hours the reaction mixtures contained equilibrium amounts of both esters in both solutions (2’-ester: 3’-ester = 1:2). Interestingly the esters started hydrolyzing if the La\(^{3+}\) was still present in the solution after complete consumption of the aminoacylating reagent. However, if the esters were isolated and stored below 0° C, dry or in solution, they were stable.
**Figure 18:** HPLC chromatograms of aminoacylation of 5’CMP with BocFPEP at pH 8 (A) and pH 6.5 (B) ([BocFPEP]=[5’CMP]=[LaCl₃]= 18 mM)
Figure 19: Aminoacylation of 5’TMP (A), 3’CMP (B) and 2’CMP (C)
We were able to aminoacylate 5’TMP and 3’CMP with BocFPhe as well (Figure 19). 2’CMP did not form any esters under the reaction conditions for 24 hours. The reaction with 5’TMP proceeded very rapidly and gave a substantial amount of a single ester product (ESI MS (-) calculated m/z 587.2, found m/z 586.1). The $^{19}$F NMR spectrum showed a pattern similar to the one observed for aminoacylation of 5’CMP; formation of the ester product resulted in a $^{19}$F NMR peak at -116.93 ppm. The reaction of 3’CMP was slower, but also gave one monoester (ESI MS (-) calculated m/z 588.2, found m/z 587), which $^1$H NMR is consistent with the 2’ ester, as the 1’ proton on the furanose ring shifted to 6.206 ppm when compared to unreacted 3’CMP.

5.3.7. Analysis of 5’CMP-BocFPhe esters

After separation by HPLC, the esters were isolated and analyzed by ESI MS as well as $^1$H and $^{19}$F NMR. Mass spectrometry identified the products as monoesters of 5’CMP with BocFPhe (ESI MS (-) found m/z 587.2). The site of esterification was determined by $^1$H NMR. The first ester to elute had a chemical shift of the 1’ H on the furanose ring at 6.1 ppm, while the chemical shift of the 1’ H of the second ester was at 5.8 ppm. Based on the difference of the chemical shifts of the 1’ H of the esters to the free 5’CMP, it was determined that the 2’-ester elutes first at 4.8 minutes, followed by the 3’-ester at 5.1 minutes. The $^{19}$F NMR spectrum of the purified and ESI MS characterized monoesters showed only one signal at -117.15 ppm.

When the same reaction was followed by $^{19}$F NMR, a signal of a third F-containing compound was observed. In the spectrum of the sample from the aminoacylation reaction there were three signals observed with chemical shifts at $\delta$ -116.96, -117.09, and -117.18 (Figure 20 A).

To determine which signal was due to the ester formation, a separate BocFPEP hydrolysis was conducted under the same reaction conditions. Both reactions were stopped by the addition of saturated EDTA solution. The $^{19}$F NMR spectrum of the hydrolysis reactions showed two signals very close to each other at -117.03 ppm and -117.18 ppm (Figure 20 B).
5.3.8. Effect of metal ion size on the esterification of 5’CMP

The effect of the size of the metal ion on the aminoacylation of 5’CMP was investigated. These reactions were carried out in 10 mM EPPS buffer and the concentrations of BocFPEP, 5’CMP and metal salts were 18 mM. The formation of esters was monitored by HPLC. The metal ions used in this study were: Mg$^{2+}$ (MgCl$_2$), Sc$^{3+}$ (ScCl$_3$), La$^{3+}$ (La(OTf)$_3$), Pr$^{3+}$ (PrCl$_3$), Nd$^{3+}$ (NdCl$_3$), Yb$^{3+}$ (Yb(OTf)$_3$). By using 18 mM 5’CMP, it was possible to monitor its conversion into esters by UV-HPLC. The effectiveness of the catalyst was measured as percent conversion of 5’CMP, i.e. decrease of the HPLC peak area. Mg$^{2+}$ did not catalyze the reaction. Sc$^{3+}$ showed only a very slight ability to provide effective coordination (0.19%), while all of the lanthanides catalyzed the ester formation (Table 7). La$^{3+}$, Pr$^{3+}$, Nd$^{3+}$, and Yb$^{3+}$ showed conversion extents of 31%, 30%, 29% and 21%, respectively.

Figure 20: $^{19}$F NMR (376 MHz, D$_2$O) spectra of (A) the 5’CMP aminoacylation reaction at 60 min. ([BocFPEP]=[LaCl$_3$]=[5’CMP]=30 mM, [EPPS]=34 mM) and (B) hydrolysis of BocFPEP at 10 min. ([BocFPEP]=[LaCl$_3$]=30 mM, [EPPS]=34 mM) (pH reaction mixture = 6.5)
<table>
<thead>
<tr>
<th>Ion</th>
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<th>Ionic radius, pm</th>
<th>Relative Conversion of 5’CMP, %</th>
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<tbody>
<tr>
<td>Mg$^{2+}$</td>
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<td>72</td>
<td>0</td>
</tr>
<tr>
<td>Sc$^{3+}$</td>
<td>21</td>
<td>75</td>
<td>0.2</td>
</tr>
<tr>
<td>La$^{3+}$</td>
<td>57</td>
<td>116</td>
<td>31</td>
</tr>
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<tr>
<td>Yb$^{3+}$</td>
<td>70</td>
<td>99</td>
<td>21</td>
</tr>
</tbody>
</table>

Table 7: Conversion of 5’CMP into esters in aminoacylation with BocFPEP in the presence of metal ions of different size

5.3.9. Aminoacylation of di- and trinucleotides with BocTEP

The capacity of the catalyst was tested in reactions with adenyl (3’→5’) cytidine (ApC) and adenyl (3’→5’) cytidyl (3’→5’) cytidine (ApCpC). The aminoacylation reagent was completely used up in the reaction time of 4 and 6 hours for di- and trinucleotide respectively.

5.3.9.1. La$^{3+}$-mediated aminoacylation of ApC

Aminoacylation of ApC was conducted with 4 mM ApC, 3 mM La(OTf)$_3$ and 3 mM BocTEP in 10 mM EPPS. The reaction was carried out at room temperature with constant stirring. The conversion of BocTEP was completed in 4 hours at room temperature. Two monoesters were eluted at 6.9 and 8.1 minutes (ESI MS (-) m/z 836.2) (Figure 21). No ester formation was observed in the absence of La(OTf)$_3$. 

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5.3.9.2. CD- study on a possible La$^{3+}$-catalyzed hydrolysis of ApC

Circular dichroism (CD) was used for detection of possible dinucleotide hydrolysis. The aminoacylation reaction mixture was prepared by the method outlined in the previous section and the measurements were taken after 4 hours reaction time. The spectra were recorded on JASCO J-710 spectropolarimeter at room temperature. In addition to spectra of the reaction mixtures (both with and without La$^{3+}$), measurements were also taken of the dinucleotide alone in buffer (with and without La$^{3+}$), as well as BocTEP and buffer alone (Figure 22).
Figure 22: CD spectra of the aminoacylation reaction of ApC with BocTEP in presence or absence of La$^{3+}$; ApC in buffer with and without La$^{3+}$ and BocTEP in buffer

Figure 23: CD spectra of adenosine and 3’CMP in 100 mM EPPS buffer at pH 8
A sinusoidal CD spectrum with a crossover at 260 nm was observed in all cases with the dinucleotide present, with the maximum at 280 nm and the minimum at 240 nm.

To be able to evaluate the extent of hydrolysis, spectra were acquired of 3’CMP and adenosine, analogues of the final products of ApC hydrolysis, 3’AMP and cytidine (Figure 23). The spectra were very different than those of ApC alone or in the reaction. 3’CMP showed a crossover at 240 nm with a minimum at 220 nm and a maximum at 270 nm, while the adenosine spectrum had two crossovers at 279 and 250 nm with the maximum at 230 nm and the minimum were at 265 nm.

5.3.9.3. La$^{3+}$-mediated aminoacylation of ApCpC

![HPLC chromatogram of La$^{3+}$-mediated aminoacylation of ApCpC](image)

The aminoacylation of ApCpC also yielded two esters (Figure 24). Stoichiometric amounts of reagents (ApCpC, BocTEP, and La(OTf)$_3$) were mixed in 10 mM EPPS, pH 8. The aminoacylating reagent was consumed completely in 6 hours. Two new compounds eluted at 6.8 and 7.0 minutes on RP-HPLC. Characterization by ESI MS gave m/z 1136.49, corresponding to the monoester of ApCpC.
5.4. Discussion

5.4.1. Productive coordination of La$^{3+}$

Ribonucleoside-5’-monophosphates provide several functionalities that are potential coordination sites for La$^{3+}$ (Scheme 5). If the metal ion coordinates to the phosphate, which will be in a mono- or dianionic form under the reaction conditions, no esterification would occur. The affinity of lanthanides toward mononucleotides increases with increase in pH, due to increased phosphate ionization. For example, at pH 6.5 the association constant for a Eu$^{3+}$-5’AMP complex is $4.2 \times 10^5$ M$^{-1}$ while at pH 1.5 – 1.8 it is only $10$ M$^{-1}$.$^{43}$ Due to the formation of such a strong complex at higher pH, the effective concentration of the metal ion is much lower.

If La$^{3+}$ coordinates to the 3’-OH group on the ribose and one of the oxygen atoms on the phosphate, it would be possible to selectively acylate the 3’-OH group. As the 2’- and 3’-O-esters are in equilibrium, a mixture of both will be obtained at the end.

If the coordination occurs at the 2’, 3’ – diol on the ribose, a mixture of 2’ and 3’-O-esters will result immediately. Lanthanides form very weak complexes with uncharged saccharides. Thus, this coordination would not inhibit the reaction in the manner that coordination to the 5’-phosphate does. For aminoacylation or acylation to occur, La$^{3+}$ must form a weak bis-bidentate complex with both the nucleotide and the aminoacyl phosphate and, after promoting the reaction, be able to dissociate.
5.4.2. **La$^{3+}$-mediated aminoacylation reactions of mononucleotides - selectivity**

Ribonucleoside-5’-monophosphates were successfully charged at the 2’- or 3’-OH groups via La$^{3+}$-mediated aminoacylation. 5’AMP, 5’UMP, 5’CMP and 5’GMP all formed monoesters. BocTEP and BocFPEP were tested as aminoacylating reagents, and both were effective in the reactions. Reactions where BocTEP was used, however, proceeded more slowly than when BocFPEP was used (see aminoacylation of 5’CMP). The greater efficiency of BocFPEP could be due to the presence of fluorine on the
aromatic ring. Thus, electronic and steric effects can be responsible for these observations.

In attempts to drive the reaction to completion, the usual ratio of BocTEP:La\(^{3+}\): nucleotide 1:1:1 was changed to 1:1.5:1. Complete conversion of BocTEP into products was not observed, but the reaction proceeded more rapidly. The conversion of BocTEP at 2 hours reaction time was over 60% for all four nucleotides. The smallest amount of esters was produced with 5’UMP. No general trend of reactivity among the nucleotides, however, is observed (Table 6). Increasing the temperature from 23°C to 35°C and 45°C also did not drive the aminoacylation reaction with BocTEP to completion.

In the reactions with ribonucleoside-5’-monophosphate it was noticed that, except for 5’AMP, the nucleotides formed only one ester. If La\(^{3+}\) coordinates to the 2’, 3’-diol, both esters should be formed at the same time. Only one ester should result from a coordination of La\(^{3+}\) to the phosphate and 3’-OH. To test this possibility we reacted 5’TMP with BocTEP and BocFPEP. Both reactions resulted in the formation of one ester – the 3’-O-ester of 5’TMP. This result confirmed that coordination of this type (to the 5’-phosphate and the 3’-OH) is possible and weak enough to allow the esterification reaction. This suggests that the esters of 5’AMP may be products from a different type of coordination than the esters of the other ribonucleotides used in the study. Different reactivity of 5’AMP and 5’CMP has been reported in the literature previously.

The effect of the position of the phosphate (5’, 3’ or 2’) on coordination and subsequent ester formation was also investigated. 2’CMP did not yield any ester products with either BocTEP or BocFPEP. 3’CMP, however, formed one ester in both cases. \(^1\)H NMR suggested that the ester formed with 3’CMP is at the 2’-OH. If the observed ester is formed as a result of coordination of the 2’-OH and 3’-phosphate groups, then a similar outcome could be expected in a reaction with 2’CMP. Multidimensional NMR detecting H-H coupling through space should be able to determine more correctly the site of acylation in 3’CMP and then conclusions could be drawn regarding the reasons for absence of an ester from 2’CMP.
5.4.3. Successful ester formation with non-protected aminoacyl phosphate (NH$_2$-TEP)

Ideally, we would like to be able to charge tRNAs with aminoacyl moieties that do not carry an amino-protecting group. The presence of a protecting group would cause synthesis of protein fragments as chain elongation would be impossible. The aminoacyl phosphates that we use carry a protecting group not only because it is required during the coupling step of their synthesis, but also for stability and storage reasons. Thus, BocTEP was treated with TFA to give an aminoacylating reagent with a free amino group, NH$_2$-TEP. This reagent was used in a successful aminoacylation reaction with 5’CMP, proving that the aminoacylation system we developed does not need the protecting group for successful coordination of the metal to the aminoacyl phosphate or acyl transfer.

5.4.4. The effect of Mg$^{2+}$ on productive La$^{3+}$ coordination

It occurred to us that aminoacylation of mononucleotides could be slow due to formation of a non-productive complex between La$^{3+}$ and 5’-phosphate. Because of the very strong coordination, the equilibrium between the complex and free metal ion and nucleotide would be shifted to the complex. This would leave very little of metal ion available.

Magnesium (Mg$^{2+}$) is known to be essential for maintaining the activity and structure of tRNAs. In a study of interactions of Mg$^{2+}$ with inorganic and nucleoside phosphates, Stuehr and co-workers showed that it coordinates exclusively to the phosphate oxygens.$^{88,89}$ With this in mind, we tested if we could “quench” the phosphate with Mg$^{2+}$ and drive La$^{3+}$ to coordinate to the diol, as Mg$^{2+}$ does not have affinity for the ribose moiety. When excess MgCl$_2$ was introduced into the reaction mixture, the percent of esters formed increased from 66% (La$^{3+}$, no Mg$^{2+}$) to 74% (La$^{3+}$ and Mg$^{2+}$). Thus, introduction of another metal ion with selectivity towards the phosphate group improves the ester yield. No reaction was observed in presence of Mg$^{2+}$ only.
5.4.5. \textit{\textsuperscript{19}F NMR as an alternative method of detection}

Based on the results obtained with \textsuperscript{19}F NMR for reactions of nucleosides with BocFPEP, we applied this technique to study aminoacylation of nucleotides. We found that BocFPEP is more effective reagent in La\textsuperscript{3+}-mediated aminoacylation reactions than BocTEP. Reactions with both 5’TMP and 5’CMP went to completion with a high yield of esters. When compared to the hydrolysis of BocFPEP, the \textsuperscript{19}F NMR spectra of these reaction mixtures indicate the formation of a new F-containing compound, with a chemical shift very close to that of BocFPEP and BocFPhe. The BocFPhe-isopropyl ester, synthesized for comparison to the esters of nucleosides and nucleotides, has a chemical shift $\delta$ -118.9 (in CD\textsubscript{3}OD), which is very close to the chemical shift of BocFPhe under the same conditions. The product formed from the reaction of BocFPEP and 5’CMP has a chemical shift similar to that of the aminoacylating reagent, BocFPEP, and its hydrolysis product, BocFPhe. Considering that there was no significant change in the environment of the fluorine atom in either of the experiments we can conclude that the third F-containing compound in the mixture is a BocFPhe-5’CMP ester.

5.4.6. \textit{pH dependence of the ester formation with BocFPEP}

Komiyama and co-workers reported selective hydrolysis of phosphomonoesters vs. phosphodiesters by Ce\textsuperscript{4+} with an increase of buffer concentration at 50 °C and pH 8.\textsuperscript{90} They found that the buffer can act as a competitive ligand to the metal ion and thus lower its catalytic ability. Phosphate monoesters are dianionic species, and are therefore better ligands for Ce\textsuperscript{4+} than the monoanionic phosphodiesters. Thus, an increase in buffer concentration would greatly affect the productive coordination to the phosphomonoester.

Oestreich and Jones noted pH dependence in metal ion catalyzed hydrolysis of acetyl phosphate.\textsuperscript{91} The metal ion does not catalyze the hydrolysis at pH where the monoanion exists but it enhances the rate of the reaction at a pH where the dianionic species are predominant.

We studied the aminoacylation of 5’CMP with BocFPEP in the presence of EPPS buffer at concentrations of 10 and 100 mM at room temperature. The pH of the reaction mixture with 10 mM EPPS was 6.5, while the reaction with 100 mM buffer was at pH 8. At lower pH, 5’CMP formed two monoesters, while at pH 8 only one ester was formed.
The selectivity of ester formation was opposite under the two sets of conditions. At higher pH, BocFPEP was completely used up in less than 60 minutes and gave 48% ester, with 52% BocFPhe. When done at lower pH, the reaction was slower but produced more esters and the amount of hydrolysis product was lower than at pH 8 – 76% esters and 24% BocFPhe. The esters were analyzed by $^1$H NMR and we concluded that, at pH 6.5, the 2’-ester is the major product. Reaction at pH 8 resulted in the formation of only the 3’-ester.

The pK$_a$’s of 5’CMP are 4.5 and 6.3.$^{92}$ At pH 8 it is primarily a dianion. At pH 6.5, however, about 1/3 is present in monoanionic form. Since dianions are better ligands for lanthanides than monoanions, complexation with phosphate is stronger at pH 8 than at pH 6.5, and the acylation reaction will be slower. The only ester formed at pH 8 is the 3’-ester, which confirms that the La$^{3+}$ is coordinated to the phosphate. The ester formation will be greater at pH 6.5 because the metal ion is available for coordination to the diol. The selectivity was observed to be driven towards the 2’-ester. This ester can be formed in such a high amount only through direct chelation of 2’- and 3’- OH-groups.

### 5.4.7. Dependence on metal ion size

Another interesting aspect of our study was the effect of the size of the metal ion on the reaction. The metal ions tested were Mg$^{2+}$, Sc$^{3+}$, La$^{3+}$, Pr$^{3+}$, Nd$^{3+}$, and Yb$^{3+}$. The effectiveness of the catalyst was measured as the percent conversion of 5’CMP in a reaction with BocFPEP. As expected, Mg$^{2+}$ did not catalyze esterification. Sc$^{3+}$ was tested because its properties are often compared to those of the lanthanides. However, in this particular reaction, Sc$^{3+}$ was not as effective as the tested lanthanide ions. All lanthanides promoted aminoacylation of 5’CMP. It was noted that the conversion of 5’CMP decreases with increase in the atomic number of the lanthanide (Table 7). The lanthanide contraction, the progressive decrease in ionic radius with increase of the atomic number, is a well known feature of these elements. The results from this study show that smaller lanthanides are less effective in aminoacylation reactions than larger lanthanides. Smaller lanthanides have a greater charge-to-size ratio, and they coordinate better to the phosphate on the nucleotide, not only because of the electrostatic interaction but also because they can not accommodate a complex with the diol on the ribose due to their...
size. In the hydrolysis of RNA or phosphodiester bonds later lanthanides, such as Tm$^{3+}$ and Ce$^{4+}$, are usually used.\textsuperscript{52, 90, 93} This process requires coordination to a terminal phosphate and the formation of a dimer of the metal ions.\textsuperscript{93} Larger lanthanides, such as lanthanum, provide better coordination of the ribose diol and are therefore better catalysts of the aminoacylation reaction.

Solvent exchange rate is also a factor here. In order a reactive complex to be formed, some of the solvent (water) molecules must be replaced from the metal coordination sphere. This process is faster for larger lanthanides and becomes slower for the smaller ones.\textsuperscript{44} Thus, the observed lower conversion of 5’CMP into esters by smaller lanthanide ions could be also due to slow ligand exchange rates.

### 5.4.8. Successful aminoacylation of ApC and ApCpC

ApC and ApCpC were used as analogues of tRNA. In the method developed by Hecht for chemical misaminoacylation of tRNAs, a dinucleotide is chemically aminoacylated and it is ligated back to a truncated molecule of tRNA.\textsuperscript{13} This method, however, requires the protection of several functional groups which are deprotected later in the synthesis. These reactions are performed in organic media and result in low yields.

The aminoacylation of both ApC and ApCpC was performed in aqueous buffer at pH 8 at room temperature. The only protecting group present was on the aminoacyl phosphate. Both substrates yielded two monoesters of the nucleotides with BocTyr (Figures 21 and 24). These reactions were completed faster than the reactions of mononucleotides probably because the phosphate is a diester, i.e. monoanionic, and is a poorer ligand for La$^{3+}$ than the dianionic phosphate of the mononucleotides.

### 5.4.9. CD-study: No hydrolysis of ApC by La$^{3+}$

Hydrolysis of ApC should proceed in the fashion presented in Scheme 6 with formation of cytidine and 3’AMP.
Scheme 6: Hydrolysis of ApC to cytidine and 3’AMP

CD spectroscopy is often used to determine the secondary structures of biomolecules. This technique is based on the different absorption of left and right handed circularly polarized light by compounds with asymmetric chromophores. In the case of nucleic acids, the CD signal measured corresponds to the electronic transitions of the bases. The bases absorb UV light from 300 nm to the vacuum UV region. The electron transitions in ribose are very weak and start at around 200 nm, while those of the phosphate begin even further into the vacuum UV (200-10 nm).

The shape of the CD spectrum of an intact ApC dinucleotide has been previously reported in the literature. A sinusoidal CD spectrum with a crossover near the normal UV absorbance maximum of the dinucleotide (260 nm) was reported to be characteristic of base stacking in aqueous solution. The CD spectrum of the monomers is sinusoidal with a crossover at 240 nm, a maximum at 280 nm and a minimum at 220 nm.

The spectra of all reaction mixtures containing ApC, with or without La\(^{3+}\), showed the same type of sinusoidal curve with a characteristic crossover at 260 nm as in the ones reported in the literature. When the aminoacylating reagent was omitted from the reaction mixture (Figure 22, ApC+La in EPPS) the maximum was slightly lower in intensity but the crossover was still at 260 nm. A significant difference was noted between the CD spectrum of the dinucleotide in the reaction (Figure 22, Reaction, with La\(^{3+}\)) and the
spectra taken of monomers similar to the products of the complete hydrolysis of ApC, adenosine and 3’CMP (Figure 23). The crossovers, as well as the minima and maxima are at very different wavelengths in both cases.

Using CD spectroscopy we determined that, under our reaction conditions and concentration of La$^{3+}$ no hydrolysis of the dinucleotide occurs.

5.5. Conclusions

Monoesters of nucleotides were produced in La$^{3+}$-mediated aminoacylation reactions in aqueous buffer. The selectivity of La$^{3+}$ towards promoting ester formation could be enhanced by the addition of a competitive cation (Mg$^{2+}$) or by a change in pH (converting the dianionic phosphate of the nucleotide into monoanionic). The change in pH of the reaction mixture also leads to the change in selectivity of ester formation, with the 2’-ester favored at low pH and the 3’-ester favored at high pH. The effective aminoacylation mediated by La$^{3+}$ with aminoacyl phosphates with free amino groups as well as the efficient esterification of the 2’ and 3’ OH-groups at the 3’ terminal of ApC and ApCpC with BocTyr, are promising results for the application of this biomimetic system for tRNA acylation and possible in vitro protein synthesis. The fact that the presence of La$^{3+}$ at this concentration does not lead to hydrolysis of the phosphodiester bond in ApC is another advantage of this approach.
CHAPTER 6: $^{19}$F NMR analysis of RNA aminoacylation

6.1. Introduction

We had attempted to study the aminoacylation of tRNA with BocTEP utilizing several conventional analytical methods. However, it was not possible to determine if the reaction took place using HPLC with UV detection, as both the nucleic acid and its aminoacylated product, have similar UV absorbances (around 260 nm) and elution times. MALDI MS did not give a definite answer due to insufficient ionization of the nucleic acid. Therefore, we examined the possibility of introducing a specific functionality (“label”) on the tRNA molecule. This “label” could be a radioactive isotope, a fluorophore, or a magnetic nucleus. After examining the alternative, we decided on an NMR-based analysis as this could give additional structural information. We chose BocFPEP as the aminoacylating reagent as it showed high efficiency in reactions with nucleosides and nucleotides, and allowed for analysis by $^{19}$F NMR.

Another problem was the limited amount of tRNA used in a reaction. Bulk RNA was chosen as an analogue of tRNA. This would allow us to study the reaction at millimolar concentrations necessary for $^{19}$F NMR. Bulk RNA is commercially available and consists of a mixture of RNAs of different types and lengths. Most important is that all have a single free diol functionality at their 3'-terminus. Aminoacylation of RNA with BocFPEP would provide us with important information about the applicability of our system in the modification of large complex molecules.

6.1.1. $^{19}$F NMR in studies of biological molecules

Several properties of fluorine make it a unique probe in protein and nucleic acid research. It is one of the most abundant elements on earth but does not occur widely in biological compounds. Due to its small size it is often used as a substitute for hydrogen in many biologically active molecules. This atom replacement is usually sterically nonperturbing. The fluorine nucleus has a spin of $\frac{1}{2}$ and 83% the NMR sensitivity of H. The $^{19}$F chemical shift is determined mostly by the fluorine electron lone pairs which exhibit a strong environmental dependence. Thus, the chemical shift is sensitive to change in the local van der Waals environment as well as electrostatic fields.
19F NMR has been used in determining protein structures and conformational changes. Incorporation of fluorinated amino acids into proteins provides a direct tool for monitoring the environment of a modified residue. Thus, these non-natural amino acids are valuable substrates in protein design and engineering. Since our synthetic method is aimed at introducing non-natural amino acids, this presented an ideal opportunity to test the system.

Fluorine can be introduced into nucleic acids as well in the form of a fluorinated heterocyclic base. 5-fluorouracil has been incorporated into tRNA as a probe to investigate protein-RNA complexes, usually the recognition of tRNA by its cognate aaRS. It was found that this modification has no major effects on the secondary structure of tRNA and it remained functional in protein synthesis in vitro.

We do not expect to see large changes in 19F chemical shifts, which are usually observed when 5-fluorouracil is introduced in tRNAs. If the aminoacylation is successful, the BocFPhe-moiety would be attached at the 3′-terminal diol. This part of the molecule is usually exposed to the solvent and the environment would not change around the F atom to any large extent.

### 6.2. Experimental

#### 6.2.1. Materials and Methods

Commercial reagents were used as received. RNA from Torula yeast, Type VI was purchased from Sigma. Nuclease free water was used in all reactions with RNA. NMR spectra were recorded at 356 MHz (19F) with D2O as a lock solvent. MicroSpin G-25 columns were purchased from GE healthcare.

BocFPEP was synthesized as previously described. LaCl3 was used as a catalyst in the reactions instead of La(OTf)3. MgCl2 was present to maintain the tertiary structure of RNA and to compete with La3+ for binding to the phosphodiester backbone.

RNA was oxidized by treatment with sodium periodate (NaIO4). 9.3 mg RNA were dissolved in 0.5 ml 100 mM MgCl2 and 0.5 ml 0.5 M EPPS pH 8. 0.1 M solution of NaIO4 was prepared immediately before the oxidation reaction. To the solution of RNA were added 5 ml 0.1 M NaIO4. The oxidation was done in the dark at room temperature.
for 20 minutes. The excess NaIO₄ was precipitated by addition of 0.1 g KCl to the mixture at 0°C. The supernatant was transferred into another vial, purified through G-25 column and the oxidized RNA (oxRNA) was precipitated from it by the addition of cold ethanol, centrifugation and drying.

6.3. Results

A typical reaction mixture contained 20 mM BocFPEP and LaCl₃, 25 mM MgCl₂, 75 mM EPPS pH 8 and 4.65 mg/ml buffer RNA or oxRNA. The nucleic acid was mixed with MgCl₂ and the buffer first. LaCl₃ was added last. The aminoacylation was conducted at room temperature with constant stirring. After 1 hour, the reaction was stopped by the addition of EDTA, filtered and ¹⁹F NMR spectra were taken. A portion of the reaction mixture was run through Sephadex G-25 column to isolate the ester of RNA/oxRNA and ¹⁹F NMR spectra were collected again.

The ¹⁹F NMR spectra of the reaction of RNA before and after purification are shown in Figure 25. The spectrum of the reaction mixture before purification showed the presence of F-containing compounds with chemical shifts at δ -117.23 and -117.46. After purification and four-fold dilution, the spectrum showed a similar pattern and intensity of the signals. The chemical shifts were slightly different, δ -117.19 and -117.51, which can be a result of the removal of salts and buffer from the solution. After treatment through a Sephadex G-25 column, the reaction mixture should be free of compounds smaller than 10 kDa, such as salts, buffer, BocFPEP, and BocFPhe.
Figure 25: Aminoacylation of bulk RNA with BocFPEP in the presence of La$^{3+}$: $^{19}$F NMR spectrum of the reaction mixture after one hour (A) and after purification and 4 times dilution (B).

Figure 26: $^{19}$F NMR spectra of the solution from the reaction of oxRNA with BocFPEP in the presence of LaCl$_3$ after 1 hour, prior to isolation of RNA (A), and the same amount of material after isolation of all oxRNA species via size-exclusion chromatography (B).
The spectra obtained from the reaction mixture of oxRNA prior to and after purification did not show the same pattern as the reaction of RNA (Figure 26). Two signals were observed, at -117.22 and -117.33 ppm, in the spectrum of the reaction mixture after 1 hour. After purification, however, the observed intensity of the signals with chemical shifts at δ -117.20 and -117.55 was greatly diminished.

6.4. Discussion

aaRS’s specifically charge the tRNA at the 3’-terminal adenosine. Esterification at any other OH-group in tRNA would not lead to incorporation of the amino acid into the growing peptide chain in the ribosome. La\(^{3+}\)-mediated aminoacylation of nucleosides and nucleotides provided important insights about the capacity of the catalyst to recognize the 2’,3’-diol moiety and effectively attach the aminoacyl group to either the 2’ or 3’ OH-group. tRNA, however, is much more complex. Taking into account that La\(^{3+}\) catalyzes the esterification of 5’TMP and 3’CMP, we must test whether the esterification of tRNA is at the 3’-terminal diol or along the backbone. Upon formation of the tertiary structure of tRNA in solution, it is possible that another OH-group is coincidentally positioned close to a phosphate. La\(^{3+}\) can potentially coordinate these groups to catalyze either the esterification of the OH-group or the hydrolysis of the phosphodiester bond.

Single type tRNA from yeast and E. coli are commercially available, but in limited amounts. We know from previous experiments that for product analysis with \(^{19}\)F NMR, concentrations in the millimolar range are required for detection at 376 MHz. In order to work with such concentrations, we used bulk RNA which provides the characteristic 3’-diol functionality.

The Malaprade oxidation of sugars with NaIO\(_4\) is a widely used reaction in carbohydrate chemistry. It converts vicinal diols into dialdehydes (Scheme 7). For our system to be effective, the presence of a 1,2-diol is essential. RNA was oxidized to convert it into an unsuitable substrate of the La-mediated aminoacylation, thus providing a negative control for the reaction.
Scheme 7: Oxidation of tRNA with NaIO₄ - conversion of a 1,2-diol into a dialdehyde

In an attempt to determine the site of esterification, we compared aminoacylation of RNA and oxRNA. ¹⁹F NMR was chosen as an analytical method because RNA molecules naturally do not contain any fluorinated subunits. Thus, if aminoacylation occurs, a signal should be observed in the ¹⁹F NMR spectrum after purification of the reaction mixture. Detection of a signal in the spectrum of the purified reaction mixture of oxRNA would suggest that the acylation does not occur at the 3’-terminal diol, but rather at another site in the molecule.

The ¹⁹F NMR spectra of the reaction solutions of RNA and oxRNA before and after purification provided very different patterns of signals (Figures 25 and 26). Before purification, both reaction mixtures showed two signals. The spectrum of the reaction solution of oxRNA before purification, however, closely resembles the pattern observed in the hydrolysis of BocFPEP.

After size-exclusion chromatography (G-25 spin column) the macromolecules (RNA, BocFPhe-RNA) were separated from other materials (BocFPEP, BocFPhe). The ¹⁹F NMR spectrum of the reaction of RNA shows the same intensity of the peaks, while the oxRNA-reaction spectrum has only traces of signals. The great difference in the spectra of the purified reaction mixtures suggests that the reaction occurs mostly at the 3’-terminal diol. The observed small signals from the oxRNA-reaction could be explained by: 1) incomplete oxidation of RNA, 2) acylation at a site other than the 3’-terminal diol, or 3) exceeding the capacity of the column. If the RNA was not completely oxidized, there could still be diol moieties present which could be aminoacylated. If the observed signals are due to acylation somewhere along the phosphodiester backbone, it is a very
small amount of the total aminoacylated compound when compared to the signals obtained from aminoacylated RNA. Some amounts of BocFPEP and BocFPhe could run through the column if the loaded volume was not in the effective range or if the column was spun for slightly too long.

6.5. Conclusions

Charging the tRNA molecules at the 3’ terminal adenosine is an essential step in the biochemical synthesis of proteins. In order to create a competent substrate for \textit{in vitro} translation, our system must be capable of recognizing and successfully delivering the aminoacyl group to the 3’-terminal diol moiety of tRNA.

The aminoacylation reactions of RNA and oxRNA were studied by $^{19}$F NMR. The results imply that the main site of acylation on RNA is the 3’-terminal diol. However, some product may be formed on a 2’-OH group of the RNA backbone if it is positioned close to a phosphate when the tertiary structure is formed.

Ideally our method will be used to aminoacylate an orthogonal tRNA that will be applied in an \textit{in vitro} system. The \textit{in vitro} system would not contain an aaRS that would correspond to this tRNA molecule. Unlike the aaRS, the ribosome verifies the tRNA identity only by recognition of the anticodon. If our system generates tRNA acylated at both the 3’-terminal adenosine and the phosphodiester backbone, protein synthesis should be successful as long as the anticodon is non-modified.
CHAPTER 7: Biomimetic aminoacylation of tRNA

7.1. Introduction

The objective of this work is to produce aminoacyl-tRNAs with any amino acid using a straightforward biomimetic approach. After the encouraging results obtained from our aminoacylation studies of small tRNA-analogues (ribose, nucleosides and nucleotides) as well as RNA, we applied the same method in reactions of tRNA.

Due to the low amount of tRNA used in each reaction, we used an alternative method or product analysis based on amino acids labeled with the fluorescent dansyl (DNS) group. This modification allows us to use a more sensitive analytical technique, fluorescence spectroscopy and antibody-enhanced fluorescence: an anti-DNS antibody is available that can be used to recognize any dansylated compound present in solution.

After the generation of several DNS-aminoacyl-tRNAs, we attempted cell-free protein synthesis using DNS-aminoacyl-tRNA^{fMet}. The N-terminal labeling of proteins with different fluorophores was discussed in Chapter 1. We chose to use tRNA^{fMet} for in vitro transcription/translation. Aminoacyl-tRNA^{fMet} initiates the protein synthesis in the ribosome. The amino group does not have to be free as this will be the N-terminal amino acid. Moreover, IF2 specifically recognizes the blocked amino group without being specific toward the side chain of the amino acid. Thus, if translation occurs, a protein with an N-terminal fluorophore will be produced. Several plasmids coding for proteins with different molecular weights (72 kDa, 30 kDa, 15 kDa and 7 kDa) were used.

7.1.1. Principles of fluorescence

Fluorescence spectroscopy is a method often used in studies of biological molecules because of its high sensitivity. Fluorescence is photon emission resulting from an initial absorption of light at a different wavelength, after vibrational relaxation, without a change in the spin state. A Jablonski diagram is presented in Figure 27.
The ground state of molecules, $S_0$, is normally a singlet state, i.e. the electron state in which all electron spins are paired. At room temperature this state represents the energy of all of the molecules in a solution. Upon absorption of a photon of energy $h\nu_{\text{EX}}$, the electrons undergo transitions that bring the molecule from a stable ground state $S_0$ to an excited vibrational state $S_1'$. The absorption is a fast process ($10^{-14} – 10^{-15}$ sec). Once at the excited state, the molecule loses energy through vibrational relaxation to a lower vibrational state, $S_1$. In solution, the excited molecules transfer energy to the solvent molecules through collisions (vibrational relaxation). This process occurs in $10^{-12}$ sec. Thus, fluorescence in solution always involves a transition from the lowest vibrational level of an excited electronic state to the ground state by emission of a photon of radiation $h\nu_{\text{EM}}$. Because of the efficiency of vibrational relaxation, the fluorescence band of a given electronic transition is displaced toward lower frequencies, or longer wavelengths from the absorption bands. This phenomenon is called Stokes shift.

Compounds containing aromatic functional groups or highly conjugated double bonds exhibit fluorescence. Fluorescence is usually due to $\pi\rightarrow\pi^*$ and $n\rightarrow\pi^*$ low-energy electron transitions. Rigid molecules have higher quantum efficiencies.

Tryptophan, tyrosine and phenylalanine are intrinsic fluorophores of proteins. The change of the $\lambda_{\text{max}}$ and its intensity give important information about the position of the fluorophore in the protein. For example, $\lambda_{\text{max}}$ of tryptophan shifts to shorter wavelengths and the intensity increases as the polarity of the solvent decreases. This can be used to

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**Figure 27:** Jablonski diagram\textsuperscript{104}: 1) Excitation; 2) Vibrational relaxation; 3) Emission
determine whether a certain tryptophan residue is buried in a non-polar pocket or is exposed to the solvent. Extrinsic fluorophores can be introduced in proteins through covalent labeling. Fluorescein, dansyl (5-(dimethylamino)naphthalene-1-sulfonyl), 1-anilino-8-naphtalene-sulfonate, rhodamine are some of the widely used fluorophores.

### 7.1.2. Methods for labeling tRNAs with fluorophores

Modification of tRNAs with fluorophores began with the studies of interactions between tRNAs and initiation or elongation factors, or with their corresponding aaRS. Direct chemical incorporation of fluorescent labels into nucleic acids is not widely used. Usually a fluorophore acceptor (amino or thiol group) is introduced into synthesized oligonucleotides prior to labeling.107-110

tRNAs carrying two fluorescent groups were prepared in studies examining the tertiary structure of tRNA by singlet-singlet energy transfer.111 This method of modification requires oxidation of the 3’ terminus of the nucleic acid prior to labeling, and therefore it cannot be used as an analogue of aminoacyl-tRNA in a translation reaction. However, after the formation of the dialdehyde, the modified RNA can be labeled at the 3’ terminus via condensation of the aldehyde and fluorescein-5-thiosemicarbazide,112 or tetramethylrhodamine hydrazine.102 This fluorophore-labeled RNA can be sequenced by enzymatic degradation.

Giovane and co-workers investigated the formation of the ternary complex of aminoacyl-tRNA with EF-Tu (prokaryotic elongation factor) and GTP. They introduced a fluorescent label, anthraniloyl, at the 3’-OH of adenosine by reacting tRNA with isatoic anhydride.113 Through fluorescence anisotropy they were able to determine the dissociation constant of the complex of the 3’-O-anthranioloyl-tRNA with EF-Tu and GTP.

The enzymatic labeling of nucleic acids involves the introduction of organic fluorophores into primers or nucleotides by synthetic means and then incorporation through PCR amplification, DNA/RNA polymerases or polynucleotide transferase.114-116
7.1.3. Antibodies and anti-dansyl antibody specificity

Antibodies, or immunoglobulins (IgG), are large glycoproteins with an average molecular weight of 150 kDa. They are produced in living organisms via immune response. Their selective binding properties toward immunogens (any agent capable of inducing an immune response) allow them to interact with molecules of a certain structure (antigens and haptens). The molecules that react with the products of a specific immune response, the antibodies, are called antigens. They can be proteins or polysaccharides found in bacteria, viruses, or other microorganisms. The site on the antigen that is recognized by the antibody is called the epitope. Haptens can elicit immune response only if they are coupled to a carrier protein. Free haptens, however, can react with specific antibodies. A variety of molecules such as simple sugars, amino acids, lipids, or peptides may act as haptens.

All immunoglobulins share some common structural features. They have two light and two heavy polypeptide chains which are bound together by disulfide bonds. The antibody molecule has a Y-shape with a central axis of symmetry (Figure 28). The antigen-binding fragments ($F_{ab}$) of the antibody are located at the N-terminal ends of the polypeptide chains. The amino acid sequence of these fragments determines the antigen-binding properties of the molecule. Antibody and antigen associations rely on hydrogen bonding, hydrophobic interactions, electrostatic and Van der Waals forces.

![Antibody structure](image)

Figure 28: Antibody structure
There are two types of antibodies: polyclonal and monoclonal antibodies. Polyclonal antibodies are isolated directly from the serum. They are a mixture of antibodies that bind to different epitopes of the immunogen. They have a wide range of selectivities and affinities.\textsuperscript{117} Monoclonal antibodies, on the other hand, are a homogeneous population of identical antibody. They have affinity for a single antigenic epitope.

The anti-dansyl antibody (anti-DNS Ab) was originally generated to study antibody/hapten interactions or changes at the binding site of the antibody.\textsuperscript{119} Parker and co-workers established that the anti-DNS Ab increased the magnitude of the fluorescence signal of an antibody-bound ε-DNS-lysine by a factor of 25 to 30, accompanied by a blue shift of the emission peak of about 20 nm.\textsuperscript{119, 120} The recognition was specific and the binding interactions occurred at the Fab portion of the antibody.\textsuperscript{119} Polyclonal rabbit anti-DNS Ab provided selectivity comparable to that of the monoclonal murine anti-DNS IgG toward DNS-labeled components of the liposomal membrane.\textsuperscript{120}

7.2. Experimental

7.2.1. Methods and Materials

tRNAs (\textit{E. coli} tRNA\textsubscript{fMet}, \textit{E. coli} tRNA\textsubscript{Val}, and yeast tRNA\textsubscript{Phe}) as well as the other commercially available chemicals were purchased from Sigma. Nuclease-free water from Fermentas was used in all experiments with tRNA and \textit{in vitro} transcription/translation. Coupled prokaryotic \textit{in vitro} transcription/translation system, EcoPro\textsuperscript{TM} T7, was purchased from Novagen. The plasmids used in the cell-free protein synthesis were kindly provided by the laboratories of Professor D. Zamble (pET22-b for \textit{E. coli} NikR and pET24-b for human carbonic anhydrase) and Professor G. A. Woolley (pET20b(+) for GCN4), Department of Chemistry, University of Toronto. S-Tag β-glucuronidase has been supplied as a control vector with the EcoPro T7 kit. Polyclonal rabbit anti-DNS Ab was purchased from Molecular Probes. MicroSpin G-25 columns were purchased from GE healthcare.

Aminoacylation reactions were carried out for one hour and performed as previously described for nucleotides and RNA. After completion of the reaction, the aminoacyl-tRNAs were purified by passing the reaction mixture through G-25 spin-columns.
Concentrations of tRNAs were determined using Beer’s law (Equation 2) with extinction coefficients obtained from literature.\textsuperscript{121}

**Equation 2:**

$$A_{260} = \varepsilon \times l \times C$$

where $A_{260}$ is the measured absorbance at 260 nm; $\varepsilon$ - the extinction coefficient of tRNA; $l$ - the light path; and $C$ - the concentration of the tRNA.

### 7.2.2. Oxidation of yeast tRNA$^{\text{Phe}}$ and *E. coli* tRNA$^{\text{Val}}$

Oxidation of tRNAs with sodium periodate was performed as previously described for RNA by the method of Proudnikov and Mirzabekov.\textsuperscript{102} A fresh solution of 0.1 M sodium periodate was prepared immediately before the reaction. 5 µl of this solution was used to oxidize 0.1 ml 5.5×10$^{-5}$ M tRNA. The reaction was carried out in the dark for 20 minutes at room temperature. The excess reagent was precipitated by addition of KCl at 0°C.\textsuperscript{103} The supernatant was removed and transferred to another vial. The oxidized tRNA was then isolated by precipitation with cold ethanol and subsequent centrifugation for 30 minutes at 13000 rpm. The resulting white pellets were dried and used in the acylation reaction.

### 7.2.3. Fluorometer settings and conditions

Fluorescence measurements were performed on a JY Horiba Fluorolog-3 spectrophotometer. The fluorophore used in these studies, the DNS-group, has an excitation maximum at 330 nm and an emission maximum at 550 nm. DNS-aminoacyl phosphates were excited at 327 nm. The instrumental settings were as follows: increment: 1 nm, excitation slit: 1 nm, emission slit: 1 nm and integration time: 1 sec. Spectra were acquired from 400 to 650 nm. All measurements were performed at room temperature in solutions containing nuclease-free water.

The anti-DNS Ab was used in fluorometric studies to determine the presence of DNS-group on the tRNA after aminoacylation. It was supplied as a solution (1 mg/ml in phosphate-buffered saline pH 7.2, containing 5 mM sodium azide). According to the manufactures’ product information, the maximum fluorescence enhancement for Ab-bound DNS is about 10-fold over the fluorescence of the free dye. Steric hindrance of covalently bound fluorophore can lower the enhancement produced by antibody binding.
7.2.4. Polyacrylamide gel electrophoresis (PAGE) analysis

The products from aminoacylation of tRNAs were determined by 10%/8M urea/Tris-borate EDTA (TBE) polyacrylamide gel electrophoresis. The gels were prepared according to standard procedure. 2.05 ml 40% solution of acrylamide / bis-acrylamide, 3.6 g urea, 1.41 ml 10X TBE were mixed with 4.1 ml dd water. After the urea was completely dissolved, 57 μl of freshly prepared 10% ammonium persulfate (APS) and 5.7 μl N,N,N’,N’-tetramethylethylenediamine (TEMED) were added to start the polymerization. Samples for loading were prepared by mixing a 1-3 μl aliquot of the DNS-aminoacyl-tRNA or free tRNA, as a control, with 10 μl of gel loading buffer (50% Glycerol, 0.02 % bromophenol blue, 1X TBE). After the samples were loaded, electrophoresis was performed in 1X TBE at a constant voltage of 200 V, 8 mA for 45 min.

Visualization of the gels was performed on a MultiImage FC™ Light cabinet controlled by AlphaEase FC™ Software (Alpha Innotech Corp.). Gels were first illuminated at 302 nm to detect DNS-products before staining with ethidium bromide (EtBr). The fluorescent bands were detected at 533 nm (fluorescein filter). For EtBr staining, the gel was immersed in a solution of EtBr (40 μl/ml) for 5 minutes. After incubation, the gel was rinsed in water for 10 minutes to remove excess EtBr. Gels were then illuminated again at 302 nm and the bands were detected at 590 nm (EtBr filter) to determine the presence of nucleic acids.

The products of the coupled cell-free transcription/translation reaction were loaded on 12% or 15% SDS-polyacrylamide gels. Protein bands were visualized through Coomassie Blue or silver nitrate staining. To detect the fluorescent bands, the gels were illuminated at 302 nm and the bands were visualized by scanning at 533 nm (fluorescein filter). When silver staining was used, the gel was scanned for fluorescence before staining. For general detection of protein bands, after staining the gels were exposed to white light and scanned.
7.3. Results

7.3.1. General procedure for aminoacylation of tRNAs

A typical aminoacylation reaction mixture consisted of tRNA (yeast tRNA\textsubscript{Phe}, \textit{E. coli} tRNA\textsubscript{Val} or \textit{E. coli} tRNA\textsuperscript{Met}) or the corresponding oxidized tRNA (oxtRNA), DNS- aminoacyl ethyl phosphate (DNSLysEP, DNSGlyEP or DNPheEP), La(OTf)\textsubscript{3} and EPPS buffer pH 8 in equimolar concentrations (~10\textsuperscript{−5} M), and MgCl\textsubscript{2} in excess (usually 10\textsuperscript{−3} M). The reaction was performed in dark at room temperature for 1 hr. After 1 hr, the tRNA/oxtRNA and DNS-aminoacyl-tRNA were isolated using a G-25 spin-column. This treatment is necessary for further use of the DNS-aminoacyl-tRNA, as it will remove any unreacted DNS-aminoacyl phosphate or hydrolysis product, the DNS-amino acid. If this step is not performed, no fluorometric studies can be done as the free DNS-amino acid or its corresponding ethyl phosphate ester, have more intense emission and quench the signal of the aminoacylated tRNA. This treatment removes Mg\textsuperscript{2+} and La\textsuperscript{3+}, as well and prevents possible metal catalyzed hydrolysis of the aminoacyl-tRNA.

7.3.2. Anti-DNS Ab fluorometric detection of modified tRNA

Yeast tRNA\textsubscript{Phe} and \textit{E. coli} tRNA\textsubscript{Val} were aminoacylated using DNSGlyEP and La(OTf)\textsubscript{3}, and the products were subjected to fluorometric studies. The property of the anti-DNS Ab to increase the intensity of the emission peak of a DNS-product was used in these studies.

Individual fluorescence spectra of DNSGlyEP, La(OTf)\textsubscript{3}, tRNAs, and buffer, both with and without the anti-DNS Ab were acquired before the measurements of the reaction products were performed. All starting solutions were tested with the same amount of antibody (5 ul). The antibody itself is not fluorescent. La(OTf)\textsubscript{3} and tRNA did not show change in the signal upon addition of the antibody.

When 5 µl of the antibody were added to a 100 µM solution of DNSGlyEP, an increase of 12.7% was observed (Figure 29). Upon addition of the antibody, the peak maximum shifted to the left from 539 nm to 526 nm.
After completion of the acylation reactions, the products were eluted through G-25 spin-columns and the fluorescence intensities of the resulting solutions were measured. 200 μl of the tRNA-reaction solution were transferred into a cuvette and topped up to 400 μl with nuclease-free water. The reaction mixture for the aminoacylation of yeast tRNA<sub>Phe</sub> consisted of tRNA<sub>Phe</sub>, DNGlyEP and La(OTf)<sub>3</sub> in equivalent molar concentrations (2.47x10<sup>-5</sup> M), excess MgCl<sub>2</sub> (42 mM), and 0.25 mM EPPS pH 8.

The observed maximum for both curves with and without antibody was around 441 nm (Figure 30). Free yeast tRNA<sub>Phe</sub> is fluorescent due to the presence of the fluorescent Y (wybutine) base and show the same emission maximum. If the intensity of the emission is compared at 488 nm, which is closer to the emission of DNGlyEP and DNGly, an increase of 26% after the addition of the antibody (10 μl) to the solution was observed.

**Figure 29:** Emission spectra of DNGlyEP in the presence (red) and absence (blue) of anti-DNS antibody
**Figure 30:** Emission spectrum of the purified products of aminoacylation of yeast tRNA$^{Phe}$ with DNSGlyEP in the presence of La$^{3+}$ at pH 8 in the absence (blue) and presence (red) of anti-DNS Ab

**Figure 31:** Emission spectra of tRNA$^{Phe}$ in the presence (red) and in the absence (blue) of the antibody
The emission spectra of tRNA^{Phe} solutions with and without anti-DNS Ab were obtained to determine if the antibody shows affinity towards tRNA alone (Figure 31). No change was observed.

The same procedure was followed with oxtRNA^{Phe}. The concentrations of the components of the reaction mixture were the same as for the aminoacylation of tRNA^{Phe}, the only difference being the oxidized form of the nucleic acid. The resulting emission spectrum is shown in Figure 32. The maximum was at 430 nm and the intensity of the signal at 488 nm increased by 8% upon addition of the antibody. The antibody did not show specificity towards oxtRNA^{Phe} alone (Figure 33).

**Figure 32:** Emission spectra of the purified products of aminoacylation of oxidized yeast tRNA^{Phe} with DNSGlyEP in presence of La^{3+} at pH 8 in the absence (blue) and presence (red) of the anti-DNS antibody
To test the reproducibility of this method, we subjected tRNA$^{\text{Val}}$ from *E. coli* and its oxidized analogue to aminoacylation. The concentrations used were: tRNA$^{\text{Val}}$ /ox tRNA$^{\text{Val}}$, DNSGlyEP and La(OTf)$_3$ in equimolar concentrations (2.5x10$^{-5}$ M), excess MgCl$_2$ (10 mM), and 0.25 mM EPPS pH 8.

The fluorescence emission spectra of the product from the acylation of tRNA$^{\text{Val}}$, with and without the antibody are shown in Figure 34. The volume of antibody solution used was 10 μl. The $\lambda_{\text{em}}$ max for the products of the aminoacylation of tRNA$^{\text{Val}}$ shifted from 505 nm to 501 nm upon addition of the antibody. The enhancement of the intensity at 501 nm was 28%. $\lambda_{\text{em}}$ max of the products from acylation of oxtRNA$^{\text{Val}}$ with and without the antibody were 528 and 523 nm, respectively, and an 8% increase in the intensity of the signal was observed at 523 nm (Figure 35).
Figure 34: Emission spectra of the purified products of the aminoacylation reaction of tRNA\textsuperscript{Val} in the absence (blue) and presence (red) of anti-DNS Ab

Figure 35: Emission spectra of the purified products of the aminoacylation reaction oxtRNA\textsuperscript{Val} in the absence (blue) and presence (red) of anti-DNS Ab
7.3.3. PAGE analysis of the products of aminoacylation of tRNA

tRNA\textsuperscript{\text{fMet}} from \textit{E. coli} was aminoacylated with ε-DNS-α-Boc-lysine. Aliquots of the reaction mixture were taken at 0, 30 and 60 min and passed through a G-25 spin-column. Nucleic acids were precipitated with cold ethanol and centrifuged for 30 min at 13 000 rpm. The supernatant was removed, and the white pellets of tRNA/aminoacyl-tRNA were air-dried. The samples were mixed with loading buffer and the presence of nucleic acid and fluorescent label were analyzed on 10%/8M urea/TBE polyacrylamide gels.

Before staining with EtBr, the gel was subjected to UV light for detection of the DNS-labeled molecules. However, DNS is a rather weak fluorophore and it is difficult to detect a strong signal. After staining with EtBr, an increase in the intensity of the fluorescence signal was observed from 0 to 60 minutes reaction time (Figure 36).

![Figure 36](image)

**Figure 36:** 10%/8M urea/TBE polyacrylamide gel, stained with EtBr, of the products from aminoacylation of \textit{E. coli} tRNA\textsuperscript{\text{fMet}} with DNSLysEP at 0, 30 and 60 min of mixing. Two aliquots were loaded of each sample with the first one being lower amount.

The acylation of yeast tRNA\textsuperscript{\text{Phe}} with 1 and 4 equivalents of the aminoacylating reagent in the presence of La\textsuperscript{3+} was also studied (Figure 37). The intensity of the signals in both reactions appears to be very similar. This suggested that the presence of higher amounts of the aminoacylating reagent in the reaction mixture did not lead to multiple incorporations of the DNS-amino acid.

As a control, tRNA\textsuperscript{\text{Phe}} was incubated with one equivalent of La\textsuperscript{3+} for one hour at room temperature. After isolation and drying, the product from this reaction was loaded onto a
gel to detect any hydrolysis products. The band migration was similar to that of tRNA\textsuperscript{Phe} and the aminoacylated product.

**Figure 37:** 10%/8M urea/TBE PAGE of the products from aminoacylation of yeast tRNA\textsuperscript{Phe} with 1 and 4 equivalents of DNSLysEP (Fluorescence (A); EtBr stained (B)). Two aliquots were loaded of each sample with the first one being lower amount.

7.3.4. **Attempts at incorporation of DNS-amino acids via *in vitro* transcription/translation**

The EcoPro T7 System is a cell-free coupled transcription/translation system derived from *E. coli*, using S30 extract from *E. coli*. The system consists of EcoPro T7 extract which contains all of the components required for coupled transcription/translation reaction except for methionine, DNA and water, and 5 mM methionine. It functions with DNA templates that contain T7 or *E. coli* promoters. Several plasmids coding for proteins of different molecular weight were tested (S-Tag β-glucuronidase – 73.5 kDa, Human Carbonic Anhydrase (HCA) – 30 kDa, NikR – 15 kDa and GCN4 – 7 kDa).
DNS-aminoacyl tRNA was prepared immediately before the \textit{in vitro} reaction, purified through a G-25 spin column, precipitated with 3 volumes of cold ethanol and dried. Just before using it in the \textit{in vitro} reaction, it was dissolved in nuclease-free water. Fractions containing up to 150 pmoles of aminoacyl-tRNA were used in the \textit{in vitro} protein synthesis. In some cases, higher concentrations were used in an attempt to limit the competition with the endogenous Met-tRNA$^{\text{fMet}}$.

A typical reaction mixture (10 μl) contained 3.5 μl T7 extract, 150 pmoles of DNS-tRNA$^{\text{fMet}}$, and 0.2 μg of the plasmid DNA. Control reactions were performed in which DNS-tRNA$^{\text{fMet}}$ was replaced with methionine, or DNA replaced with water. In all cases, the T7 extract was added last and the reaction mixtures were incubated at 37°C for 1 hour. After completion, the reaction vials were placed on ice and the proteins were precipitated with 5 volumes of cold acetone. They were collected by centrifugation at 13 000 rpm for 30 min. The supernatant was decanted and the proteins were dried. The protein pellets were then dissolved in water and equivalent amount of 2x loading buffer was added, or the pellets were directly dissolved in loading buffer. The samples were then boiled for 2 minutes and loaded onto the SDS-gel (12 or 15%).

When the DNA for S-Tag β-glucuronidase was used, no fluorescent bands with the expected 73.5 kDa molecular weight could be detected (Figure 38). Two types of \textit{E. coli} tRNA were tested – tRNA$^{\text{fMet}}$ and tRNA$^{\text{Val}}$, both acylated with the same amino acid. If incorporation is successful, these two reactions should give proteins with different molecular weight.

Three translation reactions were compared: with methionine (Lane 1), DNSLys-tRNA$^{\text{Val}}$ (Lane 2)’ and DNSLys-tRNA$^{\text{fMet}}$ (Lane 3). After staining with Coomassie blue, the gel was irradiated at 302 nm for detection of fluorescent (DNS-labeled) compounds. As expected, no such bands were observed in Lane 1. Both Lane 2 and 3 showed a fluorescent product with molecular weight around 31 kDa. However, no protein bands were detected when the gel was visualized with white light. These bands correspond to the DNS-aminoacyl tRNA’s used in the reactions.

The protein band was not readily detected (even in the control reaction, lane 1) because the lysate was still present and showed multiple large molecular weight protein bands.
Figure 38: 12% SDS PAGE: *In vitro* transcription/translation of S-Tag β-glucuronidase. Lane 1: control: DNA, Met, T7 extract; 2: DNS-Lys-tRNA$^{Val}$, DNA, T7 extract; 3: DNS-Lys-tRNA$^{fMet}$, DNA, T7 extract. S-Tag β-glucuronidase has a molecular weight of 73.5 kDa.

Phenol/chloroform extraction was attempted in order to separate the proteins from the nucleic acids. The isolated proteins, however, could not be dissolved properly to be analyzed by gel electrophoresis or by fluorescence spectroscopy. Cell-free synthesis of larger proteins labeled with fluorophores is somehow problematic. Thus, DNA plasmids for several smaller proteins were used in the further reactions.

Figure 39: 12% SDS PAGE: *In vitro* transcription/translation of NikR. (1) Reaction: DNA, T7 extract; DNSGly-tRNA$^{Met}$; (2) yeast tRNA$^{Phe}$. The expected molecular weight of NikR is 15 kDa.
The results obtained when DNA plasmids for HCA (30 kDa) and NikR (15 kDa) (Figure 39) were used, were similar to the previous example with S-Tag β-glucuronidase. In most of the cases the aminoacyl-tRNA was still present in the mixture.

Western blotting was also attempted as an alternative method to analyze the proteins synthesized via cell-free translation. The standard procedure provided by Bio-Rad was followed. The rabbit anti-DNS Ab, used in the fluorescence spectroscopy studies, was used as the primary antibody, while goat anti-rabbit antibody was the secondary antibody. After protein transfer, the membrane was developed and it was found that some of the lysate proteins were also recognized by the antibody (primary or secondary). This was observed with both HCA and S-Tag β-glucuronidase. Thus, this method was abandoned as a possible means of detecting incorporation of DNS-amino acids until a monoclonal anti-DNS Ab could be found.

Finally, in an *in vitro* transcription/translation reaction, the plasmid for GCN4, a protein with a molecular weight of 7 kDa, was used. The products of the reaction were separated on a 15% SDS polyacrylamide gel (Figure 40).

**Figure 40:** 15% SDS PAGE *In vitro* transcription/translation of GCN4. Lane 1-Ladder; 2-Reaction 1: Met, DNSPhe-tRNA<sup>Met</sup> (150 pmoles), DNA for GCN4 (200 ng), T7 extract; 3-Reaction 2: DNSPhe-tRNA<sup>Met</sup> (150 pmoles), DNA for GCN4 (200 ng), T7 extract; 4-Reaction 3: Met, DNA for GCN4 (200 ng), T7 extract; 5: DNSPhe-tRNA<sup>Met</sup> (150 pmoles).

After exposure at 302 nm, one fluorescent band was observed in lane 3 (reaction 2), with a molecular weight close to that expected for GCN4. The protein could not be detected on the gel stained with Coomassie blue after irradiating with white light.
As a more sensitive method for detection, silver staining was used. However, due to the presence of nucleic acids in the mixtures, isolated after the completion of the translation/transcription reaction, multiple bands were detected. Before staining, fluorescence bands were detected corresponding to the present DNSPhe-tRNA used in the reaction.

7.4. Discussion

aaRSs charge tRNAs only at the 3’-terminal adenosine. Several methods exist that allow for aminoacylation of tRNAs with non-natural amino acids and their further incorporation into proteins. Our approach, a direct aminoacylation of tRNA, relies on a specific recognition of the 3’-terminal diol by La$^{3+}$. The metal ion coordinates the diol and the aminoacylating reagent simultaneously to promote an intramolecular nucleophilic reaction. Protection of the functional groups on the nucleotide/nucleic acid is not necessary because La$^{3+}$ does not react with them (see Chapter 4).

Due to the very low concentration of nucleic acid used in these experiments, a detection method more sensitive than HPLC with UV detection or NMR must be used. Nucleic acids modified with a fluorescent labels are often used in studies like this. They allow for easy and fast determination of the reaction outcome.

Dansylated L-amino acids are commercially available and they are easily transformed into their aminoacyl ethyl phosphates. The presence of the fluorophore allows us to study the aminoacylation of tRNA by fluorescence spectroscopy as well as to detect the fluorescent products on polyacrylamide gels (urea or SDS).

PAGE analysis of the reaction products showed that tRNAs were successfully modified with the DNS-aminoacyl moiety. The products of reactions with longer reaction time showed stronger fluorescent bands. This result determined the reaction time to be 1 hour.

Increasing the amount of the aminoacylating reagent to four times more than the amount of the tRNA did not show significant improvement in yield.
7.4.1. Acylation occurs mostly at the 3’-terminal diol

To determine whether or not the reaction occurs at the desired site, the 3’-terminal adenosine, we oxidized tRNA\textsuperscript{Phe} and tRNA\textsuperscript{Val} with NaIO\textsubscript{4}. This treatment converts the diol into a dialdehyde. Coordination of the metal ion to the dialdehyde does not lead to aminoacylation. The products of the aminoacylation of tRNAs and ox tRNAs with DNS-aminoacyl phosphates were studied by fluorescence spectroscopy.

An important component of this study was the anti-DNS Ab, which enhances the fluorescence of the DNS-group upon binding. This was used to determine the presence of a DNS-group on the tRNAs after reaction. Both tRNA\textsuperscript{Phe} and tRNA\textsuperscript{Val} showed an increase in the signal after treatment with the antibody of 26 and 28\%, respectively (Figures 30 and 34). A small increase of 8\% was observed in the fluorescence spectra of the ox-tRNAs (Figures 32 and 35). The large difference in the fluorescence increase of the tRNA-reaction product over the ox-tRNA-reaction product, suggested that the modification is primarily at the 3’-terminal adenosine. The fact that enhancement was detected in the products of reactions of ox-tRNAs could be due to aminoacylation occurring somewhere else on the phosphodiester backbone of tRNA, or low capacity of the G-25 column. When a solution of only DNSGlyEP was subjected to treatment through a G-25 column, some fluorescence was detected in the eluting solution.

Enzymatic hydrolysis of the ox-tRNA followed by mass spectrometry would yield information as to where the modification occurred. Our attempts to analyze the prepared aminoacyl-tRNAs by MALDI MS failed due to insufficient ionization.

7.4.2. Low efficiency of DNSPhe-tRNA\textsuperscript{fMet} to initiate cell-free protein synthesis

The efficiency of incorporating biomimetically charged aminoacyl-tRNAs in a cell-free protein synthesis was tested. When N-terminal labeling of a given protein is necessary, the preferred approach is an enzymatic aminoacylation of tRNA\textsuperscript{fMet} with methionine and subsequent modification on the methionine amino group.\textsuperscript{29-31} There is an advantage in using label-Met-tRNA\textsuperscript{fMet} in an \textit{in vitro} translational system over other aminoacyl-tRNAs. To be selected from the pool of aminoacyl-tRNAs, the initiator tRNA must carry an amino acid with a blocked amino group. Studies showed that the
requirement for the methionine side chain is not specific and amino acids with shorter or longer side chains were successfully incorporated instead of fMet.

We used a coupled in vitro transcription/translation system from *E. coli*, which includes all required components for both processes. *E. coli* tRNA\textsuperscript{fMet} was charged with DNS-amino acids (DNSLys, DNSGly and DNSPhe). After isolation, DNS-aminoacyl-tRNA was used in the cell-free protein synthesis system. The products were analyzed by SDS PAGE. It was observed that the biomimetically acylated tRNA\textsuperscript{fMet} was not used up in the process (Figures 38 and 39) when larger proteins (MW of 72.5, 30 or 15 kDa) were synthesized. Fluorescent bands corresponding to the DNS-tRNA were still present. However, when the products from the synthesis of GCN4 were analyzed, a fluorescent band with molecular weight 7 kDa was detected (Figure 40, lane 3). No fluorescence from the DNSPhe-tRNA\textsuperscript{fMet} was detected on the gel (Figure 40, lane 4) probably due to the low concentration. Detection of the synthesized protein was problematic due to the presence of the lysate proteins in the samples.

The cell-free systems contain all necessary components for the translation reaction, including mixture of all amino acids, tRNAs and aaRS. Thus, the DNS-aminoacyl tRNA\textsuperscript{fMet} faces not only strong competition from the natural Met-tRNA\textsuperscript{fMet}, but also possible enzymatic hydrolysis. Generating an orthogonal tRNA/aaRS pair would solve this problem. The easier approach, however, would be to use a fluorophore with emission at longer wavelengths and with higher quantum yield. This would allow for detection of even very low concentrations of produced protein.

Western blotting was an alternative approach that we attempted to determine the presence of N-DNS-labeled protein. However, as the rabbit anti-DNS antibody is a polyclonal antibody, it recognized not only the DNS-group but also functionalities on the lysate proteins. This very sensitive method would be useful if a monoclonal anti-DNS antibody were available.

### 7.5. Conclusions and future directions

The data obtained from PAGE and fluorescence spectroscopy analysis suggested that the La\textsuperscript{3+}-mediated aminoacylation of tRNAs with DNS-amino acids was successful.
Moreover, studies of several oxtRNAs proved that the major site of reaction is the 3’-terminal adenosine. However, the fluorescence studies could report how much of the tRNA was acylated. Aminoacyl-tRNA can be isolated through interaction with immobilized EF-Tu-GTP complex. Such a treatment will separate tRNA from its aminoacyl products. This method could be used to quantify the aminoacylation of tRNA.

The aminoacyl-tRNA generated via biomimetic aminoacylation was applied in a cell-free protein synthesis system. The results show that more experiments are necessary before DNS-amino acids are successfully incorporated in proteins. Optimization of the conditions or concentrations might be helpful. However, alternative methods may be necessary to conclusively prove the incorporation of the labeled amino acids. For example, utilization of radiolabeled amino acids will allow for detection even at very low levels of incorporation. Amino acids carrying biotin can also be used as an alternative and upon its reaction with avidin, or avidin analog, the protein can be isolated. Incorporation of amino acids labeled with fluorescent groups or a functionality against which a monoclonal antibody was raised can be tested with Western blotting.

Stop-codon suppression, however, will give the most definite answer. Through site-directed mutagenesis, a stop-codon could be inserted at a specific position on the plasmid DNA and a tRNA with a corresponding anticodon could be generated. This tRNA could then be charged with the fluorescent amino acid through our biomimetic approach. The resulting aminoacyl-tRNA could then be utilized in an *in vitro* protein synthesis system. Analysis of the protein products would then be able to answer whether or not the biomimetically generated aminoacyl-tRNAs are able to transfer the amino acid.
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