Synthesis of Tritium Labeled Queuine, PreQ₁ and Related Azide Probes Toward Examining the Prevalence of Queuine

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Medicinal Chemistry) in the University of Michigan 2012

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In memory of Edward and Lillian Pederson
Acknowledgements

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As trite as such sentiments must seem to those forced to read them, my most heartfelt thanks must go to Dr. Stephanie Brooks for her patience these last five years and three months. It appears the moment has passed into memory and we all best move on, but here this record of what transpired will remain.
# Table of Contents

Dedication.................................................................................................................................ii
Acknowledgements.....................................................................................................................iii
List of Figures.............................................................................................................................vi
List of Schemes............................................................................................................................ix
List of Tables...............................................................................................................................x
List of Appendices.........................................................................................................................xi
Abstract.........................................................................................................................................xii

## Chapters

I. Queuine.......................................................................................................................................1
II. Synthesis of Queuine and PreQ\textsubscript{1}...........................................................................41
III. Investigating the Prevalence of Queuine in *Escherichia coli* RNA via Incorporation of the Tritium-labeled Precursor, PreQ\textsubscript{1}.........................66
IV. Synthesis of Azide Probes and other PreQ\textsubscript{1} analogues.................................93
V. Evaluation of Azide Probes.........................................................................................113
VI. Conclusions.........................................................................................................................132

## Appendices

........................................................................................................................................137

## Bibliography

........................................................................................................................................175
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1:</td>
<td>Structures of queuine, queuosine, preQ₁ and preQ₀</td>
<td>2</td>
</tr>
<tr>
<td>1-2:</td>
<td>Queuine biosynthetic pathways for eubacteria</td>
<td>10</td>
</tr>
<tr>
<td>1-3:</td>
<td>Structure of eubacterial and eukaryal TGT</td>
<td>15</td>
</tr>
<tr>
<td>1-4:</td>
<td>Pemetrexed</td>
<td>25</td>
</tr>
<tr>
<td>2:1:</td>
<td>Analysis of radio-labeled substrates, queuine and preQ₁</td>
<td>49</td>
</tr>
<tr>
<td>3-1:</td>
<td>Queuine biosynthetic pathways for eubacteria and eukarya</td>
<td>67</td>
</tr>
<tr>
<td>3-2:</td>
<td>Example agarose gel of total RNA preparation</td>
<td>69</td>
</tr>
<tr>
<td>3-3:</td>
<td>Total RNA isolated from TG2 and ΔqueC, in vitro</td>
<td>70</td>
</tr>
<tr>
<td>3-4:</td>
<td>Ribosomal RNA isolated from TG2 and ΔqueC, in vitro</td>
<td>72</td>
</tr>
<tr>
<td>3-5:</td>
<td>In vivo incubation of Δtgt</td>
<td>73</td>
</tr>
<tr>
<td>3-6:</td>
<td>total RNA isolated from TG2 and ΔqueC, in vivo</td>
<td>74</td>
</tr>
<tr>
<td>3-7:</td>
<td>Ribosomal RNA from TG2 and ΔqueC, in vivo</td>
<td>75</td>
</tr>
<tr>
<td>3-8:</td>
<td>5’-phosphate-dependent exonuclease treated total RNA</td>
<td>76</td>
</tr>
<tr>
<td>3-9:</td>
<td>PAGE of total RNA isolated from ΔqueC, in vivo</td>
<td>77</td>
</tr>
<tr>
<td>3-10:</td>
<td>Phosphor storage screen image of total RNA blot</td>
<td>78</td>
</tr>
<tr>
<td>3-11:</td>
<td>Phosphor storage screen image of rRNA blot</td>
<td>79</td>
</tr>
<tr>
<td>3-12:</td>
<td>Scintillation of blot of PAGE of isolated total RNA</td>
<td>79</td>
</tr>
<tr>
<td>3-13:</td>
<td>Scintillation of blot of PAGE of isolated rRNA</td>
<td>80</td>
</tr>
<tr>
<td>3-14:</td>
<td>Example gel and radioactivity of ribosomal RNA</td>
<td>84</td>
</tr>
<tr>
<td>4-1:</td>
<td>Structure of azide probes for evaluation as TGT substrates</td>
<td>94</td>
</tr>
<tr>
<td>4-2:</td>
<td>Identification of RNA by Randomized Probe Library</td>
<td>96</td>
</tr>
<tr>
<td>5-1:</td>
<td>Inhibition of Guanine Incorporation by Azide probe 18</td>
<td>119</td>
</tr>
<tr>
<td>5-2:</td>
<td>Inhibition of Guanine Incorporation by Azide Probe 19</td>
<td>120</td>
</tr>
<tr>
<td>5-3:</td>
<td>Dixon plots for each azide probe</td>
<td>121</td>
</tr>
</tbody>
</table>
5-4: Equation used to calculate the $K_i$ of a competitive inhibitor..............123
5-5: Test of azide analogues of preQ$_1$ as substrates for TGT...............124
5-6: Dibenzylcyclooctyne-PEG4-Biotin Conjugate Reagent.........................125
I-1: $^1$H NMR of 4.............................................................................138
I-2: $^{13}$C NMR of 4.............................................................................139
I-3: $^1$H NMR of 5...............................................................................140
I-4: $^{13}$C NMR of 5...............................................................................141
I-5: $^1$H NMR of 6...............................................................................142
I-6: $^{13}$C NMR of 6...............................................................................143
I-7: $^1$H NMR of 10...............................................................................144
I-8: $^1$H NMR of Queuine, 1.................................................................145
I-9: $^{13}$C NMR of Queuine, 1.................................................................146
I-10: $^{19}$F NMR of Mosher amide of racemic mixture of queuine side chain........147
I-11: $^{19}$F NMR of Mosher amide of queuine side chain..........................148
I-12: $^1$H NMR of 16.................................................................149
I-13: $^1$H NMR D$_2$O wash of 16..........................................................150
I-14: $^{13}$C NMR of 16.............................................................................151
I-15: $^1$H NMR of preQ$_1$, 17..............................................................152
I-16: $^1$H NMR D$_2$O wash preQ$_1$, of 17.................................153
I-17: $^{13}$C NMR of preQ$_1$, 17..............................................................154
I-18: DEPT 135 of preQ$_1$, 17..............................................................155
I-19: Product Data Sheet for $^3$H-labeled preQ$_1$ from Moravek................156
I-20: Product Data Sheet for $^3$H-labeled Queuine from Moravek.............158
I-21: $^1$H NMR of 20.............................................................................161
I-22: $^1$H NMR of 21.............................................................................162
I-23: $^1$H NMR of 18.............................................................................163
I-24: $^{13}$C NMR of 18.............................................................................164
I-25: High resolution mass sepectrometry spectra of 18.........................165
I-26: $^1$H NMR of 29.............................................................................166
I-27: $^1$H NMR of 19.................................................................167
I-28: $^{13}$C NMR of 19.................................................................168
I-29: High resolution mass spectrometry spectra of 19........................................169
II-1: Preliminary evaluation of aldehyde 6..............................................................171
II-2: Inactivation study of aldehyde 6.................................................................173
### List of Schemes

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1:</td>
<td>The synthesis of queuine by Goto</td>
<td>22</td>
</tr>
<tr>
<td>1-2:</td>
<td>The synthesis of queuine by Akimoto</td>
<td>24</td>
</tr>
<tr>
<td>1-3:</td>
<td>The synthesis of queuine completed by Barnett and Grubb</td>
<td>25</td>
</tr>
<tr>
<td>1-4:</td>
<td>Queuosine synthesis completed by Carell</td>
<td>27</td>
</tr>
<tr>
<td>1-5:</td>
<td>Queuine side chain synthesis completed by Ovaa</td>
<td>31</td>
</tr>
<tr>
<td>1-6:</td>
<td>Synthesis of queuine side chain by Trost and Sorum</td>
<td>33</td>
</tr>
<tr>
<td>1-7:</td>
<td>Synthesis of queuine side chain by Kim and Miller</td>
<td>34</td>
</tr>
<tr>
<td>2-1:</td>
<td>Elaboration of preQ₀ to key aldehyde</td>
<td>43</td>
</tr>
<tr>
<td>2-2:</td>
<td>Synthesis of protected queuine side chain</td>
<td>45</td>
</tr>
<tr>
<td>2-3:</td>
<td>Synthesis of Mosher amide of protected queuine side chain</td>
<td>46</td>
</tr>
<tr>
<td>2-4:</td>
<td>Reductive amination and deprotection to produce queuine</td>
<td>47</td>
</tr>
<tr>
<td>2-5:</td>
<td>Reductive amination and deprotection to produce preQ₁</td>
<td>48</td>
</tr>
<tr>
<td>4-1:</td>
<td>Attempted routes to generate preQ₁ azide analogue</td>
<td>97</td>
</tr>
<tr>
<td>4-2:</td>
<td>Synthesis of preQ₁ azide analogue</td>
<td>98</td>
</tr>
<tr>
<td>4-3:</td>
<td>Synthesis of alternate azide probe based on preQ₁</td>
<td>100</td>
</tr>
<tr>
<td>4-4:</td>
<td>Synthesis of azide probe without methylene spacer</td>
<td>101</td>
</tr>
<tr>
<td>4-5:</td>
<td>Staudinger ligation, synthesis and interaction with azide probes</td>
<td>103</td>
</tr>
<tr>
<td>4-6:</td>
<td>Synthetic routes of potential TGT inactivators</td>
<td>104</td>
</tr>
</tbody>
</table>
### List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1:</td>
<td>Stimulation of the binding of $^{14}$C-aminoacyl-tRNAs containing Q to ribosomes</td>
<td>4</td>
</tr>
<tr>
<td>1-2:</td>
<td>Queuine content from natural products</td>
<td>7</td>
</tr>
<tr>
<td>5-1:</td>
<td>Pre-incubation with Azide 18</td>
<td>117</td>
</tr>
</tbody>
</table>
List of Appendices

Appendix

I: Spectral and Other Characterization Data for Chapter II and IV............137
II: Chapter V, Kinetic Evaluation of Aldehyde 6...........................................170
Abstract

Queuine is a modified nucleotide known to occur in the anticodon of four tRNAs. The queuine modification occurs across all eukaryotes and eubacteria with few exceptions, but its function remains unclear. Prior in vitro work demonstrated that the modification can be incorporated into RNA species other than presently known tRNAs. Queuine is unusual in that, unlike the majority of modified nucleotides that result from changes to genetically encoded bases, it is incorporated into RNA by transglycosylation. Base modification by transglycosylation is unusual and represents an interesting point of entry for study. Due to this method of incorporation the modification can be studied with small molecule probes. Tritium-labeled queuine and preQ₁ were prepared to study the differences between the eukaryotic and eubacterial version of the enzyme responsible for the incorporation of the modified base, tRNA guanine transglycosylase. A concise, convergent synthesis of queuine was developed that is the shortest known route to date.

PreQ₁, the precursor to queuine incorporated by eubacteria, was used to investigate the prevalence of base modification in E. coli. Three cell lines were utilized to conduct the in vivo experiments of this study: a ΔqueC knockout of E. coli that is unable to synthesize preQ₁ so that tritium labeled compound will be incorporated exclusively, a Δtgt knockout strain of E. coli that is unable to incorporate preQ₁ and a
wild-type *E. coli* strain. As the modified nucleotide occurs through incorporation of a specially synthesized nucleotide, a study of the sites of modification by prepared probes is possible. The syntheses of two novel azide congeners were undertaken for this purpose. The evaluation of their interaction with tRNA guanine transglycosylase was undertaken to determine if they are substrates of the enzyme.

The tritium labeled preQ₁ allowed for a general evaluation of the prevalence. We determined that the four known tRNAs are the main site of incorporation *in vivo*, while many other RNAs are substrates *in vitro*. The azide probes were generated and were shown to interact with *E. coli* TGT, but not as substrates. In summary, we have gained a better understanding of queuine modification of RNAs and have developed tools that will aide in future studies.
Chapter I

Queuine

Introduction

Queuine is one of approximately one hundred modified nucleotides found in RNA.\(^1\) Since its discovery many details of the modification have been described and its potential role examined. Unlike the majority of modified nucleotides that result from modifications of the genetically encoded nucleotides, queuine is incorporated into RNA by transglycosylation.\(^2\) Eukaryae incorporate queuine obtained through their diet; whereas, eubacteria biosynthesize and incorporate the queuine precursor preQ\(_1\) before completing the modification on the RNA to yield queuine, structure given in Figure 1-1. As a result of these differences, the enzyme responsible for the incorporation of the modification into RNA, tRNA guanine transglycosylase (TGT), differs between eukaryal and eubacterial organisms\(^3\). Recently, the pathway for the synthesis of queuine was fully elucidated with all the enzymes involved identified, seen in Figure 1-2. Presently, the queuine modification is known to be present in the wobble position of the anticodon loop of tRNA\(^{\text{Tyr}}\), tRNA\(^{\text{His}}\), tRNA\(^{\text{Asp}}\), and tRNA\(^{\text{Asn}}\).\(^4\) Investigations have determined that its presence has an effect on codon recognition and has been implicated in promoting translational fidelity.
Figure 1-1: Structures of queuine, queuosine, preQ₁ and preQ₀

**Discovery of queuine**

The discovery of queuine dates to early work performed in the late 1960’s to elucidate the sequence and structure of tRNA in various species. Experiments performed to sequence yeast tRNAs⁵ and *E. coli* (Phe)⁶ had demonstrated that Crick’s wobble theory⁷, with the anti-codon recognizing redundant codons (U versus C ending), was valid. The first position of the anticodon, the wobble position, in those tRNAs was occupied by either guanosine or 2’-O-methylguanosine. When similar studies were undertaken to determine the sequence of *E. coli* tRNA<sup>tyr</sup>, it was discovered that the first position of the anticodon was occupied by a different modified guanosine not previously observed⁸. The new base of unknown structure was denoted as G* by Goodman⁸b, c, R by Doctor⁸a, and Q by RajBhandary⁸d.

The studies all employed a standard methodology to examine the sequence of tRNA. The desired tRNA was purified from other RNAs by combinations of column
chromatography procedures using DEAE-cellulose and DEAE-Sephadex. The tRNA was then digested with bovine pancreatic ribonuclease and ribonuclease T₁. Subsequent purification of the fragments allowed for their identification and when compared to products from limited hydrolysis with RNase T₁, resulting overlapping fragments provided a means to determine the overall sequence. Two dimensional electrophoretic separations of the digested nucleotides allowed for their determination and the identification of the previously unknown bases. The investigators postulated that the modification of the wobble position of the anticodon to Q must have a specific function in the decoding process⁴.

The discovery of Q, queuine, led Harada and Nishimura to examine all the other tRNAs from *E. coli* for the presence of Q⁴. They found that queuine was also present in the wobble position of three other *E. coli* tRNAs: His, Asn, Asp. The identity of the base as queuine was confirmed through two dimensional chromatography, separation retention times with DEAE-Sephadex A-25, and UV absorption spectra. The four tRNAs in which queuine was discovered are all tRNAs with redundant codons whose sole variation is that of a U or C in the third position, bases to which the wobble position binds. The authors inferred from this finding that queuine modification played a role in codon recognition, and decided to study the stimulation of binding for queuine modified [¹⁴C]aminoacyl-tRNAs to their codons.

The authors studied queuine modified aminoacyl-tRNAs without Q (− Q triplet) and with Q (+ Q triplet), including [¹⁴C]phenylalanyl-tRNA_<sub>Phe</sub> as a control for their study. The results, summarized in Table 1-1, demonstrate that when G is present there is no
codon preference; however, when Q is present in the wobble position U ending codons 
bind with a greater affinity. The results also indicate a general increase in binding when 
queueine is present. From these findings the investigators reasoned that one role of 
queueine could be to increase efficiency of protein synthesis for genes with certain 
codons and to prevent miscoding, which might occur with G. This latter conjecture 
stemmed from the authors' previous work where they demonstrated that when 
magnesium ion concentrations were raised in vitro translation errors could occur, 
showing the possible need for greater affinity under certain conditions to ensure proper 
code reading. This work also showed that all of the substrate tRNAs for the modification 
contained a U-G-U sequence in the anticodon loop, a fact that would later be 
determined to be important for recognition by the enzyme responsible for the 
modification2.

<table>
<thead>
<tr>
<th>Aminoacyl-tRNA</th>
<th>Triplet</th>
<th>- Q Triplet</th>
<th>+Q Triplet</th>
</tr>
</thead>
<tbody>
<tr>
<td>[14C]Tyrosyl-tRNA₅₃yr</td>
<td>U-A-U</td>
<td>0.11</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>U-A-C</td>
<td>0.11</td>
<td>0.24</td>
</tr>
<tr>
<td>[14C]Histidyl-tRNA₃₃his</td>
<td>C-A-U</td>
<td>0.07</td>
<td>1.22</td>
</tr>
<tr>
<td></td>
<td>C-A-C</td>
<td>0.07</td>
<td>0.78</td>
</tr>
<tr>
<td>[14C]Asparaginyl-tRNA₃₃asn</td>
<td>A-A-U</td>
<td>0.08</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>A-A-C</td>
<td>0.08</td>
<td>0.64</td>
</tr>
<tr>
<td>[14C]Aspartyl-tRNA₃₃asp</td>
<td>G-A-U</td>
<td>0.06</td>
<td>2.86</td>
</tr>
<tr>
<td></td>
<td>G-A-C</td>
<td>0.06</td>
<td>0.85</td>
</tr>
<tr>
<td>[14C]Phenylalanyl-tRNA₃₃phe</td>
<td>U-U-U</td>
<td>0.47</td>
<td>1.61</td>
</tr>
<tr>
<td></td>
<td>U-U-C</td>
<td>0.47</td>
<td>1.99</td>
</tr>
</tbody>
</table>

Table 1-1: Stimulation of the binding of [14C]aminoacyl-tRNAs containing Q to 
ribosomes by the corresponding triplets (reproduced from work of Harada and 
Nishimura⁴). The reaction was carried out in 0.1 M Tris-HCl (pH 7.5) containing 0.01 M 
magnesium acetate for 8 min at 37 °C. The amounts of [14C]aminoacyl-tRNA in the 
reaction mixture (0.05 mL) were: tRNA₅₃yr 12.5 pmoles, tRNA₃₃his 15.6 pmoles, tRNA₃₃asn 13.3 
epmoles, tRNA₃₃asp 12.2 pmoles, and tRNA₃₃phe 13.7 pmoles.
As an aside, this work into the potential for queuine modification to cause a redundant codon bias was partially the basis for a recent project in the Garcia lab. The study examined the expression of green fluorescent protein (GFP) from constructed plasmids with varied redundant codons for queuine-tRNA amino acids resulting in changes to GFP’s mRNA codon usage\(^9\). One plasmid generated an mRNA with all C ending codons and another with all U ending codons for the queuine-tRNA amino acids, which were compared to naturally occurring coding for GFP. The expression of the GFP was monitored in normal \textit{E. coli} K13 (DE3) cells as well as \textit{tgt} knockout \textit{E. coli} K12 (DE3) cells. The results indicated that synthesis was increased for U ending codons with queuine modification present and seemed to indicate that C ending codons were translated more slowly without queuine modification.

Harada and Nishimura, in their conclusions, expressed the belief that knowing the structure of the modified base would help to explain the observed changes in affinity. In 1976 the structure of queuine was reported\(^{10}\). Modified tRNA from rabbit liver was isolated as previously described and rigorously purified by subsequent paper chromatography and high pressure liquid chromatography. The authors were able to purify 0.6 mg of queuosine (the ribonucleoside of queuine) from the digested rabbit liver tRNA, and two forms were extracted. One included an alternate sugar instead of ribose as the minor constituent of the mixture. The sample proved sufficient for analysis by \(^{252}\text{Cf}\) plasma desorption mass spectrometry (PDMS) and \(^1\text{H}\) NMR, from which the structure could be determined. However, Kasai did not assign the stereochemistry of the side chain. The stereochemistry assignments could not be made until a total
synthesis of optically pure queuine was completed by Goto in 1979\textsuperscript{11}. Queuine, seen in Figure 1-1, was the first tRNA nucleoside discovered to contain a sugar-like moiety as a side chain.

In order to determine the distribution of queuine in nature, studies were conducted on various plant and animal extracts\textsuperscript{12}. A study by Katze and coworkers demonstrated the presence of queuine in bovine amniotic fluid\textsuperscript{12a} with subsequent work establishing queuine incorporation in \textit{Drosophila melanogaster}\textsuperscript{12b}. These studies verified that queuine was not limited to eubacteria, so Katze, Basile and McCloskey conducted a more exhaustive study of eukaryotic species for the occurrence of queuine\textsuperscript{12c}. The results of that study are presented in Table 1-2. The data was obtained from a whole cell assay in a serum free media involving L-M cells and monitoring the synthesized tRNA for queuine, while varying potential sources of the nutrients\textsuperscript{12b, 13}. Interestingly, the studies with the L-M cells demonstrated that the base of queuine is required, queuosine is not active in the assay. The study also included a gas chromatography-mass spectrometry method for verification of assigned structures. In addition to the data presented in the table, the authors found queuine in a limited number of samples of human amniotic fluid from normal human pregnancies (16 to 28 weeks gestation) with levels showing a mean of 29 ng/mL (ranging from 2 to 84 ng/mL).
<table>
<thead>
<tr>
<th>Source</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine amniotic fluid (third trimester)</td>
<td>2300 to 3600 ng/mL</td>
</tr>
<tr>
<td><em>Drosophila melanogaster</em> (wild type and mutants)</td>
<td>0 to 1100 ng/g</td>
</tr>
<tr>
<td>Coconut Water (ripe)</td>
<td>87 to 530 ng/mL</td>
</tr>
<tr>
<td>Bovine pineal body</td>
<td>300 ng/g</td>
</tr>
<tr>
<td>Wheat germ</td>
<td>190 ng/g</td>
</tr>
<tr>
<td>Bovine seminal vesicle (adult)</td>
<td>110 ng/g</td>
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<tr>
<td>Bovine testicle (adult)</td>
<td>58 ng/g</td>
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<td>Bovine serum (fetal)</td>
<td>33 to 54 ng/mL</td>
</tr>
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<td>Tomato (fresh, ripe)</td>
<td>21 ng/g</td>
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<tr>
<td>Bovine milk (whole and skim)</td>
<td>16 to 17 ng/mL</td>
</tr>
<tr>
<td>Bovine serum (calf)</td>
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<tr>
<td>Bovine milk (evaporated skim, canned)</td>
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<tr>
<td>Yogurt (commercial and homemade)</td>
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<tr>
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<tr>
<td>Human milk</td>
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</tr>
</tbody>
</table>

**Table 1-2:** Queuine content from natural products (reproduced from the work of Katze and coworkers\(^{12c}\)).

The data showed that queuine is found in eukaryotes in addition to eubacteria. Katze and coworkers had also demonstrated that queuine as a base was required for incorporation into eukaryotes as opposed to eubacteria that use an alternate base (later determined to be preQ\(_1\)) before subsequent elaboration to queuine\(^{14}\). The data also demonstrated that queuine was likely a dietary nutrient as it could be used in cell assays as such. A study in germ free mice was conducted to further explore the source of queuine that leads to queuosine-containing tRNA\(^{15}\). When germ free mice were fed a chemically defined diet devoid of queuine for one year, the four known modified tRNAs in the mice no longer contained queuine. The authors also determined that queuine modification of tRNA could be induced by the injection or feeding of queuine. It was also found that feeding the mice queuine-containing tRNA could restore levels of
queuine modified tRNA in the mice, implying a salvage mechanism for queuine. Katze and Gündüz went on to determine that queuine is salvaged from queuosine 5′-phosphate in eukaryotic cells\textsuperscript{16}.

From queuine's discovery in the late 1960's to the mouse studies by Katze in the early 1980's, much had been learned about the modification. Its structure, location in tRNA, and presence in eubacteria and eukarya, although via different routes of modification, had been discovered. The biosynthetic pathway, however, remained unknown as well as the enzymes responsible for its incorporation and salvage. The conducted studies did provide a rationale for undertaking further studies required to elucidate these pathways. The near universal distribution in nature and degree of complexity of queuine suggested that it has an important function. In addition, the fact that hypomodification of queuine was seen in several cancer cell lines with tumor progression halting upon queuine addition to the tumor cells\textsuperscript{16-17}, and that queuine is present in reproductive fluids, implied an importance in cell development and regulation\textsuperscript{12c}.

**Biosynthesis of queuine and incorporation**

Although queuine and some of its precursors have been known for over 40 years, its biosynthesis was not fully elucidated until recently\textsuperscript{18}. In fact until 2004 only two of the enzymes involved had been discovered and characterized, namely TGT and QueA\textsuperscript{19}. The enzyme responsible for the incorporation of queuine into tRNA, TGT, had
been extensively studied and differences between it in different organisms evaluated\textsuperscript{20}. The study of those differences had established the substrate in eubacteria and eukaryae as being preQ\textsubscript{1} and queuine, respectively. This demonstrated, along with other studies, that queuine is a dietary factor in eukaryae but synthesized in eubacteria. The synthesis of queuine early on was believed to start from guanine, which was confirmed to be from GTP as determined by a radio-labeling study\textsuperscript{21}.

The laboratory of Crécy-Lagard in 2004 identified four additional genes required for the biosynthesis of queuine: QueC, QueD, QueE and QueF. The genes were discovered by comparative genomic techniques including phylogenetic distribution of tRNA modification genes, clustering of tRNA modification genes and prediction of catalytic mechanisms\textsuperscript{22}. The study was conducted in \textit{Acinetobacter calcoaceticus} as it is naturally competent, and proves a good choice for gene knockout studies as it is efficient at homologous recombination. Their work started with identifying a GTP cyclohydrolase capable of starting the reaction sequence and examining genes encoded in proximity to these. This along with the locations of the \textit{tgt} and \textit{queA} genes was the starting point, to create an occurrence profile. The phylogenetic study of related species with known sequence led them to identify the genes they believed to be associated, due to conserved occurrences in their respective operons. The genes were validated as being involved in preQ\textsubscript{1} synthesis with knock outs constructed in \textit{A. calcoaceticus} and subsequent monitoring for queuine by HPLC traces of bulk tRNA digests from cultures of the knockout strains. Analyses of the genes by BLAST associated QueC as an ATPase, QueD as a 6-pyruvoyltetrahydropterin synthase (PTPS),
QueE as a member of a radical SAM protein superfamily and QueF showing homology to type I GTP cyclohydrolases. Studies subsequently determined that QueF, though it shares homology to I GTP cyclohydrolases, in fact catalyzes an NADPH-dependent reduction of preQ<sub>0</sub><sup>23</sup>. Additional work revealed that the cyclohydrolase required to start the synthesis from GTP was in fact FolE<sup>24</sup>. Therefore, over the past decade the picture for the overall synthesis of queuine began to take shape, as outlined in Figure 1-2.

![Figure 1-2: Queuine biosynthetic pathways for eubacteria.](image)

The synthesis begins with conversion of GTP to 7,8-dihydroneopterin triphosphate by FolE; the same first step in the biosynthesis of folic acid<sup>24, 26</sup>. In the following step, QueD catalyzes the conversion of the previous intermediate to 6-carboxy-5,6,7,8-tetrahydropterin<sup>27</sup>. The next step in the pathway is catalyzed by QueE.
It was confirmed that QueE is an enzyme of the radical SAM protein superfamily containing an iron cofactor in a [4Fe-4S] cluster. The reaction required the addition of SAM to proceed \textit{in vitro} as well as dithionite to produce the required conditions. This reaction removes what had been the 7-position nitrogen in the purine starting base through a radical mechanism, producing the carboxylic acid substituted pyrrolopyrimidine substituted at the correct position of the pyrrole ring for generating \textit{preQ}_0. The product of the QueE catalyzed reaction is the heterocyclic portion of the previously reported nucleoside analogue cadeguomycin, which had been isolated from \textit{Streptomyces hygroscopicus}\textsuperscript{28}. It has not been confirmed if this pathway is used to generate cadeguomycin, though it would appear likely. The structure of QueC was determined by X-ray crystallography\textsuperscript{29} confirming it was a member of the ATPase family, which was also demonstrated by its sequence containing the conserved PPI loop of ATPases\textsuperscript{30}. The reaction catalyzed by QueC is ATP dependent and utilizes ammonia as the nitrogen source to generate the cyano functionality of \textit{preQ}_0. The final reaction in the synthesis of \textit{preQ}_1 is catalyzed by QueF. QueF is an NADPH dependent enzyme, which performs a four electron reduction of \textit{preQ}_0 to generate \textit{preQ}_1\textsuperscript{31}. At this point in the synthesis of queuine, eubacterial TGT incorporates \textit{preQ}_1 into RNA by transglycosylation. The mechanism of tRNA guanine transglycosylase will be discussed in more detail in the following section to allow for a comparison to the eukaryal version of the enzyme. The following steps of the synthesis occur with the modified base incorporated into the RNA chain.
The first step in the synthesis of queuine after transglycosylation of preQ₁ is catalyzed by QueA. The enzyme QueA is responsible for adding the queuine side chain, but it does so by addition of an epoxycyclopentandiol ring. The enzyme was discovered as it creates a very noticeable difference, preQ₁ in place of queuine, when its activity is removed. Early on methionine was implicated, but later work demonstrated that it utilized S-adenosylmethionine as a ribosyl donor instead. The final step from the cyclopentandiol epoxide derivative has long been known to be B₁₂ dependent, but the enzyme responsible had not been identified. The enzyme was recently discovered in 2010 utilizing the Keio collection, which is a E. coli strain where each of the 4,000 non-essential genes are individually knocked out allowing for identification of the gene required for reduction of the cyclopentadiol epoxide. Each strain was grown in standard media and tRNA was isolated, digested, dephosphorylated, separated by HPLC and analyzed by UV and mass spectrometry to identify the cyclopentadiol intermediate and queuine. Two genes when knocked out yielded the cyclopentadiol intermediate during analysis: yjeS, identified as QueG; and tonB, which is known to be a periplasmic protein required for iron and cobalamin transport (further demonstrating the need for B₁₂ in the reaction catalyzed by QueG). BLAST analysis shows that QueG is most similar in sequence to corrinoid Fe-S containing proteins from anaerobic halorespiring bacteria that act as B₁₂ dependent reductive dehalogenases. The role of B₁₂ and the exact mechanism remain unknown, which is the case with many of the enzymes in this pathway. The genes required and their respective classes of protein have been identified, but work remains to fully understand the biosynthetic pathway. The
importance of TGT in the pathway, especially given it is the only enzyme of the pathway found in eukaryae, merits further review.

**tRNA guanine transglycosylase**

As mentioned earlier, queuine is an intriguing molecule as it is one of the only modifications in which a pre-synthesized modified base replaces the encoded base. In all other known cases, modification arises from a reaction with the encoded base. The enzyme tRNA guanine transglycosylase catalyzes the replacement and thus constitutes one of the most important aspects in understanding the queuine modification since this enzyme’s specificity for RNA dictates what sites are modified. The transglycosylation activity was first identified in rabbit reticulocytes during the early 1970s by Farkas\textsuperscript{34}. The enzyme was later isolated and purified from numerous organisms\textsuperscript{14, 19, 35}. Once the enzyme responsible for the activity was identified, the Garcia group prepared a recombinant form of TGT from *E. coli*\textsuperscript{36}. Once expressed and characterized, *in vitro* data demonstrated that the enzyme contained a zinc-binding domain that was important for structure and played a role in tRNA recognition\textsuperscript{37}. Further work showed that product release was the rate determining step of the reaction mechanism\textsuperscript{2, 38}.

Studies in other labs demonstrated that the eubacterial form differed from the eukaryae version not only in substrate recognition (preQ\textsubscript{1} versus queuine), but also in quaternary structure\textsuperscript{39}. The Kelly lab identified that TGT existed as a heterodimer in eukaryae and that the function of the other subunit of the dimer, QTRTD1, is yet to be fully understood. Characterization of this heterodimer by the Garcia lab through
recombinant expression validated the protein as the partner with TGT in human and demonstrated that the additional subunit was required for the enzyme to function\textsuperscript{40}.

Experiments to characterize other TGTs had shown that other substrates were accepted and incorporated into RNA by bacterial and eukaryal TGTs. These include dihydroqueuine, guanine, 7-deazaguanine\textsuperscript{41}, as well as some synthetically prepared analogues of preQ\textsubscript{0}\textsuperscript{42}. Romier \textit{et. al.} constructed models of the different TGT binding pockets in an attempt to understand the substrate specificities\textsuperscript{20}. The authors used the structure of TGT from \textit{Z. mobilis} to construct a homology model from the sequence for \textit{C. elegans}, allowing a comparison of the binding pockets for TGT from eubacteria (\textit{Z. mobilis}) and eukaryae (\textit{C. elegans}). Prior to constructing the model, seen in Figure 1-3, the authors noted that the enzymes are nearly identical in length with sequences that are 40 % identical, giving them confidence the enzymes would adopt the same fold. The model illustrates that the substitutions of Cys\textsuperscript{158} for valine and Val\textsuperscript{233} for glycine enlarge the pocket of eukaryal TGT, which allows for the occupancy of larger substrates like queuine. As it appears the pocket is amenable to accepting alternate substrates and has even evolved over time to accept queuine instead of the synthetic precursor preQ\textsubscript{1}\textsuperscript{3}, it seems likely that synthesized analogues should be amenable to incorporation by TGT as well.
Recognition for TGT was first inferred from the four known sites of queuine modification in tRNA. The anticodon sequence for tRNA\textsuperscript{Tyr,His,Asp,Asn} all contain a GUN sequence, which is preceded by a U in all four cases to give a conserved UGUN sequence. Work in the Garcia lab with isolated and prepared tRNA\textsuperscript{Tyr} demonstrated that TGT was sufficient when supplied with substrate to yield the modification\textsuperscript{43}. They also demonstrated that a mini-hairpin of RNA featuring the anticodon stem-loop sequence was also a substrate for the modification. Subsequent work in the Garcia lab demonstrated that a UGU sequence in a loop, like that of the anti-codon, is sufficient for recognition by TGT\textsuperscript{44}, and further work by Garcia and Hurt\textsuperscript{45} also demonstrated that the VirF mRNA, containing a hairpin sequence with a UGU in the loop, could be modified at
that UGU by TGT. This work implied that modification of RNAs other than the anticodon of the four known tRNAs is possible, but the question remains if this occurs in vivo.

**Observations**

Since its discovery many observations have been made concerning queuine. Some of the observations pertain to the possible role of the queuine modification and others note effects seen when it is not present. There are reports that queuine modification plays a role in avoiding translation errors\(^{46}\) and others that cite its potential importance in determining codon usage\(^{47}\). Associated with these observations are reports that looked at the outcomes of queuine modification or hypomodification. There have been many reports that associate queuine deficiency with various cancers (lung, ovarian, leukemias, lymphomas among others\(^{48}\)). Reports have also demonstrated that inhibition of queuine modification can promote carcinogenesis\(^{48g}\). Queuine modification has also been demonstrated to be required for the virulence of *Shigella flexneri*\(^{49}\). These observations are worth noting as they point to a rationale to further explore queuine modification and its potential role in cancer and *S. flexneri* infection, and may also help to elucidate what role queuine modification has in the cell.

While queuine modification has effects on the cell, it has not been linked to cell growth in bacteria as studies with *E. coli* demonstrated no change in growth, but did note a decrease in survival during stationary phase\(^{50}\). An effect on survival is also reported for *S. flexneri* as queuine-deficient *Shigella* were less virulent\(^{49}\). The effect, a
50 % reduction in virulence, was correlated with levels of \textit{virF} protein being reduced by 50 %. In these studies no effect on \textit{virF} mRNA levels were noted linking to effects on translation\textsuperscript{51}. \textit{VirF} protein is important as it is a transcriptional activator controlling expression of proteins important for intracellular spreading of the organism as well as for escaping the immune system\textsuperscript{52}. Interestingly, overexpression of \textit{virF} mRNA allows \textit{Shigella} to regain hemolytic activity further demonstrating that the loss was due to translational efficiency. Why this effect on translation occurs has been the subject of several studies.

The work of Nishimura lab, as previously described above\textsuperscript{4}, showed that the four tRNAs in which queuine is found all have redundant codons whose sole variation is that of a U or C in the third position, the base to which the wobble position binds\textsuperscript{4}. This led the authors to surmise that queuine modification plays a role in codon recognition. A study of queuine modified aminoacyl-tRNA without Q (-Q triplet) and with Q (+Q triplet), summarized in Table 1-1, demonstrated that when Q is present in the wobble position, U ending codons bind with a greater affinity. The results also indicate a general increase in binding when queuine is present. From these findings the investigators reasoned that one role of queuine could be to increase the efficiency of protein synthesis for genes with certain codons and to prevent miscoding, which might occur with G thus explaining the effect seen in the \textit{Shigella} work involving \textit{virF} translation.

Queuine modification in relation to codon usage was explored in the work of Chiari \textit{et. al.} on the differences of TGT levels in \textit{Drosophila} species. They found that
*Drosophila willistoni*, which is the only species to primarily uses U/T ending codons instead of C ending codons, had higher expression levels of TGT implying the importance of queuine modification with U ending codon usage\(^\text{53}\). The finding of queuine having a preference for U ending codons was also reported in the work of Meier *et. al.* that studied the modification *in vivo*\(^\text{54}\). The authors reported a modest preference but point out that the four modified tRNAs represent approximately 15 % of codons, which would create a cumulative preference for U ending codons that is much more pronounced. An interesting report from other work shows that the lack of queuine modification in tobacco mosaic virus increases read through of the UAG stop codon allowing for the synthesis of an alternate protein\(^\text{55}\). The work of Frey *et. al.* confirmed these findings with a study that looked at U ending or C ending codons through plasmids transformed into *E. coli* containing β-galactosidase\(^\text{56}\). Where a stop codon is placed to test if read through occurs, as measured by the activity of the resulting enzyme, the authors discovered a 2-fold increase in activity and thus an increase in read through with U ending codons when queuine was not present. This was accomplished by knocking out TGT, whereas no change was observed for C ending codons. Experiments have consistently demonstrated that queuine, when present in tRNA, effects binding to U ending codons.

Early in its discovery, queuine was found to be deficient in cancer cell lines. Work by Randerath *et. al.* demonstrated that this was the case for mitochondrial tRNA\(^{\text{asp}}\) from a Morris hepatoma compared to rat liver mitochondria tRNA\(^{\text{asp}}\). The authors pointed out this could arise for several reasons. First, it could be the result of a
general queuine deficiency in the tumor cells due to increased transcription and turnover of tRNA observed in tumor cells. Alternatively, the hypomodification of tRNA could be the result of loss of function in TGT, a decrease in its expression, or loss of transport of TGT to the mitochondria. A study by Emmerich, et. al. was later conducted examining queuine lacking tRNAs in leukemias and lymphomas. The authors found that the extent of under modification with queuine correlated with the grade of the malignancy or the cellular proliferative capacity. They also measured available amounts of free queuine in the tissue and determined the amount should be sufficient for modification, implicating another cause other than lack of queuine supply. Related work by Huang et. al. with lung cancer tissue from 38 surgically removed tumors from different patients came to the same conclusions. The authors went so far as to suggest that decreased queuine modification may be a general feature of neoplasms and be useful for grading malignancy and predicting survival of cancer patients. The finding of queuine hypomodification in cancer cell lines was also reported by Baranowski et. al. in ovarian tumors. An explanation for the cause of the deficiency has not yet been reported, but related work shows that queuine at a minimum is correlated with cancer etiology and its progression, and could prove to play a larger role in tumor suppression.

In more recent work by Pathak, Jaiswal and Vinayak, queuine modification was studied in regards to effects on levels of tyrosine phosphoprotein and Bcl2 levels. In both studies lymphoma was induced in mice by serial transplantation of live Dalton’s lymphoma ascites cells and queuine was delivered i.p. to examine what effect it has on
cancer associated protein levels as well as on the progression of the lymphoma. An increase in life span by several days was observed for higher doses of queuine, 25 µg administered daily. It was also found that Bcl2 levels decreased and queuine appeared to induce cell death. Tyrosine kinase activities were also down regulated by queuine administration. In addition, the administration of queuine has been found to inhibit the cell proliferation of Colo-DM320 cells, PC-12 cells, ascites tumor cells, NIH-3T3 cells, U87 cells, HepG2 cells and EGF supported proliferation of HeLa cells\textsuperscript{48a, 57}. The group also looked at 7-methylguanine to monitor the effects of TGT inhibition. With co-administration of 7-methylguanine, inhibitory effects on queuine levels are not seen, indicating that TGT-mediated modification of tRNA with queuine was the source of the inhibition of cell growth in the cancer cell lines. This work provided further proof that queuine has an important role in cell regulation, yet the exact role is still unknown.

**Impetus for dissertation work**

It is known that the enzyme responsible for the modification in RNA, tRNA guanine transglycosylase (TGT), recognizes a UGU sequence in an RNA hairpin loop (e.g., transfer RNA anticodon arm) as a site for modification. Previous work in our laboratory has demonstrated that modification of alternate RNA species (i.e. hairpin minihelix\textsuperscript{44b, 58} and 800 base mRNA\textsuperscript{45}) with UGU sequences in a loop can occur in vitro. Whether or not the queuine modification is limited to the four instances in transfer RNA in vivo is a question that has remained unanswered. Queuine has been demonstrated to be
important for cellular function although its exact role remains unclear. Determining if queuine modification is present in RNAs, other than the known tRNAs, is important in understanding the function of queuine modification and its role in the cell.

Previous syntheses of queuine

Before commencing our studies, a survey of the literature regarding prior syntheses of queuine was required as this would provide valuable information on strategies to prepare radio-labeled preQ₁ and queuine, and other analogues deemed critical to our research plan. There have been three previous syntheses of queuine. The first was completed by Kondo et al.⁵⁹ The synthetic route, presented in Scheme 1-1, builds upon previous work in Goto’s lab to synthesize the side chain of queuine⁶⁰. Goto’s lab undertook this work in order to determine the stereochemistry of the queuine side chain, which had not been reported in Kasai’s report on the structure of queuine¹⁰. In addition to the synthesis of the side chain, Goto had also previously synthesized queuosine to confirm its structure by total synthesis of optically pure synthetic queuine¹¹. As queuine is the substrate for incorporation in eukaryotes its synthesis was of greater interest in pursuing biochemical studies. The route is a convergent synthesis that proceeds via a Schiff base between a protected pyrrolopyrimidine aldehyde and protected queuine side chain amine, albeit in a lengthy 19 step process.
Scheme 1-1: The synthesis of queuine by Goto: part I, the synthesis of the side chain, part II, the synthesis of the aldehyde of the pyrrolo[2,3-d]pyrimidine, and part III, the Schiff base reactions and subsequent reduction and deprotection to yield queuine.
The synthesis of the side chain was the first portion completed as it was essential for the structural determination of queuosine by total synthesis\textsuperscript{11, 60}. The second part of the synthesis, the aldehyde of the pyrrolopyrimidine, was performed as shown in Scheme 1-1. The route suffers from numerous required protection and deprotection steps as well as unintentional modifications that later must be compensated for to complete the synthesis, the bromination of the pyrrole portion of the ring for example. The idea of utilizing a Schiff base formation to give the desired product was a good plan but the long route to the aldehyde is problematic from an efficiency and atom economy standpoint.

The second synthesis of queuine performed by Akimoto \textit{et al.}\textsuperscript{61} is much shorter and proceeds via a Mannich reaction to incorporate a dibenzylamine regioselectively to the C-5 position of a pyrrolopyrimidine precursor, as seen Scheme 1-2. The protected side chain of queuine was subsequently added via an amine exchange reaction. Its main drawback is the requirement of several equivalents of the side chain in a key exchange reaction. In this synthesis, Akimoto utilized the queuine side chain that had been synthesized as described previously by Goto\textsuperscript{11}. In this route, 7-deazaguanine is first protected with octanoyl chloride, likely in an effort to gain solubility given the general insolubility of the starting material. The addition of the protected pyrrolopyrimidine to a formalin solution and dibenzylamine resulted in a Mannich reaction to give the dibenzylamine substituted intermediate. The authors utilized \textsuperscript{1}H NMR chemical shifts and \textit{J} values to confirm the structure of the product. In addition, comparison to spectral data of isolated natural product ensured that the correct regioisomer had formed.
Scheme 1-2: The synthesis of queuine by Akimoto that utilizes a Mannich reaction to incorporate the queuine side chain. The side chain was prepared by the same method used by Goto$^{11}$.

The most recent synthesis of queuine by Grub et al.$^{62}$ employs a different disconnection of the molecule, proceeding via a key Mitsunobu reaction to introduce the cyclopentenylamine side chain and a subsequent cyclocondensation reaction to build up the core heterocyclic moiety, seen in Scheme 1-3. While relatively straightforward and efficient, it requires 11 linear steps. The use of a cyclocondensation of a pyrimidine to generate a queuine precursor, preQ$_0$, by Townsend$^{63}$ could have served as the inspiration for the authors’ route. The authors had previously synthesized a number of pyrrolo[2,3-$d$]pyrimidines with alternate side chains as part of a drug discovery effort at Eli Lilly around an eventual drug with a pyrrolo[2,3-$d$]pyrimidine core, Pemetrexed, seen in Figure 1-4 $^{64}$. The diol protected alcohol side chain was synthesized by a method developed by Borchardt$^{65}$. The linear nature of the route is its main drawback. The bromination step followed by the immediate pyrrolopyrimidine annulation is a low yielding two-step process proceeding in only a 45 % yield. As the
bromination is the eighth step of the sequence, the low yield represents a significant loss of material and effort.

Scheme 1-3: The synthesis of queuine completed by Barnett and Grubb. The side chain is synthesized in a procedure developed by Borchardt.

Figure 1-4: Pemetrexed is a folate antimetabolite manufactured and marketed by Eli Lilly under the brand name Alimta for the treatment of pleural mesothelioma and non-small cell lung cancer.
Syntheses of queuosine

In addition to the three previous queuine syntheses, several syntheses of molecules related to queuine (either the side chain portion of queuine, queuosine, precursors like preQ₁ and preQ₀, or related pyrrolo[2,3-ç]pyrimidine structures) have been completed. Two laboratories have synthesized queuosine. The most recent synthesis is that of the Carell lab in 2007⁶⁶ (Scheme 1-4), which also presented a new route to the queuine side chain. The side chain route is similar to that used by Barnett and Grubb⁶², which had been developed by Borchardt⁶⁵a. The lab of Goto also completed an additional synthesis of queuosine⁶⁷ in 1986 to improve upon their earlier procedure from 1979¹¹. They modified the route as their previous synthesis had required a large excess of the queuine side chain which was undesirable and also suffered from a subsequent debromination step which proved to be un reproducible. Their second synthesis of queuosine is identical to the previously reported synthesis of queuine, except instead of utilizing the benzyl protecting for the pyrrole nitrogen a ribose is installed with 2, 3-acetonide and acylated 5-hydroxyl moieties protecting the ribose. Following Schiff base formation and reduction to install the desired cylopentenylamine side chain, the protecting groups are removed after by treatment with 2N HCl. Subsequent neutralization with Amberlite IR-45 provided queuosine.
Scheme 1-4: Queuosine synthesis completed by Carell lab\textsuperscript{66}. Part I, the synthesis of the side chain, part II, the synthesis of the aldehyde of the pyrrolo[2,3-\textit{d}]pyrimidine, and part III, the Schiff base reactions and subsequent reduction and deprotection to yield queuosine.
The synthesis of queuosine by the Carell lab is presented in Scheme 1-4. Similar to the previous method by Goto’s lab they employed a Schiff Base formation as the key reaction in a convergent synthesis. Their method is shorter and more atom efficient in that unnecessary protecting and directing groups are avoided. The synthesis of the side chain borrows from the earlier work of Borchardt, which includes acetonide protection of D-ribose, PCC oxidation to excise the 5-position methylene of ribose to yield a lactone, and use of an internal Horner-Wadsworth-Emmons reaction to give the cyclopentenone. The cyclopentenone was then subjected to a Luche reduction to generate the desired alcohol stereoselectively. This is the intermediate used previously by Barnett and Grubb to generate their cyclocondensation intermediate. The Carell lab though planned on proceeding through a Schiff base intermediate to form queuosine so they performed a Mitsunobu reaction to generate an azide intermediate previously utilized by Goto.

Carell et al. made an important discovery during their synthesis that casts some doubt on previous syntheses of queuine and queuosine, as they found that the azide intermediate of the side chain can undergo a 3,3 sigmatropic shift of the allylic azide to give the enantiomer of the side chain. The same azide intermediate had been used previously by Goto and Akimoto. The Carell lab modified their procedure based on previous work of the labs of Spino, Sharpless and Fokin and Young, which had studied allylic azide rearrangements and found that cyclic allylic azides require higher temperatures to rearrange. Thus a lower temperature was used to suppress the rearrangement in the case of the queuine side chain. Carell and co-workers also found
they could suppress the undesired rearrangement if they performed the Mitsunobu reaction and subsequent Staudinger reduction in a single pot at 0 °C.

The synthesis of the heterocyclic portion of the molecule, as seen in Scheme 1-4 part II, began with the formation of the 7-deazaguanine core through a cyclocondensation with 2,4-diamino-6-hydroxy-pyrimidine, which was similar to the reaction performed by Grubb for his synthesis of queuine. It provided the same intermediate Akimoto had reported on as the starting point for his synthesis of queuine. A coupling to D-(-)-ribose occurred under Vorbrüggen conditions after chlorination of the lactam, protection of the exocyclic amine and iodination of the 3-position of the pyrolopyrimidine. Once the chloro group had been hydrolyzed back to the lactam, the molecule was subjected to a palladium catalyzed CO insertion reaction to generate the required aldehyde. With the aldehyde and amine side chain in hand the authors formed the Schiff base, reduced it to the secondary amine and removed the benzoyl and pivaloyl protecting groups to give queuosine.

Alternate syntheses of queuine side chain

In addition to the synthesis of the side chain performed by the Goto and Carell labs, which had been based on earlier work by Grubb and Borchardt, there are four alternate syntheses of the queuine side chain. The other side chain syntheses had been performed by Tanaka and Ogasawara in 1996⁷⁰, Ovaaet. al. in 1998⁷¹, Trost and Sorum in 2003⁷² as well as Kim and Miller in 2003⁷³. The synthesis of the side chain by Tanaka and Ogasawara builds upon their prior work of generating optically pure (-)-3-endo-
hydroxydicyclopentadiene by lipase-mediated transesterification. The route is lengthy and time consuming at 13 steps from the starting cyclopentadiene and proved to be low yielding. While the synthesis is of academic interest, it is not practical. The other three syntheses of the queuine side chain though merit further consideration.

The synthesis of the queuine side chain performed by Ovaa et. al. is presented in Scheme 1-5. The key features of the route are a Grubb’s ring-closing metathesis to give a cyclopentene scaffold and a [3,3] sigmatropic Overman rearrangement. The protected furanose sugar, mannitol, was procured and after selective removal of the 5,6-isopropylidene to reveal the diol, treatment with triethylorthoformate and acid catalysis afforded rearrangement to give the terminal alkene. Debenzoylation followed by a Wittig olefination gave the diene required for ring-closing metathesis by the Grubb catalyst. The imidate from the alcohol substituted cyclopentene was generated by treatment with trichloroacetonitrile and DBU. The Overman rearrangement to give the stereochemistry required for the queuine side chain was achieved by heating the imidate at reflux for 4 h. A protected version of the queuine side chain has been formed at this point. The authors, based on previous reports of glycosylated versions of queuine (D-mannose and D-galactose) being extracted from rabbit liver, also wanted to generate the side chain required for the synthesis of those molecules. Glycosylation was accomplished by hydrolysis of the isopropylidene protecting group and selective silylation the 5-hydroxyl group, followed by glycosylation of the resultant intermediate with the assistance of a catalytic amount of trimethylsilyltriflate (TMSOTf).
Scheme 1-5: Queuine side chain synthesis completed by Ovaa, et. al. The boxed structure is the protected version of the side chain. The authors went on to synthesize the glycosylated form of the queuine side chain that had been isolated from rabbit liver.

Trost and Sorum presented a synthesis, seen in Scheme 1-6, for the side chain of queuine looking to improve upon prior syntheses. They started their synthesis with the epoxidation of cyclopentadiene followed by direct formation of the acetonide based on previous work by Hancock et al. To achieve the desired epoxide opening and acetonide formation, multiple acids (Bronsted and Lewis) were tested before BF$_3$ etherate was identified as the appropriate catalyst for the transformation. The subsequent oxidation with trifluoroperacetic acid, generated from a mixture of urea-hydrogen peroxide and trifluoroacetic anhydride, and epoxide opening by elimination with lithium diethylamine gave a racemic mixture of the alcohol intermediate.
resolve their racemic mixture the authors first formed methyl carbonate derivatives with methyl chloroformate. They then performed a asymmetric allylic imidation catalyzed by (dba)\textsubscript{3}Pd-CHCl\textsubscript{3} with the ligand shown in Scheme 1-6. To increase the solubility of the potassium phthalimide the authors added tetra-\textit{n}-hexylammonium bromide. A series of solvent and additive conditions were tried until they found that one equivalent of the additive, tetra-\textit{n}-hexylammonium bromide, in DCM run for 12 h from a water ice bath to room temperature gave the best yield and ee(%), 62 % and 98 % ee. After cleavage of the phthalimide with ethylenediamine the acetonide protected version of the queuine side chain was obtained in a 17 % yield from the starting epoxide. The main drawback of this method is the need for the palladium catalyzed ligand directed asymmetric allylic imidation, which adds extra cost and effort requirements to generate the ligand and prepare the substrate for the reaction.
The final synthesis of the queuine side chain to be described here is shown in Scheme 1-7 and was completed by Kim and Miller in 2003\textsuperscript{73}. The authors sought to develop a stereoselective synthesis of the side chain based on their prior work with amino acids as chiral auxiliaries to derive an asymmetric acylnitroso Diels-Alder cycloaddition\textsuperscript{76}. Their synthesis begins with the hydroxamic acid of boc protected L-alanine. Oxidation of the hydroxamic acid forms an acylnitroso intermediate, which is trapped with cyclopentadiene to generate the Diels-Alder cycloadduct. Osmium tetraoxide provides the \textit{cis}-hydroxylation of the alkene to give the diol, which was
subsequently protected by acetonide formation with 2,2-dimethoxypropane and a catalytic amount of para-toluenesulfonic acid. Reduction with sodium borohydride removed the amino acid auxiliary and hydrogenation cleaved the nitrogen oxygen bond. All that remained was the conversion of the allylic alcohol to the allylic amine. This was accomplished by Boc protection of the amine, mesylation of the hydroxyl and base induced elimination of the mesylate. Deprotection of the Boc and acetonide was accomplished by treatment with 3M HCl to generate the side chain of queuine. This stereoselective method has the advantage of avoiding the need to resolve a racemic mixture as in the previous route or the need to suppress a side reaction as in earlier routes to the side chain of queuine.

Scheme 1-7: Synthesis of queuine side chain by Kim and Miller featuring a key amino acid derived acylnitroso Diels-Alder cycloaddition.
Syntheses of queuine precursors preQ₀ and preQ₁

In the literature there are three reported routes to preQ₀-type molecules. The first route published by the Goto laboratory in 1986⁶⁷ was based on their previous synthesis of queuine and queuosine. The authors utilized the aldehyde they had generated earlier for the Schiff base formation of queuosine. The aldehyde was treated with hydroxylamine in ethanol to give an oxime product. The authors completed the conversion to the nitrile by refluxing the oxime in acetic anhydride, which also acylated the free primary alcohol of ribose. Deprotection with methanol-ammonia and then aqueous HCl before neutralization gave preQ₀. This method for generating preQ₀ has not been used due to the length of the synthesis.

The next reported synthesis of preQ₀ was reported by the Townsend lab in 1996⁶³. The synthesis requires only two steps from commercially available starting materials and the method has been used by subsequent groups that required a route to preQ₀⁷⁷. The synthesis starts with a formylation of chloroacetonitrile with methyl formate to form chloro(formyl)acetonitrile. The reagent formed is a substrate for cyclocondensation with 2,6-diaminopyrimidin-4-one to yield preQ₀. This short straightforward synthesis provides a quick means to generate preQ₀.

One other method to generate preQ₀ has been published by the Carell lab⁷⁸. The synthesis builds on their previous synthesis of queuosine and starts from their iodo intermediate. From this intermediate the authors perform an iodine/magnesium exchange reaction with iPrMgCl-LiCl (Turbo-Grignard reagent) to form a Grignard intermediate of the iodo pyrrolopyrimidine. The authors then treated the intermediate
with tosyl cyanide to generate the nitrile and thus preQ₀. The methods by Carell and Goto have the advantage of generating the nucleoside directly. However, the method by Townsend et al. produces the form needed for most biochemical experiments, and it can be further elaborated to the nucleoside by standard methods if so desired. Their method is also amenable to elaboration to generate preQ₁ as well as other pyrrolopyrimidines such as cadeguomycin, kanagawamycin, archaeosine, and echiguanine A or B.

The queuine precursor preQ₁ has been synthesized by several methods since its discovery. The first synthesis was performed by the Goto lab and is based on their synthesis of queuine. The authors used their previously described bromination reaction of the methyl substituted pyrrolopyrimidine, seen in Scheme 1-1, which was subsequently displaced by azide by treatment with sodium azide in DMF. After treatment with ammonia-methanol to remove acetyl protecting groups from the exocyclic amine, the authors reduced the azide to the desired amine and removed the other bromine with H₂ and Pd/C. The authors also published a method to generate the ribose substituted nucleoside version of preQ₁ that used the same route with the ribose in place of the benzyl group on the pyrrolopyrimidine. Akimoto et al. also described a method to generate preQ₁ based on their synthesis of queuine. They were making a series of analogues to study the effects of queuine analogues on mouse L5178Y cells, a leukemia cancer model. They generated their common dibenzylsubstituted intermediate and treated it with methanol-ammonia to give preQ₁.
In addition to these routes, other authors who had published syntheses of queuine have offered their own routes to preQ$_1$.

Grubb and Barnett describe a route to get a protected form of preQ$_1$ based on the cyclocondensation method they had developed to generate queuine$^{64}$. Their strategy for queuine, as described in Scheme 1-3, was altered in that instead of using the queuine side chain the authors used phthalimide to substitute the 1,3-propane diol. The authors could then oxidize the remaining alcohol to an aldehyde and brominate α to it, forming the reagent for the cyclocondensation to give the phthalimide version of preQ$_1$. The authors did not describe the method to give preQ$_1$ from this intermediate, but one can envision a cleavage of the phthalimide with hydrazine or ethylenediamine similar to the reaction used by Trost in Scheme 1-6. That method was later reported by the Carell lab$^{77b}$. In that same publication Carell described a method from preQ$_0$ that had been generated by the method first described by the Townsend lab$^{63}$. The attempted to reduce the nitrile or preQ$_0$ with H$_2$ (50 psi) over Pd/C, but only obtained a 10% yield of the product. In 2010, the Carell lab described a synthesis to the nucleoside version of preQ$_1$, which likely could be used to generate the heterocycle as well. From their iodo intermediate, that had been generated first for their synthesis of queuine, the authors formylated the ring via a palladium catalyzed CO insertion reaction. The aldehyde was then converted to an oxime by treatment with hydroxylamine. The authors unsuccessfully attempted to convert the oxime to the amine with sodium borohydride, but were eventually successful by treating with NiCl$_2$-H$_2$O and sodium
borohydride. Having the amine, the authors deprotected the intermediate with sodium hydroxide to give the nucleoside form of preQ₁.

In addition to synthetic organic chemistry methods to generate preQ₁ two groups have described enzymatic reactions to generate the product. Both groups describe a nitrile oxidoreductase that reduces preQ₀ to the amine of preQ₁. The group of Iwata-Reuyl reported the study of the enzyme QueF from Bacillus subtilis that is part of the biosynthetic pathway to generate queuine²³. In addition, another group reported on the enzyme CinQ from Pseudomonas putida, its version of QueF⁸¹. Iwata-Reuyl reports that QueF is an NADPH dependent enzyme, which performs a four electron reduction of preQ₀. The authors propose future studies to test the substrate scope of the enzyme pointing to it as a novel reaction type that could prove important as a biocatalytic agent.

**Design of dissertation research to examine the prevalence of queuine**

The project was designed with two distinct phases and objectives. The first phase of the project involved synthetic work in the Showalter lab to generate the target molecules required for the study. For this project and to complete ongoing work in the Garcia lab studying differences between eubacterial and eukaryal TGT, tritium labeled substrates for each target molecule, queuine and preQ₁, needed to be synthesized. Thus, designing and completing a synthesis of each was the starting point for the first objective of the project. The route to queuine and preQ₁ needed to be both efficient and allow for facile incorporation of a tritium label. Having access to tritium-labeled
preQ₁ would allow me to complete the first objective of the project, namely determine the prevalence of queuine in *E. coli* in a general sense, the degree of queuine modification and types of RNA that are substrate for the modification.

In addition, the first phase of my work in the Showalter lab required the design and synthesis of azide analogues of preQ₁ to commence the second objective of this work, namely further exploring the prevalence of queuine through developing a means to identify specific sites of queuine modification. Azide probes based on the structure of preQ₁ were designed to be substrates for the TGT. Incorporation of the azide probes would allow for subsequent identification of modified RNAs since the azide would allow for subsequent elaboration as a means to isolate the modified RNA. As each target molecule was synthesized, I transitioned my percentage of effort from the Showalter lab to the Garcia lab to evaluate each molecule in appropriate biological experiments. My final year was spent solely in the Garcia lab.

During the second phase of my graduate career, I worked primarily in the Garcia lab. Initial experiments with the tritium-labeled preQ₁ afforded me the opportunity to examine the prevalence of queuine modification in *E. coli* by tracking incorporation of the radio-label into RNA. To complete the first objective, three cell lines were utilized to conduct the *in vitro* and *in vivo* experiments of this study: a ΔqueC knockout of *E. coli* that is unable to synthesize preQ₁ so that tritium labeled compound could be incorporated exclusively, a Δtgt knockout strain of *E. coli* that is unable to incorporate preQ₁ and a wild-type *E. coli* strain. To complete the second objective, developing a
means to identify sites of queuine modification with the azide probes, the azide probes would be examined.

Analyses of the azide probes interactions with tRNA guanine transglycosylase were undertaken. This provided the information required to examine if the azide probes are substrates for TGT, which would present an opportunity to discover novel sites of queuine incorporation into RNA. Incorporation of a probe with an azide would allow for subsequent Staudinger ligation or click reaction to a tethered biotin. The attached biotin would allow for isolation and concentration by streptavidin affinity chromatography. Experiments to determine conditions for Staudinger ligation and click reaction with the synthesized azide congeners of preQ₁ thus were also an aspect of the second objective. Evaluating the generated azide probes as a means to identify specific sites of queuine would complete the second phase of the project.
Chapter II

Synthesis of Queuine and PreQ₁

The first objective of our work was the synthesis of queuine and preQ₁ as both were required for the desired kinetic analysis and prevalence determinations: preQ₁ as substrate for bacterial TGT and queuine as substrate for the human version of the enzyme. The overall routes required a design that would provide a straightforward means of incorporating tritium into the molecule in the final steps to provide the desired radio-labeled substrates. A survey of the literature, as fully outlined in first chapter, shows that there are three previous reports on the synthesis of queuine. That of Kondo et al.⁵⁹ proceeds via a Schiff base between a protected pyrrolopyrimidine aldehyde and protected queuine side chain amine in a total of 19 steps. The synthesis of Akimoto et al.⁶¹ is much shorter and proceeds via a Mannich reaction to incorporate the protected side chain of queuine regioselectively to the C-5 position of a pyrrolopyrimidine precursor. Its main drawback is the requirement of several equivalents of the side chain in a key exchange reaction. The more recent synthesis of Grub et al.⁶² employs a different disconnection of the molecule, proceeding via a key Mitsunobu reaction to introduce the cyclopentenylamine side chain and a subsequent cyclocondensation reaction to build up the core heterocyclic moiety. While relatively straightforward and efficient, it requires 11 linear steps. Our strategy was to incorporate the best features of previous syntheses toward developing a convergent synthesis in which easily accessed side chain amine and core heterocyclic moieties
would be condensed via a reductive amination step. Use of the queuine side chain in the reductive amination would provide queuine; whereas, the use of a protected amine as an ammonia surrogate would provide preQ$_1$. Such a scheme would not only provide an efficient route to queuine and preQ$_1$ (and other amine congeners) but allow a simple means for incorporation of a tritium radiolabel at the end stage of the synthesis, a feature lacking in earlier routes.

The most direct route to the desired heterocyclic moiety of queuine was determined to be via elaboration of readily available preQ$_0$ (3)$^{63}$ (Scheme 2-1). The synthesis of preQ$_0$ had been accomplished previously in the Townsend and Gangjee Laboratories$^{63}$, 77c. The respective procedures differ principally in the method of synthesis of chloro(formyl)acetonitrile, 2. The Gangjee lab utilized toluene as the solvent, and isolated chloro(formyl)acetonitrile by extraction before proceeding to the subsequent cyclization. The procedure used by the Townsend lab generated the reagent in THF and then proceeded directly without isolation to the cyclization. Both groups reported satisfactory yields, so each procedure was examined to determine which would best provide preQ$_0$ for our overall synthesis. The procedure of the Townsend lab proved to be the more reproducible method and therefore was used.

Thus, in a slight modification of the procedure of Migawa et al.$^{63}$, condensation of in situ-generated chloro(formyl)acetonitrile (2) with 2,4-diamino-6-hydroxypyrimidine (1) gave preQ$_0$, which was difficult to enter into further reaction due to its poor organic solubility. We decided to address this by evaluating different methods of silylation. Initial attempts with neat hexamethyldisilazane catalyzed by ammonium sulfate were
unsuccessful, likely due to limited silylation. A solution to this was drawn from the procedure of Barnett and Kobierski\textsuperscript{82} to first silylate 3 with excess $N,O$-bis(trimethylsilyl)acetamide (BSA) and after removing volatiles in vacuo, treat the residual oil with trityl chloride in pyridine to provide tritylated preQ\textsubscript{0} (4). The site of tritylation, while not important for subsequent reactions, was determined by $^1$H NMR to be on the N-2 position.

\textbf{Scheme 2-1:} Elaboration of preQ\textsubscript{0} to key aldehyde

During procedure optimization for the formation of chloro(formyl)acetonitrile, it was discovered that the reagent could be isolated as the stable sodium salt. Employing toluene as opposed to THF afforded the salt as a precipitated product. The salt remains stable for at least a year when stored in a desiccator. This was confirmed by standard analysis and utilizing the reagent in the synthesis of preQ\textsubscript{0}, which is accomplished in good yield (96\%). Toward assessing general utility, a methodology study with the salt of reagent 2 was carried out. Reactions with 6-aminouracil, cytosine and 6-amino-2-(methylthio)-4-pyrimidinol were subjected to various conditions evaluated for the formation of preQ\textsubscript{0}. These included several solvent systems (water, DMF, acetonitrile, 50\% aqueous DMF) as well as variations in monomer equivalents and temperature. Reaction with 6-amino-2-(methylthio)-4-pyrimidinol returned starting material; whereas, attempted reactions with cytosine resulted in complex mixtures. Experiments
with 6-aminouracil typically returned starting material but did yield product (19 %) in 50% aqueous DMF buffered with sodium acetate. The reaction was not reproducible with several runs resulting in recovered starting materials. A review of the literature showed only one example (2,4,6-pyrimidinetriamine⁶) besides that of pyrimidine 1 (or pyrimidine 1 modified with an N-4 alkyl chain) which cyclized in the same manner. Hence, the reagent appears to have limited utility in the synthesis of annulated pyrrole heterocycles.

Several trials were attempted to transform nitrile 4 to desired aldehyde 5 with DIBAL-H before a reproducible method with an acceptable yield was found. Our initial trials attempted to reproduce the results of Olgen et al.⁸³, but yields were poor and quite variable. Additional conditions such as preforming lithium or sodium salts were evaluated, as well as catalytic methods (PtO₂, Raney nickel) described earlier⁷⁷b. None were satisfactory. A key breakthrough was to utilize silylation of the heterocycle to impart sufficient solubility such that reduction could be conducted at a lower temperature. Hence, protected nitrile 4 was silylated with HMDS in refluxing toluene, and upon removal of volatiles the remaining glass-like solid was dissolved in dichloromethane and cooled to -78 ºC. DIBAL-H was then added to complete the transformation to 5 after mild acidic workup. Acid hydrolysis of 5 then provided highly insoluble aldehyde 6.⁴²a The chemistry shown in Scheme 2-1 completed a three-pot sequence in an overall 28 % yield to key heterocyclic aldehyde 5 for subsequent entry into a reductive amination reaction.
With the aldehyde in hand, our attention focused on synthesizing the amine side chain of queuine. A recent synthesis by Klepper et al.\textsuperscript{66} was deemed to be the best available approach and was utilized in our synthesis with some modifications. Our decision to follow this route was based on its length, overall yield and the fact that it avoids a [3,3] sigmatropic rearrangement of an allylic azide intermediate that leads to racemization, which occurs in earlier routes employing similar intermediates\textsuperscript{60}. Klepper et al. were able to suppress this by conducting the Mitsunobu reaction and subsequent Staudinger reduction in the same pot at a reduced temperature.

\textbf{Scheme 2-2:} Synthesis of protected queuine side chain

As shown in Scheme 2-2, the synthesis of the cyclopentenylamine side chain begins with compound 7, which is readily derived from D-ribose\textsuperscript{66}. This contains the requisite chirality for the two alcohol functions in queuine. The reactions from 7 to alcohol 10 were conducted similarly as previously described\textsuperscript{65a, 84} although minor adjustments were made in several steps (see Experimental Procedures). One key modification was in the Horner-Wadsworth reaction (from 8 to 9), which was found to be more reproducible when a shorter reaction time was used as demonstrated earlier
by Smith et al.\textsuperscript{84b} For the key final step (10 to 11), we modified the procedure of Klepper et al. to avoid the use of the hazardous hydrazoic acid in the Mitsunobu reaction. Utilizing diphenylphosphoryl azide (DPPA) instead, 11 was procured in good yield with careful control of temperature followed by \textit{in situ} Staudinger reduction. The four-pot route from 7 to 11 proceeded in 14 % overall yield and was highly reproducible.

Before completing the synthesis, the chiral purity of the amine needed to be verified due to possible racemization of the azide intermediate to 11. As shown in Scheme 2-3, this was accomplished by generating the Mosher amide under standard amidation conditions\textsuperscript{85}. Subsequent \textsuperscript{19}F NMR demonstrated that only one enantiomer was present. This was confirmed by comparing the spectrum of the Mosher amide of 11 to that of racemic 11, synthesized via an alternate route that failed to suppress the [3.3] sigmatropic rearrangement of the allylic azide (see Spectra Data).

\textbf{Scheme 2-3:} Synthesis of Mosher amide of protected queuine side chain

With key reaction partners aldehyde 5 and amine 11 in hand, the synthesis of queuine was completed. As shown in Scheme 2-4, this was done under standard reductive amination conditions to provide penultimate adduct 14 in near quantitative yield. This was entered directly into a global deprotection with methanolic HCl to provide queuine which precipitated out of solution as the monohydrochloride salt in 87% yield.
Earlier methods used to generate preQ\(_1\) (17) have suffered from poor yields\(^{59, 77b}\). Therefore, in Scheme 2-5 a synthesis building on prior experience with aldehyde 5 was developed. The reductive amination of aldehyde 5 with trityl amine was determined to be the best way to proceed after considering a variety of ammonia surrogates. Reductive aminations with benzyl amine, 4-methoxybenzylamine (PMB) and 2,4-dimethoxybenzylamine (DMB) all proved successful but their respective adducts were difficult to deprotect. For these adducts, attempts at acidic deprotection failed as only the trityl was removed leaving the other amine still protected with the PMB or DMB. Hydrogenolysis techniques using palladium on carbon appeared successful, but the resulting product, preQ\(_1\), was difficult to isolate from the reaction mixture due to its insolubility. Given the small scale for the reaction that is requisite for radiolabeling, this was not a plausible scenario. Therefore, trityl amine was employed to accomplish our goal, as depicted in Scheme 2-5. The reductive amination required heating to 70 °C to achieve reaction but proceeded well giving a 79 % yield. Global deprotection was possible with methanolic-HCl in high yield (89 %), and afforded a simple isolation of the product as the monohydrochloride salt.
Scheme 2-5: Reductive amination and deprotection to produce preQ₁

With convergent routes developed for the syntheses of queuine and preQ₁ which shared aldehyde 5 as a common intermediate, all that remained was to synthesize each molecule with a tritide source as opposed to the hydride source used previously in the reductive amination step. Moravek Biochemicals was contracted to complete the synthesis of tritium labeled versions of the molecules. The intermediate imines of each were prepared and purified by silica gel flash chromatography. Moravek Biochemicals utilized NaBT₄ to complete the reductive aminations, which tritium labeled the molecules. The previously described global deprotection gave the desired products, which were verified by NMR and mass spectrometry.

The generated tritium labeled queuine and preQ₁ were utilized in a study to determine differences in human and E. coli TGT³. During that work, investigations of each substrate versus wild-type enzyme verified previously reported kinetics and therefore validated our syntheses of the substrates, with data presented in Figure 2-1. This validation allowed for the subsequent results to be reported with confidence and demonstrated the utility of our concise, convergent synthesis of queuine and our short synthesis of preQ₁.
In summary, we developed a short, concise syntheses of queuine and preQ₁, which we believe addresses limitations of earlier syntheses. The synthesis of queuine proceeds in an overall 24 % yield in a five-pot linear sequence from starting pyrimidine 1, and the synthesis of preQ₁ is completed in an overall 20 % yield. Our routes demonstrate the utility of silylation to facilitate reactions of various pyrrolo[2,3-<i>d</i>]pyrimidine intermediates, which otherwise would be difficult to conduct reliably. Furthermore, our syntheses, especially in the facile generation of aldehyde 5, offers the
possibility of easily accessing related pyrrolo[2,3-\textit{d}]pyrimidines as well as making further analogues of queuine.

**Methods and Materials**

Glassware was oven-dried before use for reactions run under anhydrous conditions. Melting points were determined in open capillary tubes on a Laboratory Devices Mel-Temp apparatus and are uncorrected. The NMR spectra were recorded on a Bruker instrument at 500 MHz for $^1$H and 125 MHz for $^{13}$C spectra. Chemical shift values are recorded in $\delta$ units (ppm). Mass spectra were recorded on a Micromass TofSpec-2E Matrix-Assisted, Laser-Desorption, Time-of-Flight Mass Spectrometer in positive ESI mode unless otherwise noted. TLC was performed on EM Science aluminum baked silica gel (SiO$_2$) plates. Visualization was performed with 254 nm UV light, potassium permanganate stain to identify olefins, and 2,4-dinitrophenylhydrazine (2,4-DNP) stain to identify aldehydes. Toluene and pyridine were distilled over CaH$_2$. All other reagents were commercially available and used as received.

**Experimental Procedures:**

\[
\begin{align*}
\text{Cl} & \quad \text{CHO} \\
\text{CN} & \quad 2
\end{align*}
\]

**Chloro(formyl)acetonitrile (2).** A dry RB flask with a stir bar and under a blanket of N$_2$ was charged with a suspension of NaOMe (7.14g, 132 mmol) in THF (90 ml). The suspension was cooled to $-5 \, ^\circ$C in a water ice-salt bath. Methyl formate (9.0 mL) was
added in a slowly over about 60-90 sec and the solution temperature was maintained for 15-20 min. Chloroacetonitrile (8.33 mL, 132 mmol) was added drop-wise over ~45 min from an addition funnel with each droplet resulted in a local discharge of yellow color. The mixture was maintained at –5 °C for 2 h during which a very fine suspension developed and the solution color became a pale orange. The bath was removed and the mixture was allowed to come to rt over about a 1 h period. TLC (EtOAc) of an aliquot quenched with a few drops of concd HCl showed a dominant product spot (Rf 0.45). The mixture was ice-cooled and treated drop-wise with concd HCl (12 mL) during which the color deepened, eventually becoming cherry red. The suspension was then filtered over a small pad of Celite to collect the NaCl and the pad was washed with EtOAc until the wash was nearly colorless. The collected filtrate was concentrated in vacuo at 40–45 °C until most of the volatiles had been removed. The solution of 2 was used immediately or after being well purged with N₂ stored in the freezer for future use.

**Chloro(formyl)acetonitrile (Sodium salt of 2).** A dry 250 mL 3-neck RB flask was equipped with a mechanical stirrer and an addition funnel. The flask was charged with a suspension of NaOMe (10.8 g, 200 mmol) in toluene (100 mL), which was cooled to –5 °C in a water ice-salt bath. Methyl formate (37.0 mL, 600 mmol) was added slowly over 5 min and the solution temperature was maintained for 15-20 min. Chloroacetonitrile (12.66 mL, 200 mmol) was added drop-wise over ~1 h from the addition funnel with each droplet resulting in a local discharge of yellow color. The mixture was kept at –5 °C
for 3 h and then allowed to come to room temperature, becoming a thick buttery yellow suspension after 16 h. The suspension was filtered on a glass Buchner fritted funnel and the collected white solid was washed with EtOAc and dried under vacuum overnight to give 23.2 g (93 %) of product: $^1$H NMR (DMSO-$d_6$) $\delta$ 8.50, 8.16 (result of two possible isomers of salt; 7:1 ratio of integrals for peaks 8.16:8.50); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 168.23, 126.55, 67.78; The material was stored in a desiccator at room temperature. The material maintained its physical and chemical properties for over a year, giving comparable yields in subsequent reactions.

![chemical structure](image)

2-Amino-4,7-dihydro-4-oxo-3H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile (3; PreQ$_0$ from Sodium salt of 2). A RB flask was charged with a stir bar, anhydrous NaOAc (0.065 g, 0.793 mmol), 2,6-diaminopyrimidin-4-one (0.10 g, 0.793 mmol), the sodium salt of 2 (0.249 g, 1.982 g), glacial acetic acid (0.124 mL) and distilled water (3.2 mL). The rapidly stirring suspension was warmed to 50 °C for 16 h and then refluxed for 1 h. The resulting suspension was cooled to room temperature and collected on a glass Buchner fritted funnel. The light brown solid was washed with portions of water and then acetone. Vacuum drying provided 0.133 g (96 %) of 3: mp 360 °C (dec); $^1$H NMR (DMSO-$d_6$) $\delta$ 11.98 (br s, 1H) 10.74 (br s, 1H), 7.59 (s, 1H), 6.43 (s, 2H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 158.0, 154.3, 152.1, 128.2, 116.4, 99.2, 86.0.
2-Amino-4,7-dihydro-4-oxo-3H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile (3; PreQ<sub>0</sub> from free form of 2). A RB flask was charged with a stir bar, anhyd NaOAc (18.2 g, 222 mmol), 2,6-diaminopyrimidin-4-one (1; 13.32 g, 105.6 mmol) and distilled H<sub>2</sub>O (170 mL). The rapidly stirring suspension was warmed to 50 °C, and then treated drop-wise with the aqueous solution (~40 mL) of chloro(formyl)acetonitrile 2 (from previous step) over a ~1.5 h period. The suspension became viscous and beige in color during the addition. Total volume at the end of the addition was 210-220 mL. The mixture was stirred at 50 °C for ~16 h, brought to reflux over an hour, and then refluxed for 1 h. During this time, the suspension darkened a little and became thinner. The mixture was cooled to rt and the solids were collected onto a Buchner fritted funnel. The light brown solid was washed with portions of H<sub>2</sub>O (~300 mL) and then acetone (~200 mL). The solids were then taken up in 300 mL 20 % aq KOH. After dissolution (~15 min), the deep brown solution was treated with charcoal (Norit 211). After stirring for 30 min, the mixture was filtered over Celite and the pad was washed with H<sub>2</sub>O. The filtrate was cooled in an ice bath and acidified to pH 2-3 with drop-wise addition of concd HCl. After stirring for 2 h, the viscous light beige precipitate was collected on a Buchner funnel and washed well with H<sub>2</sub>O. Vacuum drying over P<sub>2</sub>O<sub>5</sub> provided 12.62 g (68 %) of 3: mp 360 °C (dec; lit<sup>63</sup> >360 °C); <sup>1</sup>H NMR (DMSO-<i>d</i><sub>6</sub>) δ 11.98 (br s, 1H) 10.74 (br s, 1H), 7.59 (s, 1H), 6.43 (s, 2H);<br> <sup>13</sup>C NMR (DMSO-<i>d</i><sub>6</sub>) δ 158.0, 154.3, 152.1, 128.2, 116.4, 99.2, 86.0.
2-Amino-4,7-dihydro-4-oxo-3H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile (3; PreQ₀ from Sodium salt of 2). A RB flask was charged with a stir bar, anhyd NaOAc (0.065 g, 0.793 mmol), 2,6-diaminopyrimidin-4-one (0.10 g, 0.793 mmol), sodium salt of chloro(formyl)acetonitrile (0.249 g, 1.982 g), glacial acetic acid (0.124 mL) and distilled H₂O (3.2 mL). The rapidly stirring suspension was warmed to 50 °C for 16 h and then refluxed for 1 h. The resulting suspension was cooled to room temperature and collected on a glass Buchner fritted funnel. The light brown solid was washed with portions of H₂O and then acetone. Vacuum drying provided 0.133 g (96 %) of 3.

4,7-Dihydro-4-oxo-2-[(triphenylmethyl)amino]-3H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile (4). A suspension of nitrile 3 (2.10 g, 12 mmol) and DMF (24 mL) under nitrogen was treated drop-wise with N,O-bis(trimethylsilyl)acetamide (BSA; 8.9 mL, 36 mmol). The suspension was stirred until complete solution was attained (1.5 h) and then for a further 1.5 h, and concentrated in vacuo overnight to remove DMF and volatiles. The residual oil was diluted with pyridine (24 mL) and the solution was treated with trityl chloride (4.01 g, 14.4 mmol). The mixture was stirred under N₂ at 40 °C for 24 h and then poured into 5 % aq NaHCO₃ (100 mL). The precipitated solid was collected,
washed with H₂O, air dried for 1 h, triturred in CH₃CN, and oven dried to leave 3.22 g (64 %) of 4: mp 195-197 °C; Rf 0.57 (EtOAc); ¹H NMR (DMSO-d₆) δ 11.85 (br s, 1H), 10.69 (br s, 1H), 7.61 (s, 1H), 7.45– 7.20 (m, 15H), 6.46 (s 1H); ¹³C NMR (DMSO-d₆) δ 157.5, 151.6, 150.3, 14145.1, 134.3, 129.0, 128.2, 127.1, 116.1, 100.1, 85.9, 70.6; MS m/z 418.5 (M+H)+, 440.5 (M+Na)+.

4,7-Dihydro-4-oxo-2-[(triphenylmethyl)amino]-3H-pyrrolo[2,3-d]pyrimidine-5-carboxaldehyde (5). A suspension of tritylated nitrile 4 (2 g, 4.8 mmol), 1,1,3,3,3-hexamethyldisilazane (HMDS, 2 mL), a few mg of (NH₄)₂SO₄, and toluene (4 mL) was heated at reflux until solution was achieved and then for 1 h more. The solvent and excess reagent were removed in vacuo and the resulting oil was dissolved in CH₂Cl₂ (20 mL). The mixture was then cooled in a dry ice-acetone bath and DIBAL-H (6.4 mL of a 1.0 M solution in CH₂Cl₂) was added drop-wise over ~15 min. A TLC taken after 3 h (spraying the plate with 2,4-DNP solution to monitor aldehyde formation) showed about a 75 % conversion to 5 and the initial solution had turned into a light-colored suspension. Another 2.4 mL of DIBAL-H solution was added and stirring was continued for an additional 3 h. The suspension was quenched with 3.5 mL of 9:1 H₂O:HOAc and the bath was allowed to come to rt. The suspension was diluted with 60 - 80 mL of 1:1 EtOAc:H₂O and stirred until the solids had broken up and two layers separated on standing. The layers were separated and the aq phase was further extracted with
EtOAc. After washing the combined extracts with brine and drying over MgSO₄, the EtOAc extract was slowly filtered over a pad of flash SiO₂ (on a frit) which was eluted with EtOAc until TLC showed the absence of product. The combined product fractions were concentrated to a solid residue that was triturated in minimal hot EtOAc. After cooling, the solids were collected by filtration and dried to leave 987 mg (49%) of 5 as a crème-colored solid: mp 257 °C (dec); Rₓ 0.33 (EtOAc); ¹H (DMSO-d₆) δ 11.86 (br s, 1H), 10.66 (br s, 1H), 10.02 (s, 1H), 7.57 (s, 1H), 7.31 (s, 15H), 7.22 (s, 1H); ¹³C NMR (DMSO-d₆) δ 185.4, 158.5, 151.5, 151.2, 145.2, 129.1, 128.2, 127.1, 124.3, 120.1, 99.3, 70.6; MS m/z 421.2 (M+H)⁺, 443.2 (M+Na)⁺; HRMS (ESI) m/z 443.1485 [(M+Na)⁺; calcd for C₂₆H₂₀N₄NaO₂⁺: 443.1484]. Processing of the mother liquor by flash SiO₂ column chromatography, eluting with EtOAc, provided 322 mg (16%) of a second crop of slightly less pure 5, which was suitable for use in subsequent reactions.

2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidine-5-carbaldehyde (6). (a) From deprotection of 5: A suspension of 100 mg of aldehyde 5 and 2 mL of 9:1 HOAc:H₂O was stirred at rt for 20 h. The initial orange mixture became a lighter shade of orange as the reaction progressed. The suspension was diluted with ~3 mL H₂O and the solids were collected, washed well with H₂O. The solids were triturated in MeOH, collected and dried to leave 30 mg (71 %) of 6 as a pale orange solid: mp 280 °C, dec (lit² >300 °C, dec); Rₓ 0.3 (20:2:2:1, EtOAc:EtOH:acetone:H₂O); ¹H (DMSO-d₆) δ 12.01 (br s, 1H), 10.69
(br s, 1H), 10.06 (s, 1H), 7.52 (s, 1H), 6.35 (br s, 2H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 185.5, 159.0, 153.8, 153.4, 124.5, 120.4, 98.4; MS (negative mode) $m/z$ 177.0 (M-1)$^+$; 

(b) From DIBAL-H reduction of 3: Silylation of nitrile 3 (3.0 g, 17.1 mmol) with (NH$_4$)$_2$SO$_4$ (0.23 g, 1.7 mmol), HMDS (25 ml, 120 mmol) and toluene (50 mL) followed by DIBAL-H (22.3 mL of CH$_2$Cl$_2$ solution; 22.3 mmol) reduction in CH$_2$Cl$_2$ (40 mL) was carried out as described above for the synthesis of aldehyde 5. After 2 h TLC was performed to check reaction progress. Additional DIBAL-H (22.3 mL, 22.3 mmol) was added after 2 h and the reaction was allowed to come to rt overnight. The reaction was quenched with 9:1 HOAc:H$_2$O, and the precipitated solids were collected and washed with H$_2$O. The solids were suspended in H$_2$O and treated with 5N aq KOH until solution was achieved. The solution was clarified with charcoal, and then filtered over a pad of Celite. The filtrate was acidified to pH 3 with 6N aq HCl to precipitate solids, which were collected, washed with H$_2$O, and dried to leave 1.38 g (45%) of pure 6.

1-Methoxy-2c2-isopropyldien-α/β-D-ribofuranose (6α/6β) (7). A solution of D(-)-ribose (16.3 g; 108 mmol) and 2,2-dimethoxypropane (33 mL; 269 mmol) in acetone (150 mL) was cooled in a water ice-salt bath and perchloric acid (aq. 70 %, 46 73 mmol, 6.5 mL) was added dropwise. The water ice bath was removed and the solution was stirred at room temperature for 2 h. Methanol (23 mL; 568 mmol) was added and the solution was stirred overnight during which it turned orange. The solution was cooled in
an water ice-salt bath and a solution of sodium bicarbonate (12.8 g in 40 mL H₂O) was added. The resulting salt was filtered off and the filtrate was evaporated to approximately 30 mL and extracted with dichloromethane. The organic layers were combined and washed with brine and dried over MgSO₄. This was then rotary evaporated to an oil and vacuum pumped to give the product as a yellow oil, 20.5 g (93%). \( R_f = 0.65 \) (pentane: EE, 7:1) NMR data were identical to those previously reported\(^6^6\).

\[ \text{(1R,5S,8R)-8-Methoxy-3,3-dimethyl-2,4,7-trioxabicyclo(3.3.0)octan-6-one (8).} \]

The following is a slight variation of a previously reported procedure\(^6^6\). Benzene (300 mL) was added to a 3-neck RB flask equipped with a mechanical stirrer, Dean-Stark tube, and a reflux condenser. Following a 1 h benzene reflux to remove any H₂O, 1-methoxy-2,2-isopropyliden-\( \alpha/\beta \)-D-ribofuranose (6\( \alpha/\beta \)) (7; 8.0 g, 3.6 mmol) was added to the flask followed by pyridinium chlorochromate (PCC; 33.6 g, 156 mmol). The mixture was refluxed for 16 h, cooled and the benzene was decanted off. The PCC residue was then stirred with CH₂Cl₂ and the mixture was allowed to stand before the CH₂Cl₂ layer was decanted off. The combined organic phases were concentrated to 50 mL, filtered through Celite, and further concentrated to 10 mL. The solution was filtered through a SiO₂ plug, using 1:1 hexanes:EtOAc as the eluant. The filtrate was concentrated to an oil that was pumped in vacuo until a solid formed. The solids were crystallized from hot
hexanes to leave 3.21 g (46 %) of 8: mp 82-83 °C (lit\(^3\) 75-76 °C); R\(_f\) 0.63 (EtOAc); NMR data were identical to those previously reported\(^6\).

![Chemical structure of 9](image)

**(4R,5S)-4,5-Isopropylidenedioxy-3-cyclopentenone (9).** The following is a slight variation of a previously reported procedure\(^6\). Dimethyl methylphosphonate (0.66 mL, 6.17 mmol) was added to a flask containing THF (35 mL). The flask was cooled to −78 °C and n-BuLi (3.86 mL; 1.6 M in hexanes) was added drop-wise over 10 min. After stirring for 30 min, compound 8 (1.16 g, 6.17 mmol) was dissolved in THF (5 mL) and added in one portion to the reaction solution. The reaction was stirred at −78 °C for 2.5 h then plunged into a water ice bath for 30 min. The reaction was quenched with the successive addition of 10 mL each of EtOAc followed by H\(_2\)O, and the mixture was stirred for 10 min. The mixture was then diluted with H\(_2\)O and the aq phase was saturated with NaCl and extracted with EtOAc (7x). The combined extracts were washed with brine, dried over Na\(_2\)SO\(_4\), and concentrated to a yellow oil. The oil was filtered through a plug of SiO\(_2\), washing with 7:3 hexanes:EtOAc. The eluant was concentrated to leave 0.44 g (47 %) of a clear oil that formed white needles of 9 upon standing: mp 70 °C (lit\(^3\) 71 °C; NMR data were identical to those previously reported\(^6\).
(3R,4R,5S)-3-Hydroxy-4,5-isopropyldenedioxy-cyclopentene (10). The following is a slight variation of a previously reported procedure. A solution of enone 9 (0.25 g, 1.62 mmol) and CeCl$_3$·7H$_2$O (0.604 g, 1.62 mmol) in MeOH (6.5 mL) was cooled in a water ice bath. The solution was then treated portion-wise with NaBH$_4$ (0.123 g, 3.24 mmol) and the bath was removed. The suspension was stirred for 1 h, cooled, and quenched by drop-wise addition of 1N aq HCl such that solution was achieved and pH >4 was maintained. The quenched solution was diluted with H$_2$O and extracted with EtOAc (7x). The combined extracts were washed with brine, dried (MgSO$_4$), and concentrated in vacuo to leave 0.243 g (96 %) of 10 as a pale yellow oil: NMR data were identical to those previously reported.

(35,4R,5S)-3-Amino-4,5-isopropyldenedioxy-cyclopentene (11). Triphenylphosphine (0.369 g, 1.41 mmol) in THF (5 mL) was cooled to -20 °C in a dry ice acetonitrile bath. Diisopropyl azodicarboxylate (DIAD; 0.277 mL, 1.41 mmol) was added to the reaction drop-wise and stirred for 10 min, during which time the reaction became cloudy white. A solution of alcohol 10 (0.2 g, 1.28 mmol) in THF (2 mL) was added to the mixture, which was then reaction stirred for 30 min. The mixture was warmed to 0 °C in a water
ice-salt bath and diphenylphosphoryl azide (0.331 mL, 1.54 mmol) was added drop-wise. The mixture was stirred for 7 h in the water ice-salt bath after which time additional triphenylphosphine (0.369 g, 1.41 mmol) was added. The mixture was stirred for an additional 12 h in the water ice-salt bath before being quenched with H₂O (0.5 mL) and stirring was continued for 8 h. The mixture was concentrated \textit{in vacuo} and then taken up in hexanes with a minimal amount of CH₂Cl₂ to precipitate triphenylphosphine oxide byproduct, which was removed by filtration. The filtrate was then purified by two successive SiO₂ column chromatographies (eluting with CH₂Cl₂:MeOH, 7:1) to give 0.137 g (69 %) of 11 as a yellow oil: Rₓ 0.5 (3:1, CH₂Cl₂:MeOH; stained by aq KMnO₄ solution); NMR data were identical to those previously reported⁶⁶.

![Structure of compound 14](image)

5-\[\[(3aR,4S,6aS)-3a,6a-Dihydro-2,2-dimethyl-4H-cyclopenta-1,3-dioxol-4-yl]amino\]methyl]-2-[(triphenylmethyl)amino]-1,7-dihydro-4H-pyrrolo[2,3-d]pyrimidin-4-one (14). Aldehyde 5 (0.030 g, 0.071 mmol), amine 11 (0.017 g, 0.107 mmol) and Na₂SO₄ (0.041 g, 0.285 mmol) were suspended in MeOH (0.6 mL) and the mixture was stirred at rt for 6 h. When complete the reaction mixture was filtered and concentrated \textit{in vacuo} to give the imine, which was verified by mass spectrometry. The imine was added to MeOH (0.6 mL) and the suspension was cooled in a water ice-salt bath. NaBH₄ (0.0054 g, 0.143 mmol) was added and the bath removed shortly after the
addition. The reaction was stirred for 4 h and then quenched with saturated aq NaHCO₃ (0.6 mL) followed by stirring for 1 h. The mixture was diluted with H₂O and extracted with EtOAc (3x). The combined organics were washed with brine, dried (Na₂SO₄) and concentrated in vacuo to leave 0.057 g (99 %) of 14 as beige crystals: mp 175 °C (dec); R₆ 0.15 (9:1, EtOAc:MeOH); ¹H NMR (DMSO-d₆) δ 10.63 (br s, 1H), 10.34 (br s, 1H), 7.42 (s, 1H), 7.30 (d, J = 3.0, 12H), 7.21 (d, J = 3.1, 3H), 6.36 (s, 1H), 5.82 (d, J = 4.9, 1H), 5.76 (d, J = 5.6, 1H), 5.13 (d, J = 4.7, 1H), 4.35 (d, J = 5.6, 1H), 3.65 (d, J = 13.7, 1H), 3.55 (d, J = 7.7, 1H), 1.25 (s, 3H), 1.23 (s, 3H); MS m/z 560.2 (M+H)+; HRMS (ESI) m/z 560.2673 [(M+H)+; calcd for C₃₄H₃₄N₅O₃+-560.2662].

2-Amino-5-[[1S,4S,5R]-4,5-dihydroxy-2-cyclopenten-1-ylamino)methyl]-1,7-dihydro-4H-pyrrolo[2,3-d]pyrimidin-4-one, monohydrochloride (15; Queuine). Compound 14 (0.020 g, 0.036 mmol) in an oven dried flask charged with a stir bar was dissolved in 1.25 M methanolic HCl (0.3 mL). The solution was stirred at rt for 2.5 h during which a solid precipitated out. The solid was collected, washed with EtOAc, and dried in a vacuum oven to give 9.8 mg (87%) of 15 as a monohydrochloride salt: R₆ 0.49 (3:1, MeCN:0.2 M NH₄Cl); mp 210 °C (dec).; ¹H NMR (DMSO-d₆) δ 11.38 (br s, 1H), 11.07 (br s, 1H), 9.46 (d, J = 19.3, 2H), 6.88 (s,1H), 6.61 (br s, 2H), 6.15 (d, J = 6.3, 1H), 5.97 (d, J = 6.1, 1H), 4.46 (d, J = 4.0, 1H), 4.32 (d, J = 14.0, 1H), 4.27 (d, J = 13.9, 1H), 4.13 (t, J = 5.3, 1H), 4.04 (d, J = 4.5, 1H); ¹³C NMR (DMSO-d₆) δ 160.5, 153.1, 139.1, 128.8, 118.1, 109.1, 98.8, 74.1,
73.1, 66.9, 42.0, 29.4; MS m/z 278.2 (M+H)^+, 300.2 (M+Na)^+; HRMS (ESI) m/z 278.1254 [(M+H)^+; calcd for C_{12}H_{16}N_{5}O_{3}: 278.1253].

2-(Tritylamino)-5-((tritylamino)methyl)-3H-pyrrolo[2,3-d]pyrimidin-4(7H)-one (16). A mixture of aldehyde 5 (100 mg, 0.24 mmol), trityl amine (74 mg, 0.29 mmol) and sodium sulfate (68 mg, 0.48 mmol) was added to an oven-dried flask charged with a stir bar. To this was added THF (1.2 mL) and the reaction mixture was stirred at reflux for 6 h. The cooled mixture was filtered and concentrated in vacuo to give imine 6. The imine was resuspended in THF (1.2 mL) and cooled to 0 °C in a water ice bath. Sodium borohydride (18 mg, 0.48 mmol) was added portion-wise and the bath removed shortly after the addition. The mixture was stirred for 3 h, carefully quenched with water (1.2 mL) and stirred for an additional 1 h. The quenched reaction mixture was then further diluted with water and extracted three times with ethyl acetate (adding a few drops of methanol to help resolve the layers). The combined extracts were washed with brine, dried over sodium sulfate and concentrated to a residue that was purified by flash silica gel chromatography. Elution with ethyl acetate (~100 mL) followed by concentration in vacuo of product fractions left 125 mg (79 %) of 16 as a yellow solid: mp 175 °C (dec); R_f = 0.7 (ethyl acetate); ^1H NMR (DMSO-d_6) δ 10.64 (br s, 1H, exchanges D_2O), 10.20 (br s, 1H, exchanges D_2O), 7.31-7.16 (m, 31H), 6.38 (s, 1H), 3.79 (t, J = 8.1, 1H, exchanges D_2O) 2.98 (d, J = 8.0, 2H); ^13C NMR (DMSO-d_6) δ 159.52, 150.45, 149.93, 146.71, 145.48,
2-Amino-5-(aminomethyl)-3H-pyrrolo[2,3-d]pyrimidin-4(7H)-one, monohydrochloride (17; preQ₁). A suspension of protected preQ₁ (7; 25 mg, 0.04 mmol) and methanolic HCl (2 mL of 1.25 M solution) was heated at reflux for 2 h. After reaching initial solution, crystalline product precipitated out as the hydrochloride began to form. The suspension was cooled to room temperature and the precipitate was collected onto a frit, washed with methanol, and dried to leave 6 mg (74 %) of 1 as a white solid: mp 225 °C (dec); ¹H NMR (DMSO-d₆) δ 11.29 (br s, 1H, exchanges D₂O), 11.06 (br s, 1H, exchanges D₂O), 8.26 (br s, 3H, exchanges D₂O), 6.79 (s, 1H), 6.61 (br s, 2H, exchanges D₂O), 4.02 (d, J = 5.2, 2H; s with D₂O wash); ¹³C NMR (DMSO-d₆) δ 160.41, 153.09, 151.55, 116.77, 111.04, 98.79, 35.81; MS m/z 180.2 (M+H)⁺, 202.2 (M+Na)⁺.

2-Amino-5-(aminomethyl-²H)-3H-pyrrolo[2,3-d]pyrimidin-4(7H)-one (17; ²H-labeled preQ₁). Reaction of preformed imine 16 was carried out with ²H-sodium borohydride as described above for the synthesis of cold-labeled 16. The derived 16 was then subjected to the same deprotection reaction conditions as described above for the
synthesis of 17. The collected product 17 had a specific activity of 7.0 Ci/mmol. It showed a chemical purity of 99.5 % by HPLC using UV detection at 256nm and a radiochemical purity of 96.1 % by HPLC using radiochemical detection. See Appendix for further characterization data. Note: this reaction was carried out for a fee by Moravek Biochemicals.

2-Amino-5-[[[1S,4S,5R]-4,5-dihydroxy-2-cyclopenten-1-yl]amino]methyl-^3H]-1,7-dihydro-4H-pyrrolo[2,3-d]pyrimidin-4-one, monohydrochloride (15; ^3H-labeled queuine). Reaction of preformed imine 14 was carried out with ^3H-sodium borohydride as previously described for the synthesis of cold-labeled 14^86. The derived 14 was then subjected to the same deprotection reaction conditions as previously described for the synthesis of 15^86. The collected product had a specific activity of 7.3 Ci/mmol. It showed a chemical purity of 94.1 % by HPLC using UV detection at 259 nm and a radiochemical purity of 99.4% by HPLC using radiochemical detection. See Appendix for further characterization data. Note: this reaction was carried out for a fee by Moravek Biochemicals.

Chapter III
Investigating the Prevalence of Queuine in *Escherichia coli* RNA via Incorporation of the Tritium-labeled Precursor, PreQ₁

It is known that the key enzyme responsible for the queuine modification in *E. coli* RNA, tRNA guanine transglycosylase (TGT), recognizes a UGU sequence in an RNA hairpin loop (e.g., transfer RNA anticodon arm) as a site for modification. Previous work in our laboratory has demonstrated that modification of alternate RNA species (i.e. hairpin minihelix⁴⁴b,⁵⁸ and 800 base mRNA⁴⁵) with UGU sequences in a loop can occur *in vitro*. Whether or not the queuine modification is limited to the four instances in transfer RNA *in vivo* is a question that has remained unanswered. The interest in addressing this question has increased given that it is now appreciated that 60-70% of the human genome codes for “non-coding RNA”⁸⁷. Determining if the queuine modification is present in RNA other than the known tRNA, is important in understanding the function of the queuine modification and its role in the cell.

The incorporation of queuine into RNA via transglycosylation provides an interesting and unique point of entry to study the prevalence of this base modification as it lends itself to the utilization of designed queuine analogues. Synthetic radio-labeled versions of the TGT substrate can be utilized to track the incorporation of queuine into RNA. Eukaryae incorporate queuine obtained through their diet; whereas, eubacteria biosynthesize and incorporate the queuine precursor preQ₁ before completing the modification on the RNA to yield queuine (Figure 3-1). As a result of these differences, the enzyme (TGT) responsible for the incorporation of the modification into RNA differs between eukaryal and eubacterial organisms¹³. Tritium-
labeled preQ₁ ([³H] preQ₁) was synthesized along with tritium-labeled queuine (described in Chapter II) and both were used in a study that explored differences between human and *E. coli* TGTs, which concluded that divergent evolution from a common progenitor led to the eubacterial and eukaryal enzymes³, ⁸⁶.

**Figure 3-1**: Queuine biosynthetic pathways for eubacteria and eukarya. Note the key role of TGT and the heterocyclic substrate difference between eubacterial and eukaryal TGTs. G(34) tRNA refers to tRNA with guanine in the 34 position, the anticodon wobble base. Q refers to queuine and oQ refers to epoxy-queuine²⁵.

A number of *in vitro* and *in vivo* experiments were conducted utilizing three *E. coli* strains: a ΔqueC knockout (see Figure 3-1) that is unable to synthesize preQ₁ allowing tritium-labeled compound to be incorporated exclusively, a Δtgt knockout that is unable to incorporate preQ₁ and a wild-type *E. coli* strain. The queC gene knockout strain was chosen as queC is the first committed step in the queuine biosynthesis pathway. Deletion of queC prevents the biosynthesis of any potential TGT-substrate.
intermediates, thereby eliminating any competition for incorporation of our labeled compound by endogenous unlabeled preQ\(_1\) or other intermediates. The \(\Delta\text{tgt}\) strain served as an excellent negative control to ensure that the incorporation observed was TGT-dependent. Our studies clearly demonstrate TGT-dependent incorporation of radiolabel into ribosomal RNA and non-ribosomal/non-transfer RNA species.

Results

Total RNA was prepared from \(E.\ coli\) cultures utilizing Epicentre Biotechnologies, MasterPure™ RNA preparation kit. Development of a method to separate the RNA and detect sites of incorporation of radio-labeled preQ\(_1\) was then undertaken. We separated the RNA species on a polyacrylamide gel and attempted to quantitate incorporated radioactivity via phosphorimaging of the dried gel; however, this method was not successful in our hands (data not shown), as we were unable to detect any bands. Given the fact that digestion of agarose polysaccharide chains by the enzyme Gelase allows for isolation of gel-separated oligonucleotides (via cutting gel bands, Gelase digestion and precipitative recovery of the RNA), our focus shifted to utilizing agarose gels. Total RNA was successfully fractionated via excising bands from a low-melting agarose gel after separation by electrophoresis. After Gelase digestion of the agarose, the RNA would be ethanol precipitated and collected by filtration. The collected RNA could then be subjected to liquid scintillation counting for radioactivity quantitation. Several standards were used as rough size markers for band excision, most significant of which is tyrosine transfer RNA that had been prepared \textit{in vitro}\(^{43}\).
Gels were visualized before cutting by UV shadow onto a silica TLC plate containing a fluorescent dye; this method was utilized to avoid potential interference of liquid scintillation counting by RNA stains (e.g., ethidium bromide). Figure 3-2 shows an example of a gel (stained with ethidium bromide for illustrative purposes) with the excision grid depicted.

![Figure 3-2](image)

**Figure 3-2:** Example agarose gel of total RNA preparation. Lane A: 1 kb ladder, B: virF mRNA, C: minihelix/hairpin RNA, D: tyrosine tRNA, E: total RNA, and F: total RNA with grid showing gel bands for radioactivity determinations.

Total RNA isolated from *E. coli Δtgt* and *E. coli ΔqueC* was used for *in vitro* studies. The RNA from *E. coli Δtgt* cannot contain queuine, preQ₁ or any intermediate incorporated due to the lack of TGT. The RNA from *E. coli ΔqueC* should not contain queuine, preQ₁ or any intermediate incorporated as the biosynthesis of preQ₁ has been interrupted at the first committed step (Figure 3-1). To ensure that preQ₁ and other intermediates were not provided to the ΔqueC cultures as a nutrient, the culture media,
2xTY, consisted of a yeast extract and trypsin hydrolysate of milk casein. Neither should contain preQ₁ or queuine, as yeast does not utilize either and the milk protein casein was shown by manufacturer assay to be free of oligonucleotides. Milk protein, even if contaminated with nucleotides, would only contain queuine, which is not a substrate for the eubacterial TGT. These suppositions were borne out as both RNA preparations were substrates for pure His-tagged *E. coli* TGT *in vitro* as shown in Figure 3-3. Results are presented as a bar graph representing the radioactivity in each excised band. Band 1 is the slowest migrating RNA at the top of the gel, and band 8 is the fastest migrating RNA at the bottom of the gel. Band 7 co-migrates with authentic transfer RNA. Interestingly, radioactivity is incorporated in RNA across all bands with the majority being in bands that correlate with higher molecular weight RNAs (Figure 3-3).

**Figure 3-3:** Radioactivity in gel bands of total RNA isolated from *E. coli* TG2 and *E. coli ΔqueC* incubated *in vitro* with TGT and [³H] preQ₁. A bar graph of DPM for each band excised from enzyme and no enzyme controls (n=2). *E. coli* strains ± enzyme as depicted in inset.
As a control, incubations of total RNA with $[^3\text{H}] \text{preQ}_1$ without TGT were conducted and exhibited far lower counts (Figure 3-3), which may be due to a small amount of $[^3\text{H}] \text{preQ}_1$ non-covalently associating and co-precipitating with the RNA. Control experiments were also performed to ensure labeled tRNA would only be detected where seen in visualization. A sample of tRNA$^{\text{Yr}}$ prepared in vitro was labeled and migrated on an agarose gel. From the visualization, bands of the sample were excised and subjected to Gelase digestion followed by ethanol precipitation and scintillation. As expected the band determined to be tRNA contained counts. A small number of counts above background were observed for the adjoining slices, and all other bands gave counts indistinguishable from background.

In an effort to determine if ribosomal RNA is a substrate for TGT, a method to exclusively isolate ribosomal RNA was undertaken with the $\Delta\text{queC strain}$. The isolation was accomplished by pelleting the ribosome via ultracentrifugation. This RNA was similarly incubated in vitro with TGT and $[^3\text{H}] \text{preQ}_1$. Figure 3-4 shows that ribosomal RNAs are also substrates for TGT with low counts in bands 7 & 8. Treatment of the ribosome preparation with proteinase K shifted the counts to lower migrating bands, 4-6, consistent with dissociation of the 70S ribosome into the constituent RNAs.
Figure 3-4: Radioactivity in gel bands of ribosomal RNA fractions isolated from *E. coli ΔqueC* separated on an agarose gel from an *in vitro* incubation with TGT and [³H] preQ₁ (n=1).

With data from *in vitro* experiments indicating that the prevalence of preQ₁ and thus queuine extended beyond the previously known tRNA to be affected, efforts to verify the finding *in vivo* were carried out. As a control for the *in vivo* incubations, we used the *E. coli Δtgt* cell line. Results of total RNA isolated from *in vivo* incubations of [³H] preQ₁ with *E. coli Δtgt* were similar to standard background counts of 60 to 150 DPM (Figure 3-5). Therefore, it is clear that any radioactivity detected above 150 DPM results from TGT-catalyzed enzymatic modification of RNA (i.e., “TGT-dependent”). For a reference, counts performed during inspections of a lab are considered clean for counts under 150 (counts at those levels are a result of background radiation sources; materials in building and cosmic radiation).
**Figure 3-5:** Radioactivity (DPM) of total RNA fractions separated on an agarose gel from an *in vivo* incubation of *E. coli Δtgt* with [$^3$H] preQ₁. This experiment establishes the background for the *in vivo* experiments at less than 150 DPM and that counts in other experiments are a result of TGT-dependent incorporation of $^3$H labeled preQ₁.

Studies were then conducted under the same conditions with *E. coli ΔqueC* and TGT2 cell lines. The results shown in Figure 3-6 reveal that the greatest amount of incorporation is detected in the transfer RNA band (band 7). The experiment demonstrated that preQ₁ can be scavenged by *E. coli* and used as a nutrient. Given that the known site of TGT modification is transfer RNA and that transfer RNA is very abundant in the cell, it was not surprising to see the majority of the counts in that size range. The fact it is clear that radioactivity was present in the other bands verified the *in vitro* data and indicated that the prevalence was more widespread than previously known. Interestingly, similar results were observed with the TG2 cell line (see Discussion).
Figure 3-6: Radioactivity in gel bands of total RNA isolated from *E. coli* TG2 and *E. coli ΔqueC* incubated *in vivo* with [³H] preQ₁. Data presented is the average (n=4) DPM. Inset: data from bands 1-5 rescaled.

Ribosomes were then isolated from cultures of *E. coli ΔqueC* incubated *in vivo* with [³H] preQ₁. Half of the ribosome preparation was treated with proteinase K to digest the ribosomal proteins and dissociate the ribosomal RNAs prior to gel electrophoresis. As seen in Figure 3-7, the untreated ribosomes exhibit significant radioactivity in all bands with the lowest levels in bands 7 & 8. As for the *in vitro* experiments, treatment of the *in vivo* ribosome preparation with proteinase K shifted the counts to lower migrating bands, consistent with dissociation of the 70S ribosome into the constituent ribosomal RNAs.

To further probe if RNA species other than ribosomal RNA are modified by TGT, total RNA was isolated from an *in vivo* incubation of *E. coli ΔqueC* with [³H] preQ₁ and treated with a 5’-phosphate-dependent RNA exonuclease, which degrades ribosomal
RNA but not other RNAs. The results presented in Figure 3-8 compare the exonuclease digestion data to total RNA results from ΔqueC total RNA collected previously. The detected radioactivity decreases for bands 1-5; however, the counts remain substantially above background.

**Figure 3-7:** Radioactivity in gel bands of ribosomal RNA isolated from *E. coli* TG2 and *E. coli* ΔqueC incubated *in vivo* with [³H] preQ₁. Data presented is the average (n=4) DPM for each gel band for both isolated ribosome as well as proteinase K digest of ribosome.
Figure 3-8: Radioactivity in gel bands of 5‘-phosphate-dependent exonuclease treated total RNA isolated from E. coli ΔqueC incubated in vivo with [3H] preQ1. Data presented is the average (treated: n=2, untreated: n=4 ) DPM for each gel band. Inset: data from bands 1-5 rescaled. 5‘-Phosphate-dependent exonuclease selectively digests rRNA.

All studies to this point were conducted using agarose gels to separate RNA strands. A potential problem with this method is that it is not a denaturing system, so there is the possibility that modified tRNA could associate with other RNA species and give misleading data. To verify the previous results, the total RNA experiment was repeated using denaturing gel conditions. For separating the RNA, a 6 % polyacrylamide gel with 8 M urea was determined to be best. The total RNA was prepared as before and after isolation was diluted to a concentration of 700 ng/µL and loaded onto the gel. Visualization consisted of staining the gel with ethidium bromide for UV transillumination. Based on a tRNA standard, bands were excised for extraction of RNA and detection. Extracting the RNA from the urea polyacrylamide gels was accomplished
by soaking the bands in a 0.5 M ammonium acetate, 0.1 mM EDTA and 0.1 % SDS buffer for 12 hours. After extraction the samples were ethanol precipitated and washed with 70 % ethanol to remove impurities. The RNA was resuspended and dried onto filters for scintillation. The data in Figure 3-9 casts doubt on the previous results as the counts are near background for all but the tRNA band and adjacent bands. The polyacrylamide gel method did not allow for loading as much RNA onto the gel as in the agarose experiments.

![8 M Urea Polyacrylamide Gel, Total RNA](image)

**Figure 3-9:** Radioactivity in gel bands of total RNA isolated from *E. coli ΔqueC* incubated *in vivo* with $[^{3}H]$ preQ$_{1}$. Data presented is the average (n=2) DPM for each band excised (n=2).

In order to better analyze the PAGE experiments, two additional methods were attempted. Instead of extracting the RNA by soaking in buffer, the polyacrylamide gel was blotted onto a Zeta-Probe membrane. The resulting blot was placed in an exposure cassette with a TR phosphor storage screen in an attempt to visualize the radioactivity
from the blot. The visualization of the total RNA blot is seen in Figure 3-10 and rRNA in Figure 3-11. Only one band is visible for the total RNA isolation, and its size indicates that it is tRNA. In the visualization of rRNA two bands are visible near one another. To verify these results, the blotting membrane was cut into bands and scintillated to determine where radio-labeled preQ₁ was present on the blot. The results of these experiments for total RNA are seen in Figure 3-12 and for rRNA are seen in Figure 3-13. The result of the scintillation experiments match what is seen in the visualization.

**Figure 3-10**: Phosphor storage screen image of total RNA blot with TR screen; visualized after screen exposed to blot for 7 days.
Figure 3-11: Phosphor storage screen image of rRNA blot with TR screen; visualized after screen exposed to blot for 7 days.

Figure 3-12: Radioactivity in bands cut from Zeta-Probe membrane blot of PAGE for isolated total RNA; isolated total RNA was analysed at three concentrations named in legend, 1300 ng/µL, 650 ng/µL and 350 ng/µL; negative control consisted of blotting membrane.
Figure 3-13: Radioactivity in bands cut from Zeta-Probe membrane blot of PAGE for isolated rRNA; isolated rRNA was analysed at three concentrations named in legend, 1000 ng/µL, 550 ng/µL and 275 ng/µL; negative control consisted of blotting membrane.

Discussion

The ability to investigate the prevalence of the queuine modification was made possible by having a means to separate and detect RNA modified by TGT with tritium-labeled preQ1. The use of low-melting agarose gel electrophoresis provided a straightforward means to separate RNA by the extent of migration into the gel (correlating with RNA size), allowing us to resolve the known modified transfer RNA species from RNAs of other sizes. The low-melting agarose could then be digested to allow for precipitation and isolation of RNA from a particular band excised from the gel. As shown in Figure 3-2 by the overlaid grid, all lanes with labeled RNA were divided into 8 equal bands with band 7 representing transfer RNA as determined by control tRNATyr electrophoresed in an adjacent lane. Once band 7 was identified and excised, others
were divided and subjected to digestion, RNA precipitation/collection and detection by liquid scintillation counting. This method was determined to give sufficient resolution for our purposes by initial studies with in vitro labeled tRNA\textsuperscript{Tyr}. It was demonstrated that counts were limited in these control experiments to band 7 with a small portion in band 6. Access to \[^3\text{H}\] preQ\textsubscript{1} with this separation and detection method made the study of queuine prevalence possible.

Results of initial in vitro studies demonstrate that modification of sites other than the known transfer RNA species are indeed possible. Interestingly, while there are a significant number of counts in the transfer RNA band (Figure 3-3, band 7), the majority of the radioactivity is found in bands corresponding to RNAs that migrate into the gel to a much lower extent than transfer RNAs (e.g., bands 1-5). The reason for this is unclear. Regarding the possibility that the total RNA preparation may not capture transfer RNA efficiently, the results of the in vivo studies discussed below clearly show high levels of radioactivity incorporation in the transfer RNA band (e.g., band 7 in Figure 3-6).

The results of in vitro incubations of isolated ribosomes from \textit{E. coli ΔqueC} show that the fully formed ribosome is also a substrate for TGT (Figure 3-4). The low counts in lane 7 confirm that the counts detected are not due to transfer RNA that may have co-purified in the ribosome preparation. The proteinase K-treated sample exhibited a shift in counts from lanes 1 & 2 (likely the intact or partially dissociated 70S particle) mostly to lanes 4-6 (likely the dissociated ribosomal RNAs). Note that the \textit{E. coli} 5S rRNA is 105
bases long, similar in size to transfer RNAs (~75 bases), possibly accounting for the counts in lanes 6 & 7 of the proteinase K treated sample.

The results from the in vivo experiments on total RNA as shown in Figure 3-6 give data on one hand more consistent with expectations and yet on the other also surprising. Firstly, they show that preQ$_1$ can be taken up and utilized by E. coli as a nutrient from the media. The results also show that transfer RNA is modified to the greatest extent. This is not surprising as transfer RNAs are the species known to be modified and exist in a greater abundance than many other individual RNA species. The surprising finding is that modification occurs in RNAs from essentially all other bands (e.g., size ranges) at counts significantly above background. Background was determined with the use of TGT knockout cell line, with the data presented in Figure 3-5. The fact the modification appears to be much more widespread than the four known transfer RNA species encouraged us to attempt to identify the other types of RNA that are targeted for modification, rRNA in particular.

The isolation of 70S ribosomes followed by their subsequent separation and detection (as well as ribosomes treated with proteinase K to free the constituent rRNA) indicated that rRNA is indeed a substrate for queuine modification. The culture volumes were increased by necessity so that ribosomes could be isolated; preparations from cultures smaller than 100 mL were unsuccessful. The larger culture size also required a lower concentration of the $[^3]$H preQ$_1$ due to the scarcity and cost of the material; however, the length of the overnight incubation and the concentration of $[^3]$H preQ$_1$ slightly above its $K_M$ for TGT were evidently sufficient to result in efficient incorporation.
The data in Figure 3-7 shows the results of these experiments; additionally, Figure 3-14 shows ethidium bromide visualization with associated DPM charted. The total counts for the bands are well above background and the distribution helps to understand the pattern of results seen in the total RNA experiments. For total RNA experiments it is possible that rRNA species represent the majority of the modification seen in the upper bands of the gel, due to the fact that rRNA species are more abundant than other species, accounting for 80% of RNA. However, this would indicate that only a small portion of rRNA is modified as the counts are well below that of tRNA.

To further verify the rRNA as substrate for queuine modification, the use of a 5′-phosphate RNA exonuclease that exclusively digests rRNA was employed. The data given in Figure 3-8 compares the results to those of the total RNA experiments for the ΔqueC cell line as total RNA from ΔqueC was utilized in these experiments. It clearly shows that the counts in the slower migrating RNA species are reduced, consistent with the digestion of the rRNA to smaller fragments that might be included in bands 6-8. The fact that counts remain at slower migrating bands (e.g., 1-4) could indicate that other RNA species, such as mRNA, are sites for modification. It is unclear if there is a large number of RNAs of differing sizes that are modified in vivo or if there is a smaller number of larger RNAs that are modified in vivo and we are seeing them as well as their degradation products throughout bands 1-5. Further studies to isolate and identify the modified RNAs are in process.
**Figure 3-14:** Example gel and radioactivity of ribosomal RNA (untreated and proteinase K treated) fractions separated on an agarose gel from an *in vivo* incubation of *E. coli ΔqueC* with [3H] preQ1. I: 2 % low-melting agarose gel stained with EtBr to visualize bands; A: 100 bp ladder, B: 70s ribosome, C: proteinase K digested 70s ribosome, D: tyrosine tRNA standard. Bands were excised based on this visualization as represented by the grid on the gel and quantified by liquid scintillation counting. II: Chart presents results in DPM for each band.

However, attempts to confirm these results via denaturing PAGE proved problematic. Denaturing PAGE conditions were not utilized for the entire study as they do not allow for loading as much RNA, which reduces the counts seen for each band making it more difficult to detect low abundant species that are modified. The results from the agarose experiments could be the result of association of modified tRNA with other RNA species such as rRNA, which to some extent might be predicted. The results from PAGE analysis followed by soaking the excised bands to detect the presence of radio-label gave counts above background for all the bands but not significantly above background except in the case of the tRNA and adjacent bands, as seen in Figure 3-9.
Subsequent experiments with PAGE were conducted in an attempt to find a more definitive confirmation of the results from the agarose experiments. The polyacrylamide gels were run at three concentrations of isolated RNA (either total or rRNA) and were blotted onto a Zeta-Probe membrane. With the RNA on the surface of the blotting membrane, visualization of the radio-labeled RNA was carried out with a TR phosphor storage screen. The total RNA visualization showed one distinct band that is tRNA. The rRNA visualization showed two faint bands. It is possible that only tRNA is modified, as would be indicated by the total RNA visualization, but the presence of two spots in rRNA visualization is problematic. Unless, the 8 M urea denaturing condition were not sufficient to disrupt the interaction of rRNA and modified tRNA in this rRNA enriched sample. The blots were subsequently cut into bands, as the agarose gels had been excised into bands for scintillation counting. The scintillation results match those of the visualization and do not help resolve the uncertainty. The counts in these experiments, even for the visualized bands, are lower than seen in the agarose experiments and might indicate there is just insufficient material loaded with the PAGE methods to detect modified low abundance RNAs.

An interesting observation from our in vivo work with total RNA, as seen in Figure 3-6, is that observed incorporation of radio-labeled preQ₁ is nearly identical for the TG2 and ΔqueC cell lines. It is known that queuine biosynthesis is controlled by a riboswitch. A riboswitch is an RNA element that responds to binding by a small molecule by affecting the biosynthetic pathway. The work of the Breaker lab has demonstrated that there are riboswitches that bind preQ₁ affecting the biosynthesis of
queuine by having regulating among other the following genes: $queD$, $queE$, $queC$ and $queF^{89a}$. When the riboswitch is bound by its small molecule, $\text{preQ}_1$, biosynthesis is stopped. Our in vivo studies with total RNA confirmed those findings, as our exogenously provided radio-labeled $\text{preQ}_1$ must be halting $E.\ coli$’s own synthesis to give us the nearly identical counts for the TG2 and $\Delta queC$ cell lines. In fact, our results show that the $\Delta queC$ line was likely not required for this portion of our work based on how well our provided $\text{preQ}_1$ is incorporated in TG2 cells. However, it is important that we had the knockout cells as the azides we will work with next are unlikely to have the same effect on the $\text{preQ}_1$ riboswitch.

This study has led to several interesting observations and defined certain questions further that ultimately will require further experimentation. Our in vitro studies with labeled $\text{preQ}_1$ confirmed previous reports that TGT can recognize and modify RNA other than the four known affected tRNAs. We also demonstrated that $E.\ coli$ utilizes exogenous $\text{preQ}_1$, verifying it can enter the cell. In addition, it was observed that the provided $\text{preQ}_1$ binds the $\text{preQ}_1$ riboswitch. We have also demonstrated that the four known queuine modified tRNAs utilize the majority of the queuine in the cell. It remains possible that queuine modification occurs at locations other than tRNA, but those species are either low in abundance or the instances of modification are regulated by some other means, possibly localization of the enzyme$^{39}$. As modification with queuine is possible in vitro for many species of RNA as demonstrated in our in vitro experiments, especially given they were conducted in the presence of tRNA in those
experiments, a means of regulating modification other than recognition by TGT must occur in the cell.

**Materials and methods**

Unless otherwise specified, all reagents were ordered from Sigma-Aldrich. Yeast extract and agar were ordered from Fisher Scientific; tryptone from Acros Organics; Seaplaque low-melting agarose was from Lonza. The MasterPure™ RNA Purification Kit and Gelase™ Agarose Gel-Digesting Preparation were procured from Epicentre Biotechnologies. The Biodegradable Liquid Scintillation Counting Cocktail Bio-Safe II™ was ordered from Research Products International Corporation. Fluorescent silica chromatography plates were acquired from Analtech. The [³H] preQ₁ was obtained from a convergent synthesis with radio-labeling as outlined in Chapter II and isolation of final compound was carried out by Moravek Biochemicals³.

**Experimental Procedures:**

**Isolating total RNA.** *E. coli* cell strains TG2 and Δtgt were grown overnight at 37 °C on LB plates (5.0g tryptone, 2.4 g yeast extract, 7.5 g agar, 5 mL 1M NaOH) while the ΔqueC strain was grown overnight at 37 °C on L-Kan plates (50 μg/mL kanamycin). Individual colonies were then isolated and incubated in 3 mL 2xTY broth (8.0 g tryptone, 5.0 g yeast extract, 2.5 NaCl) overnight at 37 °C with shaking. Subsequently, the total RNA was isolated following the vendor’s (Epicentre MasterPure™ RNA Purification Kit) protocol.
Isolation of Ribosomal RNA. *E. coli ΔqueC* was grown overnight at 37 °C on L-Kan plates (50 μg/mL kanamycin). Individual colonies were then isolated and incubated in 3 mL 2xTY broth overnight at 37 °C with shaking. One mL of overnight culture was added to 100 mL 2xTY broth and incubated at 37 °C for 3 h. The cells were pelleted at 5,000 x g for 15 min at 4 °C. The pellet was resuspended in 4 mL of buffer (50 mM Tris-HCl, 10 mM MgCl$_2$, 100 mM NH$_4$Cl, 0.5 mM EDTA). The cell lysis was achieved by four rounds of a freeze thaw cycle with addition of 1 µL of PMSF (100 mM in isopropyl alcohol) and 1 µL of lysonase bioprocessing reagent, from Novagen, after the last round and incubation for 20 min. Debris was pelleted via centrifugation at 13,000 rpm in a table-top microcentrifuge for 10 min. The supernatant was transferred to a clean tube, treated with 5 µL of DNase I (1 unit/µL) and held at room temperature for 10 min. Debris was pelleted via centrifugation at 13,000 rpm for 10 min. The supernatant was transferred to an ultra centrifuge tube and 1 mL of 2 M NH$_4$Cl was added. The remaining volume of the tube was filled with microbiology grade mineral oil. The ribosome pellet was obtained by centrifugation at 40,000 rpm with a Beckmann Ti70 rotor (100,000 x g) for 4 h at 4 °C. The supernatant was discarded and the pellet rinsed twice with 0.5 mL of buffer (50 mM Tris-HCl, 10 mM MgCl$_2$, 100 mM NH$_4$Cl). Subsequently, 0.5 mL of the buffer was added to the tube and the pelleted ribosomes were resuspended overnight at 4 °C.

Proteinase K Digestion of Ribosomal Proteins. Ribosomes collected from the above-described ribosome isolation were treated with 1 µL of proteinase K (50 µg/µL). The
sample was incubated at 65 °C for 15 min in a heating block. The sample was vortexed for 10 sec every 5 min during the incubation. The protein was precipitated with 175 µL MPC protein precipitation solution from Epicentre Biotechnologies. The debris was pelleted via centrifugation at 13,000 rpm for 10 min. The supernatant was decanted and the rRNA precipitated by addition of 500 µL of isopropanol. The tube was inverted 30 times and then centrifuged at 13,000 rpm for 10 min to pellet the rRNA. The rRNA pellet was washed with 70 % ethanol twice and then resuspended in 5 µL of water.

**5’-Phosphate-Dependent RNA exonuclease treatment of total RNA.** Total RNA was isolated as described above. The total RNA was resuspended in 16.5 µL RNase-free water. The exonuclease digestion was conducted following the vendor’s (Epicentre mRNA-ONLY™ Prokaryotic mRNA Isolation Kit) protocol. To the RNA solution 2 µL of the supplied buffer and 0.5 µL of RNase inhibitor (20 units) were added. The 5’-Phosphate-Dependent RNA exonuclease (1 µL, 1 unit) was then added, and the reaction was heated at 30 °C for 60 min in a water bath. To terminate the reaction 1 µl of 100 mM EDTA (pH 8.0) was added. The RNA was subsequently ethanol precipitated, washed with 70 % ethanol and resuspended in 5 µL of water. The sample was ready for analysis by agarose gel separation.

**In vitro incubations with TGT and [³H] preQ₁.** Total RNA isolated from *E. coli Δtgt* and *E. coli ΔqueC* and ribosomal RNA prepared from *E. coli ΔqueC* were incubated *in vitro* with TGT and [³H] preQ₁. The reaction conditions were as follows: 5.65 µL enzyme TGT (5
μM), 10 μL total or ribosomal RNA, 20 μL 5X bicine reaction buffer, 14 μL [³H] preQ₁ (100 μM, 1 Ci/mmol) and 50.35 μL deionized water. A no-enzyme control reaction was also conducted where the enzyme was deleted and 56.0 μL deionized water was used. The reactions were incubated for 2 h at 37 °C. Water was removed from the solutions via vacuum centrifugation for 2 h and the resulting pellets were resuspended in 10 μL of deionized water and then analyzed via agarose gel separation as described below.

**In vivo incubation with [³H] preQ₁, total RNA.** *E. coli Δtgt, ΔqueC* and TG2 cell lines were cultured with [³H] preQ₁ added to the media. These incubations were conducted under the same conditions as described above for total RNA isolation and detection. Three mL cultures in 2xTY with 30 μL of [³H] preQ₁ (650.6 μM; 1 Ci/mmoll) were incubated for 12 h at 37 °C. Cell pellets from two, 1 mL samples from each 3 mL culture were obtained via centrifugation at 13,000 rpm in a table-top centrifuge. The pellets were then subjected to total RNA isolation as described above.

**In vivo incubation with [³H] preQ₁, ribosomal RNA.** *E. coli ΔqueC* was incubated in 3 mL culture of 2xTY 12 h at 37 °C. One mL of this overnight culture was used to inoculate 100 mLs of 2xTY with 30 μL of [³H] preQ₁ (650.6 μM; 1 Ci/mmoll). The culture was allowed to grow at 37 °C with shaking until an OD₆₀₀ of 0.6 was achieved, approximately 3 h. The cells were pelleted via centrifugation at 5,000 x g for 15 min. The pellet was stored at -20 °C prior to ribosome isolation as described above.
Low-melting Agarose Gel Electrophoresis and Excision of Bands. The reaction samples were separated by electrophoresis using 2% SeaPlaque low melting agarose gel at 50 V for 120 min. Three RNA species prepared in vitro were used as controls: tyrosine transfer RNA (~80 nt), VirF mRNA (~800 nt), and tyrosine transfer RNA anticodon arm minihelix (ECYMH, ~25 nt). Subsequently, the gel was positioned on a silica gel chromatography plate and 250 nm UV light was used to visualize the bands via shadowing of the fluorescence of the silica plate. Sample bands were excised (8 per lane) and the agarose was digested with Gelase™ following vendors’ protocol. The radioactivity in the resulting samples was determined by collecting the ethanol precipitated RNA on Whatman GF/C filters with subsequent liquid scintillation counting in 4 mL of BioSafe II cocktail.

Urea Polyacrylamide Gel Electrophoresis and Excision of Bands. The reaction samples were separated by electrophoresis using 6% polyacrylamide, 8 M urea gel in TBE at 200 V for 45 min. Tyrosine transfer RNA (~80 nt) was used as a control. The gel was stained for 2 h with ethidium bromide diluted into TBE. The bands were visualized and excised by UV transillumination at 254 nm.

Recovery of RNA from Polyacrylamide Gel. Excised bands were macerated and soaked for 12 h in a solution of 0.5 M ammonium acetate, 0.1 mM EDTA, and 0.1 % SDS. The Buffer was decanted from the tube and ethanol precipitated. The precipitate was washed twice with a 70 % ethanol solution. The RNA samples were resuspended in
water and dried onto Whatman GF/C filters with subsequent liquid scintillation counting in 4 mL of BioSafe II cocktail.

**Blot of Polyacrylamide Gel.** Isolated total RNA or rRNA from previously described methods was diluted to approximately 1000 ng/µL. A serial dilution was performed to create two additional samples; one with half the concentration and the final with a quarter of the initial concentration. The samples were then separated as previously described by PAGE. When electrophoresis was complete the gel was transferred to a Zeta-Probe membrane by semi-dry transfer at 10 V for 40 min. The membrane was then dried overnight. Once dry the blot was placed in an exposure cassette with a TR phosphor storage screen for seven days to develop. The image was visualized using a Typhoon scanner in phosphor storage mode.

**Scintillation of Blotting Membrane.** After visualization, the blot of the PAGE experiment was cut into eight equal bands per lane. The bands were subjected to liquid scintillation counting in 4 mL of BioSafe II cocktail.
Chapter IV  
Synthesis of Azide Probes and other PreQ₁ analogues

With prior work demonstrating that the prevalence of queuine extends beyond the previously known four transfer RNAs, our attention turned to finding a means to identify the specific RNA species modified. To that end, azide probes were designed (seen in Figure 4-1) based on the heterocyclic core of queuine and preQ₁ with the intent they would be substrates for tRNA guanine transglycosylase (TGT). If substrates, the azide of the probe would allow for a bio-orthogonal reaction. A reaction to a biotin containing reagent would allow for isolation and concentration by affinity chromatography. In this kind of study there are two main concerns for potential probes. First, it must be determined that the probe will be recognized by TGT and incorporated into RNA similarly to preQ₁ and queuine. Second, the probe must also be stable to the conditions of the incorporation and ligation as well as the method of incorporation by TGT (e.g., deprotonation of the pyrrole nitrogen to facilitate its attack on the 1′ carbon of the RNA-TGT covalent intermediate). The heterocyclic portion of queuine and preQ₁ is retained in the probe design as it is important for recognition by TGT². The first azide probe, 18, with a single methylene between the heterocycle and the azide is the most desirable as it is the most analogous to preQ₁. The second probe, 19, features an additional methylene spacer and was planned due to stability concerns for, 18, which with a single methylene between the azide and the heterocycle may be unstable due to the azide being able to act as a leaving group given its benzylic like connection to the
heterocycle. The additional methylene unit insulates the azide from the heterocycle thus removing the possible instability issue, while hopefully remaining compact enough for the active site of TGT.

![Figure 4-1: Structure of azide probes for evaluation as TGT substrates](image)

Once the probes are completed it will be determined if they interact with TGT through kinetic analysis. In addition, mass spectrometry will be utilized to verify if the probes are in fact substrates. If substrates for TGT, they will be introduced to the ΔqueC E. coli strain, so that the probe may be taken up without competition from endogenous preQ₁. Once the RNA from cells grown with an azide probe as nutrient in the media is isolated, the bio-orthogonal nature of the azide functionality will be utilized to isolate affected RNA strands from unaffected RNA. The Staudinger ligation reagent described by Bertozzi and other will allow for ligation and subsequent isolation by biotin strepavidin affinity. Similarly, work by Bertozzi and others has also validated using click chemistry, both in a copper catalyzed reaction to a terminal alkyne as well as a copper free click reaction to a cyclooctyne, to allow for modification that provides a means to isolate and concentrate the modified species. The purification is accomplished utilizing the biotin on the opposite end from the Staudinger ligation part of the reagent.

Once purified, several methods could be attempted to identify the RNA strands modified. For larger RNA strands DNA microarray chip analysis is possible, the limitation
being the gene must be present on the microarray to be identified\textsuperscript{91}. For smaller non-coding strands, amplifying amounts and purifying for sequencing is a possible solution. This would be accomplished by first adding a poly A tail to the isolated RNA using poly A polymerase. The poly A tail could then be used as a degenerate priming site to amplify all the isolated RNA strands by PCR to give the complimentary DNA. To sequence, the resulting DNA would be further isolated so strands could be determined by pyrosequencing individually\textsuperscript{92}. Another possible approach for determining the sequence of smaller RNA strands has also been devised. The azide-linked strands of RNA could be exposed to a randomized pool of DNA oligonucleotides that contain restriction sites on either end. The primers that bind to the matching sequence of RNA would be pulled out of the mixture by utilizing the biotin of the ligated azide probe. These then could be PCR-amplified and subcloned into a plasmid for sequencing. The resulting sequence would be used to probe genome sequence databases for the gene responsible for the RNA. The proposed method is shown in Figure 4-2.
Results and Discussion

The first two routes explored for the synthesis of probe 18 are shown in Scheme 4-1. Aldehyde 5 was the starting point for both pathways. In the initial attempt alcohol 20 was generated from aldehyde 5 by sodium borohydride reduction in methanol. The first conditions for azide introduction examined were standard mesylation/azide displacement. However, not surprisingly mesylation failed. A Lewis acid catalyzed method shown was attempted and also failed to yield the desired azide. Small amounts of the product were generated, but the majority of material decomposed under the reaction conditions. Several other Lewis and mineral acids were evaluated, but all fared worse than the original conditions. It is likely the acidic reaction conditions are responsible for the decomposition, resulting in a benzylic-type cation from expulsion.
of the hydroxyl, which readily leads to polymerization. This called into question the stability of the target compound 18. Another approach, with precedence based on the work of Hoops, et. al.\textsuperscript{94}, was attempted. This involved starting with previously generated aldehyde 5, which was transformed to amine 22 in reasonable yield by reductive amination\textsuperscript{95}. Further functionalization by methylation to form the quaternary salt 23 was designed to facilitate the subsequent substitution by azide to give product 21. However, the reaction was low yielding and contained a large amount of a polymeric side product as in earlier reactions. In addition, attempts to accomplish this synthesis from the unprotected aldehyde, which has been synthesized from preQ\textsubscript{0} (3) directly, were also unsuccessful.

![Scheme 4-1: Attempted routes to generate preQ\textsubscript{1} azide analogue](image)

An alternate method for substituting a benzylic-type alcohol with an azide was discovered in the literature and served as the basis for another route to 18\textsuperscript{96}. The reaction pathway, as shown in Scheme 4-2, also started with alcohol 20. The alcohol
was treated with diphenylphosphoryl azide (DPPA) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) to effect the transformation to the azide. The reaction likely proceeds through a diphenylphosphoryl intermediate with the released azide displacing the now activated alcohol. Azide 21 was then converted at low temperature to probe 18 by removal of the trityl protecting group with TFA in the presence of triethylsilane, acting as a scavenger. This scheme yielded probe 18 in an overall yield of 18 % from the starting pyrimidine 1.

![Scheme 4-2: Synthesis of preQ₁ azide analogue](image)

The synthesis of the alternate azide probe 19, which features an additional methylene spacer between the heterocyclic ring and the azide, was based largely on a reported synthesis of the alcohol intermediate 28 by Hammond and coworkers. Its completion was important due to the concerns about the stability of probe 18 with respect to TGT’s mechanism of incorporation into RNA. The synthesis, shown in Scheme 4-3, begins with 1,4-butanediol (23), which was treated with one equivalent of sodium hydride for monoprotection with benzyl chloride. The remaining alcohol group of 24 was then oxidized to aldehyde 25 with PCC. Alternate methods for the oxidation were attempted that utilized TEMPO. Oxidation with TEMPO and bleach failed to give a
good yield of aldehyde 25. Another set of conditions using TEMPO and trichloroisocyanuric acid\textsuperscript{98b} were only successful on a small scale. As the oxidation to the aldehyde with PCC proceeded in good yield and at minimal cost, it was retained in the route. Next, aldehyde 25 was brominated $\alpha$ to the aldehyde with bromotrimethylsilane and DMSO. Bromotrimethylsilane can be difficult to work with as it is extremely sensitive to atmospheric moisture. Another method of bromination with 5,5-dibromobarbituric acid and acetic acid in dichloromethane\textsuperscript{99} was evaluated as a replacement for the bromotrimethylsilane method. This method failed to give the desired product, so the earlier transformation with bromotrimethylsilane was retained. The resulting product, 27, was used without purification in the subsequent cyclization step to give the deazaguanine core, compound 28, with the appropriate linker.

This synthesis differed from the previously published report in several ways. First, chromatographic purifications after the benzylation and oxidation steps were found to be necessary to provide good yields in subsequent reactions. In addition, by changing the solvent concentration of the cyclization reaction, the product can be collected as a precipitate, instead of having to employ difficult extraction and chromatography steps. Another difference in our route from the literature procedure was in the removal of the benzyl protecting group. The authors had utilized boron trichloride for the deprotection. This method was found to be cumbersome due to the resulting boronates that made extracting the product difficult. It was determined that a transfer hydrogenation with ammonium formate and palladium on carbon was a superior method for removal of the benzyl protecting group.
Standard conditions to generate probe 19 from alcohol 28 failed. Attempts were made to mesylate the alcohol for substitution with sodium azide, but mesylation did not proceed cleanly to a single product. Mitsunobu conditions were attempted but also failed likely due to competing side reactions with the heteroatoms of the pyrrolopyrimidine. Finally, to generate probe 19 from the alcohol we employed a similar method as had been used in installing the azide of 18. As the alcohol of 19 is a primary alcohol of an alkyl nature rather than a benzylic type, modifications to the reaction conditions were necessary. Treatment of 19 with DPPA and DBU gave the phosphoryl ester of the alcohol instead of going directly to the azide. The phosphoryl intermediate can be isolated and purified. It is likely that this intermediate also forms in the earlier reaction with 20 to activate the otherwise unreactive alcohol into a suitable leaving group. However, in this example the alkyl alcohol is not as activated to spontaneous displacement with azide as the benzylic-like alcohol had been previously. To complete this displacement, we utilized a literature method that describes the transformation of the 6-position alcohol of a sugar to an azide\(^\text{100}\). Hence, after the
alcohol of 28 was activated by the formation of the phosphoryl ester, this intermediate was treated with sodium azide, 15-crown-5 ether and tertabutylammonium iodide to complete the substitution to the desired azide probe 19 in an overall 11% yield.

Scheme 4-4: Synthesis of azide probe without methylene spacer

The generation of an additional alternate azide probe was investigated. A probe with the azide connected directly to the heterocycle was envisioned as a way to reduce the size of the probe and avoid steric interactions that could encumber its incorporation by TGT into RNA. Two proposed routes to a key halogenoazido acetaldehyde intermediate are presented in scheme 4-4. The first route, substitution of starting 1,4-dibromo-2-butene with sodium azide followed by ozonolysis as based on a procedure from the laboratory of Chi-Huey Wong101, was designed to generate 2-azidoacetaldehyde. Unfortunately, we were unable to carry out the synthesis as we did not have access to a functioning ozonizer. An alternate route was attempted built on our prior experience with forming reagents for ring closures. Dichloroacetaldehyde diethyl acetal was procured but attempts to mono-substitute one chlorine for an azide
failed. Our attempts to hydrolyze the species to the aldehyde also proved problematic and thus the route was abandoned. An additional option considered was to replace the ozonolysis of the first route with an osmium tetroxide oxidation and subsequent sodium periodate cleavage to give azidoacetaldehyde. This was attempted on the dibromo starting material, but failed to give desired product. An additional route contemplated a Pinnick oxidation of aldehyde 5 (see Scheme 2-1) to the carboxylic acid followed by a Curtius rearrangement with DPPA to generate the amine substituted heterocycle. A Sandmeyer-type reaction would then provide the desired azide. This latter route was not explored due to time and financial constraints.

In order to use the probes as intended, the Staudinger ligation reagent had to be prepared. The synthesis, seen in Scheme 4-5, was performed as described in the literature from commercially available materials\textsuperscript{102}. With the synthesis of the Staudinger ligation reagent, all molecules required for the second phase of the project had been made. Experiments to determine if the azide probes interact with TGT can be undertaken, and the Staudinger ligation reaction can be attempted to purify and concentrate modified RNA for identification.
In addition to the azide probes, the syntheses of several potential inactivators of TGT were planned. An irreversible inhibitor of TGT would be a useful tool in the study of queuine prevalence as well as in future work studying the role of queuine. Routes to several possible inactivators were designed, seen in scheme 4-6. The potential inactivators were designed as preQ₁ analogues, with leaving groups in place of the primary amine of preQ₁. The molecules would benefit from the recognition of the pyrrolopyrimidine core for the binding site and have the capability of inactivating TGT through a substitution reaction with an amino acid residue in the active site of the enzyme. It would be of interest to know if the cysteine in the active site of the eubacterial version of TGT, but not found in eukaryotic version\(^3\), would make for inactivators that are selective for the eubacteria TGT. The potential inactivators were
based on previous work performed in the Garcia lab in which the fluoro analogue of preQ₁ was generated and tested\(^9\). The route described in their paper was low yielding and difficult to carry out due to solubility issues, thus we thought that our experience with generating preQ₁ would provide us with a better route.

Scheme 4-6: Synthetic routes of potential TGT inactivators

The first route attempted was identical to that described in the previous paper\(^9\) with the exception we utilized our trityl protected aldehyde 5 whereas previously the unprotected form had been used. The route started with a reductive amination of 5 to the dimethylamine derivative. Further methylation of the dimethylamine derivative formed in situ the quaternary ammonium salt as a leaving group for subsequent substitution with fluorine from addition of KF. The reaction did not yield product, but
instead gave a complex mixture. A modern method of installing fluorine was then examined via conversion of a primary alcohol to a fluoride with diethylaminosulfur trifluoride (DAST). This reaction also produced polymeric mixtures without any trace of the desired product. The same transformation was also examined with X-talE, the tetrafluoroborate salt of DAST. This reaction too resulted in multiple products and polymer. Based on these results, the fluorine substituted preQ₁ analogue, if formed, was deemed too reactive to be useful for subsequent enzymatic reaction and thus was abandoned.

Although the chloro analogue would likely be more reactive than fluoro, the previous success we had experienced with DPPA gave us reason to attempt the formation of this potential inactivator with diphenylphosphoryl chloride (DPPCl). The reaction conditions appeared to produce a new product, seen by TLC analysis, so NMR of the crude mixture was attempted. The NMR appeared to contain two molecules one of which was potentially the desired product. A silica gel flash column was performed to separate the two molecules. Upon loading the dissolved mixture to the column, it turned pink and no material was collected even after extensive flushing with polar solvent, indicating the acidity of the silica gel had broken down the product. It would appear that the chloro analogue, if could be generated and isolated, would likely be too unstable for biological conditions; therefore, further experiments to generate it were not attempted.

The deprotected alcohol was generated according to the approach in shown in Scheme 4-6. Aldehyde 5 was first reduced to the alcohol with sodium borohydride, as
had been performed in the generation of azide probe 18. The trityl protecting group was removed under the same conditions used to yield azide probe 18. The product precipitated under those condition as had the azide analogue. Analysis by mass spectrometry and $^1$H NMR indicated the product had formed, but it decomposed in solution during further NMR analysis. The alcohol had been generated to offer alternate routes to analogues or to use as an inhibitor as the alcohol is capable of hydrogen bonding with heteroatom residues in the active site or potentially be an inactivator given the reactive nature of the benzylic like connection. Biological evaluation was not attempted as the molecule was too unstable in solution: DMSO, H$_2$O, or buffered aqueous conditions. The fact the alcohol analogue was too unstable to use ruled out any further attempts to make the fluoro, chloro or related analogues.

Methods and Materials

Methods and materials used are identical to those of Chapter II.

Experimental Procedures:

![Structural formula of 5-(Hydroxymethyl)-2-(tritylamino)-3H-pyrrolo[2,3-d]pyrimidin-4(7H)-one (20).](image)

5-(Hydroxymethyl)-2-(tritylamino)-3H-pyrrolo[2,3-d]pyrimidin-4(7H)-one (20). An ice cold suspension of 4-oxo-2-(tritylamino)-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidine-5-
carbaldehyde\(^1\) (0.10g; 0.24 mmol) in methanol (1 mL) was treated with sodium borohydride (0.027 g; 0.71 mmol). The reaction was monitored by TLC, aided by 2,4 DNP visualizing agent to determine when no aldehyde remained. After 3 h, the mixture was quenched with 3 mL of brine, and extracted with dichloromethane. The combined extracts were dried over magnesium sulfate and concentrated to leave 0.099 g (98.6 \%) of a white powder as the product. \(R_f\) 0.33 (EtOAc); \(^1\)H NMR (DMSO-\(d_6\)) \(\delta\) 10.69 (br s, 1H), 10.51 (br s, 1H), 7.41 (br s, 1H), 7.31-7.20 (m, 15H), 6.34 (s, 1H), 4.95 (br s, 1H), 4.41 (d, \(J = 5.6\), 2H); MS \(m/z\) 445.1 (M+Na)\(^+\).

5-(Azidomethyl)-2-(tritylamino)-3H-pyrrolo[2,3-d]pyrimidin-4(7H)-one (21).

A solution of alcohol 20 (0.05 g, 0.12 mmol) in THF (1 mL) was treated with diphenylphosphoryl azide (DPPA) (0.031 mL, 0.142 mmol). The reaction was cooled to \(0^\circ\text{C}\) and 1,8-diazabicycloundec-7-ene (DBU) (0.021 mL, 0.142 mmol) was added drop-wise. The reaction was stirred for 4 h and an additional equivalent of DPPA and DBU each was added after 4 h. After 18 h total reaction time, the reaction was quenched with a 1:1 mixture of diethyl ether and water (5 mL). Additional ether and water were added and the organic layer was extracted and washed with water (2x) and brine. The organic layer was dried over sodium sulfate and concentrated to give a yellow powder, which was purified by SiO\(_2\) chromatography (gradient from 8:2, hexanes:ethyl acetate, to 1:1, hexanes:ethyl acetate). Product fractions were concentrated in vacuo to leave
0.050 g (94 %) of the product as a white powder. Rf 0.52 (1:1, hexanes:ethyl acetate); 1H NMR (DMSO-\textit{d}_6) δ 10.94 (br s, 1H), 10.36 (br s, 1H), 7.42 (br s, 1H), 7.31-7.20 (m, 15H), 6.58 (s, 1H), 4.31 (br s, 2H); MS m/z 459.1 (M+Na)$^+$, 895.3 (2M+H)$^+$.

2-Amino-5-(azidomethyl)-3H-pyrrolo[2,3-d]pyrimidin-4(7H)-one (18). Starting azide (0.10 g, 0.022 mmol) and triethylsilane (3.57 µL, 0.022 mmol) were dissolved in dichloromethane (0.5 mL) and stirred until solution was achieved. The mixture was cooled to -78 °C, and treated with trifluoroacetic acid (3.32 µL, 0.045 mmol). The reaction mixture was stirred at -78 ºC for 3 h, and then allowed to warm to room temperature. A formed precipitate was collected and dried to leave the product (0.039 g, 85 %). as an off white powder. 1H NMR (DMSO-\textit{d}_6) δ 11.11 (br s, 1H), 10.44 (br s, 1H), 6.76 (s, 1H), 6.20 (br s, 2H), 4.38 (s, 2H); 13C NMR (DMSO-\textit{d}_6) δ 158.69, 157.95, 152.39, 116.95, 111.07, 98.32, 45.65; MS m/z 228.0 (M+Na)$^+$, 433.1 (2M+Na)$^+$; HRMS (ESI) m/z 228.0611 [(M+Na)$^+$; calcd for C$_7$H$_9$N$_5$ONa$^+$: 228.0610].
**4-Benzyloxybutan-1-ol (24).** The product was prepared as previously described\(^97\). The product was purified by SiO\(_2\) chromatography (gradient, hexanes to ethyl acetate) to afford 8.4 g (71\%) of product as an oil. \(R_f\) 0.26 (8:2, hexanes:ethyl acetate). The \(^1\)H NMR was the same as previously described\(^97\).

**4-benzyloxybutanal (25).** The product was prepared as previously described\(^97\). The reaction solution was separated from the insoluble PCC by celite filtration. The solution was distributed between ether and water. The combined organic layers were filtered through a plug of SiO\(_2\) to give a clear yellow oil. The product was purified by SiO\(_2\) chromatography (gradient, hexanes to ethyl acetate) to afford 2.75 g (69\%) of product. \(R_f\) 0.67 (3:2, hexanes:ethyl acetate). The \(^1\)H NMR was the same as previously described\(^97\).
2-Amino-5-(2-(benzyloxy)ethyl)-3H-pyrrolo[2,3-d]pyrimidin-4(7H)-one (27). Aldehyde 25 (0.9 g, 5.05 mmol) was dissolved in acetonitrile (10 mL) and cooled to 0 °C. Bromotrimethylsilane (0.784 mL, 6.06 mmol) and DMSO (0.430 mL, 6.06 mmol) were added drop-wise. The reaction mixture was stirred at room temperature for 4 h. A suspension of 2,6-diaminopyrimidin-4-one (0.701 g, 5.55 mmol) in water (12 mL) was added as a single charge. The mixture was stirred for 18 h, during which a white precipitate formed. The precipitate was collected by vacuum filtration on a glass Buchner fritted funnel and triturated in acetonitrile to obtain 0.75 g (52 %) of product as a cream colored powder. \( R_f 0.44 \) (4:1, ethyl acetate: methanol). The \(^1\)H NMR was the same as previously described\(^{97}\).

2-amino-5-(2-hydroxyethyl)-3H-pyrrolo[2,3-d]pyrimidin-4(7H)-one (28). A mixture of benzyl protected alcohol 27 (0.5 g, 1.76 mmol) and ammonium formate (1.109 g, 17.6 mmol) was dissolved in methanol (7 mL). Upon heating to reflux, 10 % Pd/C (0.187 g, 0.176 mmol) was added to the mixture. After 8 h the reaction mixture was filtered
through a pad of celite and concentrated in vacuo to a grey solid. The solid was trituted in hot ethyl acetate to give 0.2 g (59 %) of the product as a white powder. \( R_f \) 0.40 (4:1, ethyl acetate: methanol). The \(^1\)H NMR was the same as previously described.97

![Diagram of compound 29]

2-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl diphenyl phosphate (29). A solution of alcohol 28 (0.162 g, 0.834 mmol), diphenyl phosphoryl azide (0.539 mL, 2.5 mmol) and DMF (6.7 mL) was cooled to 0 °C in a water ice bath, and then treated with DBU (0.374 mL, 2.5 mmol). The reaction mixture was allowed to warm to room temperature, stirred for 16 h, and then distributed between ethyl acetate and water. The combined organic extracts were washed with brine, dried over sodium sulfate, and concentrated to an off-white solid. Trituration in ethyl acetate with a small amount of methanol gave 0.350 g (98 %) of the product as a white solid. \( R_f \) 0.76 (4:1, ethyl acetate: methanol); \(^1\)H NMR (DMSO-\(d_6\)) 10.80 (br s, 1H), 10.25 (br s, 1H), 7.39 (t, \( J = 7.7 \), 3H), 7.24 (t, \( J = 7.7 \), 2H), 7.17 (d, \( J = 7.7 \), 3H), 6.46 (s, 1H), 6.04 (br s, 2H), 4.52 (q, \( J = 6.8 \), 2H), 2.96 (t, \( J = 6.8 \), 2H); MS \( m/z \) 449.0 (M+Na)^+, 875.0 (2M+Na)^+.
2-Amino-5-(2-azidoethyl)-3H-pyrrolo[2,3-d]pyrimidin-4(7H)-one (19). A mixture of 29 (0.186 g, 0.436 mmol), sodium azide (0.142 g, 2.181 mmol), tetra-n-butyrammonium iodide (0.016 g, 0.044 mmol), 15-crown-5 ether (10 µL, 0.044 mmol) and DMA (3.5 mL) was stirred at 80 °C for 18 h. The reaction mixture was concentrated in vacuo to give a brown solid that was dissolved in methanol and prepared for dry loading onto a chromatography column. The product was purified by SiO₂ chromatography (ethyl acetate to 4:1 mixture of ethyl acetate: methanol, gradient). Product fractions were pooled and concentrated in vacuo to give the product (0.060 g, 61 %) as a yellow-white powder. Rf 0.63 (4:1, ethyl acetate: methanol); \(^1\text{H} \) NMR (DMSO-\(d_6\) \(\delta\) 10.77 (br s, 1H), 10.22 (br s, 1H), 6.48 (s, 1H), 6.03 (s, 2H), 3.56 (t, J = 6.6, 2H), 2.82 (t, J = 6.6, 2H); \(^{13}\text{C} \) NMR (DMSO-\(d_6\) \(\delta\) 159.69, 152.74, 151.99, 115.20, 114.52, 100.00, 51.53, 26.42; MS m/z 220.1 (M+H)+; HRMS (ESI) m/z 220.0949 [(M+H)+; calcd for C₈H₁₀N₇O+: 220.0947].
Chapter V

Evaluation of Azide Probes

Both azide probes were designed as analogues of preQ₁, the precursor of queuine that eubacteria incorporate into RNA with TGT before elaboration to queuine, with the intent they would be substrates. The basis for this came from previous work in the Garcia lab⁴²a and others⁴¹, which had demonstrated that TGT could accept alternate molecules as substrates that shared the pyrrolopyrimidine core. The work of Romier²⁰ and our own work³ demonstrated, through comparative kinetic studies of the TGT enzymes with radio-labeled substrates, that the eukaryal and eubacterial TGTs had evolved from a common ancestor to accommodate their respective substrates. These studies indicated TGT was amenable to changes of the side chain, provided the core pyrrolopyrimidine remained.

Our interest in preparing azide probes originated from the knowledge that queuine modification occurs across eukaryotes and eubacteria with few exceptions, but its function remains unclear. In addition, it had been observed that preQ₁ could be incorporated into alternate RNA species in vitro other than the four identified tRNAs. The azide probes, if substrates, would provide a means to identify other species of RNA that are substrates for queuine modification. As azides have been demonstrated to be bioorthogonal, after incorporation into RNA they can be subjected to Staudinger ligation or click chemistry conditions to be linked to a biotin-tethered reagent for subsequent isolation and concentration⁹⁰a, b, ⁹⁰e. Therefore, we would be able to utilize the unique
method of queuine modification, transglycosylation, as a point of entry to study queuine prevalence more specifically. The azide probes also have the potential to allow for sites of modification within the substrate RNAs to be identified.

The first aspect of studying the two azide probes was to determine their apparent $K_i$ against TGT in order to establish if they interact with TGT. The apparent $K_i$ value can guide future experimental design, indicating appropriate concentrations for evaluation of the azides’ incorporation. The kinetics of a competitive substrate and competitive inhibitor are identical in kinetic analysis with our experimental design, so a $K_i$ apparent would be identical to the $K_m$ if the azides were indeed alternate substrates. The azide probes were initially tested with E. coli TGT in a guanine exchange assay at various concentrations ranging from a control with no azide to 1 mM concentration of the azide in a “decade search”. This information was then used to determine an appropriate range of inhibitor concentrations to more precisely determine the $K_i$ of each probe as had been performed previously. A range that was deemed appropriate for each was selected and those azide concentrations were tested against three different concentration of labeled substrate, $[^{14}\text{C}]$ guanine. For azide probe 18 the following concentrations, as seen in Figure 5-1, were used: 5, 50, 100, 250, 500 $\mu$M. The following concentrations of azide probe 19, as seen in Figure 5-2, were used for the guanine exchange assay: 10, 100, 175, 250, 500, 1000 $\mu$M. We elected to perform the Dixon analyses with three concentrations of guanine to confirm the competitive nature (competitive inhibitor or competitive substrate) of the azide interaction with TGT.
Initial experiments with these concentrations of azide probes were examined with different $[^{14}\text{C}]$ guanine concentrations to determine conditions for analysis of the azide probes. Ideally, a concentration below the $K_m$ of guanine, at $K_m$ and above $K_m$ would have been utilized, but this was not possible for our experiment. It was determined that although the $K_m$ of guanine for TGT is 0.35 µM that concentration or any lower concentration of guanine would not work since sufficient DPM counts would not be achieved to characterize the enzyme's interaction with the azides. The lowest concentration examined that gave adequate DPM counts was 0.75 µM. The labeled guanine substrate was next examined at 1.00 µM and 1.50 µM. The values obtained by these two values were nearly identical and prompted concerns they were too close for differentiation and would lead to difficulty in analyzing the data. Therefore, 5.00 µM was chosen as the third substrate concentration for the $K_i$ determination of the azide probes.

Experiments to confirm the azide probes as substrates for TGT would be undertaken when the apparent $K_i$ was determined. With that value determined incorporation experiments could be designed and the resulting RNA analyzed for modification with the azide analogues of preQ$_1$. The confirmation experiments planned included mass spectrometry with the mini-hairpin of RNA and tRNA$^{\text{Tyr}}$. In addition, trials to work out conditions for the Staudinger ligation and click chemistry with a copper free click reagent would allow for an alternate means to test for incorporation and validate our design to pull down the resulting modified RNA. The azide probes would be tested and monitored by HPLC at several time points with the reagents at various
concentrations of azide and reagent to determine conditions for the Staudinger ligation or click reaction. Once the sample was tethered to the biotin of those reagents, it could be incubated in streptavidin coated wells, which would be subsequently tested for the presence of RNA with RiboGreen® fluorescent to confirm modification and ligation or click reaction with a respective reagent could be performed. If validated as substrates for *E. coli* TGT, future studies to identify alternate sites of queuine modification could be conducted *in vitro* and *in vivo* based on the methods developed with the cell lines used in our previous work to determine the prevalence of the queuine modification with radio-labeled preQ₁.

**Results and Discussion**

Before starting the experiments to determine *Kᵢ*, an inactivation study with azide probe 18 was carried out. With the difficulty in generating certain probes as described in Chapter IV, the benzylic-like connection of azide 18 could cause it to act as an inactivator. Therefore a set of experiments were performed, as had been described in previous work by Hoops⁹⁴, to evaluate azide 18 as an inactivator. Evaluation occurred at 150 µM concentration of azide 18. Pre-incubation times of 15 min, 30 min, 1h and 2h were used. Each experiment was run in duplicate and compared to a control run simultaneously. In every case, the control counts collected were identical to those obtained for reactions using pre-incubated enzyme, as seen in Table 5-1. As azide 19 had been designed and generated as a less reactive alternative to 18, it was not tested
in this manner. The results indicated that the azide probes were not inactivators and experiments to determine the $K_i$ commenced.

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<th>Control</th>
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**Table 5-1:** Pre-incubation with azide 18. Azide data compared to control, pre-incubation preformed with 150 μM of azide 18, 5 μM TGT and tRNA at 20 μM. Data presented is average of two experiments.

Once the DPM data was obtained from the guanine exchange assays by liquid scintillation counting, the data was converted into pmol of guanine using its specific activity (46.4 mCi/mmol). The pmol of guanine were then plotted versus time, as seen for azide probes 18 and 19 as shown in Figure 5-1 and 5-2 respectively, to determine the rate of incorporation. The rate and the concentrations were used to construct Dixon plots\textsuperscript{103} that are presented in Figure 5-3. In a Dixon plot if the molecule under investigation is a competitive inhibitor (or models as such), the lines for data where substrate concentration is held constant should intersect in the second quadrant at $-K_i$. If the interaction is non-competitive they should intersect at the x-axis between the second and third quadrant. If the lines do not intersect at one of these locations, the interpretation is that a more complicated binding relationship is occurring. In
constructing the Dixon plots, the reactions with the highest concentration for each azide probe were removed from the analysis as the most error is associated with those data points. The $K_i$ of each could be estimated from the plots, but the analysis did not give the expected data pattern.
Figure 5-1: Inhibition of guanine incorporation by azide probe 18. Trials were run with the following conditions: 0.1 μM TGT; 10 μM tRNA^{Tyr}; 0.75 μM, 1.50 μM, or 5.00 μM ^{14}C guanine. Experiments were performed in duplicate. Data was obtained by quenching and precipitating 70 μL of reaction mixture in 5 % TCA, and the resulting precipitate was collected by filtration. Liquid scintillation counting provided raw data for analysis.
Figure 5-2: Inhibition of guanine incorporation by azide probe 19. Trials were run with the following conditions: 0.1 μM TGT; 10 μM tRNA\textsuperscript{Tyr}; 0.75 μM, 1.50 μM, or 5.00 μM \textsuperscript{14}C guanine. Experiments were performed in duplicate. Data was obtained by quenching and precipitating 70 μL of reaction mixture in 5 % TCA, and the resulting precipitate was collected by filtration. Liquid scintillation counting provided raw data for analysis.
Figure 5-3: Dixon plots for each azide probe; based on guanine exchange assay data.

The first unexpected pattern in the data occurred in the guanine exchange assay charts. While the data fit would be expected to curve over time, it was not expected that enough reaction progress would be made for the data in our reaction design to go beyond the linear range, but the data clearly curves. Based on the pmol incorporated at the final time point, an estimate of the percent completion can be calculated to
determine if the reaction had gone beyond the linear region of the conditions. It was determined that by the final time point of the reaction that approximately 20% of the labeled guanine had been incorporated in the reactions, which would explain the loss of linearity of the data as the substrate concentration was dropping significantly. Therefore, to fit the data to determine the initial rate of reaction the final time point was excluded.

The patterns generated by the three line fits in the Dixon plots were also not as expected. The lines for a competitive inhibitor or substrate would be expected to intersect in the second quadrant. This is not the case for our azides. The three fits do not arrive at the same value and give an intercept in first quadrant or on the y-axis, which indicates that the azides are likely displaying mixed inhibition. The data pattern could also be the result of a different, additional binding mode being present at higher concentrations of the azides or for the substrate. If the elevated levels lead to binding to the free enzyme as well as the enzyme-tRNA_{tyr} complex, a more complicated pattern would arise. To fully characterize the azides, further experimentation would be required; however, our primary interest in performing the experiments had been to determine a concentration to run the subsequent incorporation studies to validate the azides as substrates. From the data we determined, using the equation in Figure 5-4, that the K_i for each azide is approximately 70 µM, which is greater than that for guanine, 0.35 µM, or its natural substrate preQ_1, 0.05 µM. Observing the expected pattern for a competitive inhibitor or substrate was desired, but obtaining an apparent K_i value for
subsequent experiments allowed us to continue our evaluation of the azide probes as alternate substrates.

\[ K_i = \frac{K_m}{K_{cat} [S]v} \]

**Figure 5-4:** Equation used to calculate the \( K_i \) of a competitive inhibitor or substrate; rates determined in the Dixon plots, \( K_m \) of guanine with TGT is 0.35 \( \mu \)M, \( K_{cat} \) is 0.00629 \( \mu \)M.

In order to determine if the azides are substrates for TGT, experiments were designed to modify and isolate RNA post treatment with TGT and an azide probe. The azide probes at 200 \( \mu \)M (approximately 3x Ki apparent) were incubated overnight with RNA and TGT was added every 6 h. Two RNAs were used. First, a mini-hairpin of the tRNA\(_{tyr} \) was used as had been utilized in earlier work to demonstrate preQ\(_1 \) incorporation by mass spectrometry\(^{38} \). Later, full length tRNA\(_{tyr} \) was utilized under the same conditions. A representation of the experiments is presented in Figure 5-5. The reactions were quenched by ethanol precipitation and the samples were desalted and submitted for mass spectrometry analysis, utilizing electron spray ionization. In neither analysis with either RNA substrate was the incorporation of the azide observed; intact mass of starting RNA was observed instead. The mass spectrometry analysis of an untreated sample of mini-hairpin and tRNA was performed concurrently. The small mass change made detection difficult for experiments with full length tRNA, so attempting the ligation with the Staudinger ligation reagent to make the mass difference between product and starting tRNA greater was attempted. Only mass to charge values smaller than the starting tRNA was observed, demonstrating a breakdown
of the tRNA likely due to the incubation for the ligation reaction and extra handling of the tRNA. These experiments failed to show that the azide probes were substrates for TGT, but as questions remained about using mass spectrometry to verify incorporation (a low percentage of RNA modification might not be detected) another set of experiments were undertaken to examine if the azide probes were substrates for TGT.

![Diagram](image)

**Figure 5-5:** Test of azide analogues of preQ$_1$ as substrates for TGT. Prepared tRNA$^{Tyr}$ was used as well as 25 base stem loop for analysis by mass spectrometry. Biotin-streptavidin affinity experiments used prepared tRNA$^{Tyr}$.

The final attempt to determine if the azides were substrates for TGT started with first testing the ligation and click reactions with each probe to ensure that the proper chemistry was occurring. The azide probe and Staudinger ligation reagent were incubated at various concentrations and assayed by HPLC at various time intervals to monitor reaction progress. It was determined that the Staudinger ligation reagent needed to be at a concentration of at least 2.5 µM for the reaction to proceed, and an
excess of ligation reagent was required for the reaction to go to completion, as observed by HPLC at 24 h. It was also determined that the ligation reagent had to be dissolved in DMF as it is oxidized by DMSO and has inadequate solubility in H$_2$O. An alternative to the Staudinger ligation reagent was also examined. A copper free click reagent, a dibenzylcyclooctyne with a PEG-4 linker to biotin from Jenna Bioscience (Figure 5-6), was also examined. This reaction proceeded at lower concentrations (0.1 µM) of reagent, could be run in 2 h, and would not be reactive with DMSO. However, the reaction still required an excess of reagent to go to completion.

![Figure 5-6: Dibenzylcyclooctyne-PEG4-Biotin Conjugate Reagent, Jenna Bioscience](image)

With conditions for the Staudinger ligation and copper free click reaction worked out, the azide incorporation experiments were performed again with prepared tRNA$^{tyr}$. The isolated tRNA$^{tyr}$ was subjected to the ligation or click reaction for 24 h before isolation of the RNA. The RNA was then suspended in Milli-Q H$_2$O and incubated in a 96 well plate coated with strepavidin. Each reagent is tethered to biotin, so any modified RNA would be bound to the plate through biotin’s interaction with strepavidin. The wells were washed and analyzed for RNA concentration with RiboGreen® fluorescent
dye. The wells with tRNA isolated from the ligation or click reaction gave values equal to negative control, unmodified tRNA. Therefore, this set of experiments also failed to demonstrate that the azides are substrates for eubacterial TGT, although the possibility remains that they are substrate for human TGT or even eubacterial TGT under yet to be identified conditions.

In conclusion, the azide probes interact with eubacterial TGT. The type of interaction has yet to be determined, but analysis indicates that the apparent $K_i$ of the interaction is approximately 70 $\mu$M. The azide probes do react with the Staudinger ligation probe and copper free click reagent. Alternate methods to observe the incorporation might be more successful or conditions described here could be optimized for detection of the modified species. Evaluation with the human version of TGT with its larger binding pocket that accommodates queuine could prove more successful with the presented methods. Work with the human form of the enzyme is desirable and should be the focus in the future given the yet to be understood nature of queuine’s role in cancer progression.

**Experimental Procedures:**

**TGT $[^{14}\text{C}]$ guanine exchange assay of azide probe 18.** Assays were performed with azide probe 18 under the following conditions: 0.1 $\mu$M TGT; 10 $\mu$M tRNA$^{\text{tyr}}$; 0.75 $\mu$M, 1.50 $\mu$M, or 5.00 $\mu$M $^{14}$C Guanine at six concentrations of azide probe 18: control (no azide), 5, 50, 100, 250, 500 $\mu$M. Experiments were performed in duplicate in bicine buffer. Data was obtained at time points for 2, 4, 6, 8, 10 min by quenching and precipitating 70 $\mu$L of
reaction mixture in 5 % TCA. The resulting precipitate was collected by filtration. Liquid scintillation counting provided raw data for analysis.

**TGT \([^{14}C]\) guanine exchange assay of azide probe 19.** Assays were performed with azide probe 19 under the following conditions: 0.1 μM TGT; 10 μM tyr tRNA; 0.75 μM, 1.50 μM, or 5.00 μM \(^{14}C\) Guanine at six concentrations of azide probe 18: 10, 100, 175, 250, 500, 1000 μM. Experiments were performed in duplicate in bicine buffer. Data was obtained at time points for 2, 4, 6, 8, 10 min by quenching and precipitating 70 μL of reaction mixture in 5 % TCA. The resulting precipitate was collected by filtration. Liquid scintillation counting provided raw data for analysis.

**Pre-incubation study of azide 18 with TGT \([^{14}C]\) guanine exchange assay.** Azide 18 at 150 μM was incubated with 5 μM TGT in the presence of tRNA at 20 μM in bicine buffer for 15, 30, 60, 120 min. A control pre-incubation of TGT without any of azide 18 was also performed. Following incubation, TGT was diluted into a \([^{14}C]\) guanine exchange assay with the following conditions in a bicine buffer: 20 μM tyr tRNA, 0.1 μM TGT (from pre-incubation), 20 μM \([^{14}C]\) guanine. Experiments were run in duplicate and time points were taken at 2, 4, 6, 8, 10 min.

**Azide incorporation into tyrosine tRNA.** TGT mediated incorporation of azide probes 18 and 19 was attempted with the following conditions: tyr tRNA 80 μM, azide probe 200
μM, TGT 1.4 μM in bicine buffer. An additional aliquot of TGT was added at 6 h and 12 h, and the reaction was quenched by ethanol precipitation at 18 h. The resulting tRNA was pelleted by centrifugation at 13k rpm for 10 min in a table top eppendorf centrifuge. The pellet was washed twice with 70 % ethanol and air dried 10 min before being suspended in solvent for subsequent use.

**Staudinger Ligation of azide probe 18.** Ligation reaction of azide probe 19 was examined in carbonate-bicarbonate buffer at a pH of 9.0 in a 1:10 ratio of azide to Staudinger ligation reagent 31. The Staudinger ligation reagent 31 was suspended in DMF at a concentration of 5 mM for a stock solution. The reaction was attempted at the following concentrations of azide probe: 0.1, 1.0 and 2.5 mM. The progress of the reaction was monitored by HPLC at 1h, 2h, 4 h, 18 h, 24 h. HPLC experiments were run as a gradient from 10% to 90 % of acetonitrile (0.1 % TFA) with water (0.1 % TFA) over 10 minutes.

**Copper free click reaction of azide probes.** Click reaction of azide probe 18 and 19 was examined in carbonate-bicarbonate buffer at a pH of 9.0 in a 1:1, 1:10, 1:50 ratios of click reagent with respective azide being analyzed. The click reagent from Jenna Bioscience was suspended in DMSO to give a 6.67 mM stock solution. The reaction was attempted at the following a concentration of azide probe of 1.0 mM and monitored by HPLC at 1h, 2h, 4 h, 18 h, 24 h. HPLC experiments were run as a gradient from 10% to
90 % of acetonitrile (0.1 % TFA) with water (0.1 % TFA) over 10 minutes. The progress of the reaction was monitored by HPLC at 1h, 2h, 24 h. HPLC experiments were run as a gradient from 10% to 90 % of acetonitrile (0.1 % TFA) with water (0.1 % TFA) over 10 minutes.

**Mass spectrometry sample preparation.** Samples of mini-hairpin RNA and tyrosine tRNA were purified by one of two methods. The first method utilized was to ethanol precipitate the sample with a 10 % volume to volume addition of 5M NH₄OAc at pH 5.3 and then 250 % addition of ethanol. Samples were stored at -20 ºC for 8 h. Precipitated RNA was pelleted by centrifugation at 13,000 rpm for 10 min in a table top eppendorf centrifuge. The pellet was washed twice with 70 % ethanol and air dried 10 min before being suspended in H₂O. The process was repeated twice before the sample was submitted for analysis.

The alternate method was to use a Millipore™ Zip-Tip® C₁₈ resin to desalt the sample. The Zip-Tip® was attached to a 10 µL pipetman and washed twice with 10 µL of a wetting solution consisting of a 1:1 ratio of acetonitrile to Milli-Q H₂O. The tip was then equilibrated by washing three times with 10 µL of a 0.1 M triethyl ammonium acetate at pH 7.0 solution. The sample of RNA in solution was purified by adhering RNA to the resin by taking up 10 µL of the sample into the tip followed by aspirating the sample and repeating the procedure ten times. The sample on the resin was then washed with 10 µL of a 0.1 M triethyl ammonium acetate at pH 7.0 solution three times.
The sample was then washed with 10 µL Milli-Q H₂O three times. To elute the sample for analysis, 10 µL of a 1:1 ratio of acetonitrile to Milli-Q H₂O was added to a eppendorf tube. The solution was taken into the Zip-Tip® and aspirated and the cycle repeated 10 times.

**Azide probe modified tRNA with Staudinger ligation reagent.** After allowing an azide probe to interact with TGT and tyrosine tRNA as described previously, the pelleted sample was suspended in carbonate-bicarbonate buffer at a pH of 9.0 to a volume of 20 µL. Ligation stock solution (DMF) was added to give a final concentration of 2.5 mM of reagent. The reaction was run at room temperature for 24 h until quenched by ethanol precipitation.

**Azide probe modified tRNA with click reagent.** After allowing an azide probe to interact with TGT and tyrosine tRNA as described previously, the pelleted sample was suspended in carbonate-bicarbonate buffer at a pH of 9.0 to a volume of 100 µL. Click reagent stock solution (DMSO) added to give a final concentration of 2.5 mM of reagent. The reaction was run at room temperature for 24 h until quenched by ethanol precipitation.

**Biotin streptavidin affinity analysis of azide probes with Ribogreen®.** A 96 well black flat bottom streptavidin coated plat was washed three times with TE buffer (pH 7.5). The RNA solution was resuspended in 150 µL TE buffer and added to a well of the 96
well plate. In addition, 150 µL of unmodified tyrosine tRNA, at the same concentration (determined by nanodrop) was added to a well to serve as a control. The plate was incubated at 37 °C for 30 min. The sample was removed and the plates washed three times with TE buffer. The samples were suspended in 75 µL of TE buffer and 75 µL of Ribogreen fluorescent dye, working stock prepared by vendor’s protocol, was added. In addition, tyrosine tRNA at five known concentrations was added to another lane of wells with ribogreen dye to serve as a standard curve: 0.16, 0.32, 0.64, 1.28, 2.56 ng/µL of RNA (determined by nanodrop).
Chapter VI

Conclusions

In the course of completing this dissertation project many of the objectives set at the start were completed. The proposed target molecules for the project were all generated. In doing so, we developed a concise, convergent synthesis of queuine that is the shortest route developed to date. Our route features a short high yielding synthesis of a key intermediate, aldehyde 5, which allowed for the facile synthesis of preQ₁ and queuine. This aldehyde could also be used as an intermediate for generating many other yet-to-be synthesized pyrrolopyrimidine analogues. The designed route also worked as planned in allowing for the straightforward generation of tritium-labeled versions of queuine and preQ₁. Having those labeled substrates has allowed the lab to study the kinetics of TGT directly and has provided a means to examine the differences between eukaryal and eubacterial TGT. Our route to the aldehyde 5 also demonstrated the use of silylation to enhance reactant organic solubility, which allowed for the straightforward reduction of a heterocyclic nitrile with DIBAL-H that was otherwise difficult to achieve.

In addition to generating unlabeled and labeled versions of the substrates for eubacterial and eukaryal TGT, we also completed the synthesis of two novel azide congeners of preQ₃. We demonstrated the utility of diphenylphosphoryl azide for installing azides by substitution of both a benzylic like alcohol (giving azide 18), as well as an alkyl alcohol to give azide probe 19. The means of generating a stable salt form of
chloro(formyl)acetonitrile provides an improvement in the synthesis of pyrrolopyrimidines. Even though limited attempts at condensations of related heterocyclic substrates with it suggests that the reagent may have precise reactivity requirements, further reaction conditions need to be examined to determine if it has broader utility.

Our biological experiments with the target molecules also presented us with interesting findings. The use of radio-labeled preQ₁ allowed us to confirm several previous observations. First, we confirmed that RNA other than tRNA can be modified in vitro by TGT. The in vivo work with TG2 and ΔqueC cell lines verified previous experiments examining the preQ₁ riboswitch. The work demonstrated that exogenously supplied preQ₁ can enter the cell. In addition, we observed in vivo a high level of [³H]preQ₁ incorporation into the RNA of the TG2 cells, consistent with prior findings that preQ₁ will bind to the riboswitch and halt biosynthesis of preQ₁, resulting in the bacteria utilizing the provided preQ₁. It was also demonstrated that the four known tRNAs are the main site of incorporation in vivo. Our experiments did not rule out other naturally occurring sites of queuine modification, but they did demonstrate that the vast majority of the modification occurs in tRNA with any other modification occurring either in low abundancy species of RNA or being the result of a regulated means of access to the modifying enzyme affording only a small percentage to be modified.

The designed azide probes did work in Staudinger ligation and copper free click reactions and at least act as competitive inhibitors of guanine exchange by the E. coli TGT, but it was not determined if they are TGT substrates. More analysis is likely
required and experiments with the human version of the enzyme may work more easily
given the increased size of the binding pocket. A study of queuine modification sites in
human tissue would also be of greater interest, given the correlation of queuine levels
and cancer progression described in the literature. This work has also presented some
ideas for alternate experiments that merit further study.

As described in the appendix to chapter V, aldehyde 5 has the potential to be an
interesting inhibitor of TGT given its structure seems perfectly suited to bind in the
active site of TGT. It also would seem to have a built in preference for the eubacterial
TGT given it contains a cysteine residue the eukaryal form lacks that normally interacts
with the amine of preQ$_1^3$. That cysteine residue is where the aldehyde would be located
if its binding is directed by the pyrrolopyrimidine core, which would create a selectivity
that could prove interesting in TGT studies. In addition, our ability to synthesize
queuine and preQ$_1$ also allows for some new experiments to study the redundant codon
bias theory long postulated and supported by some data. With the ability to prepare
our own tRNA and modify it ourselves with prepared queuine and TGT, one could
imagine a single molecule experiment with alternate mRNA that would allow for a
binding study like that recently performed by the Cornish lab$^{104}$ to determine if amino
acids required their respective tRNA for binding to the ribosome. A study with mRNA
biased in varying degree for U ending and C ending codons could allow for such a study.
The study would be more direct and controlled than the experiment run in the 1970s by
Nishmura and Harada$^4$. 
One other study that comes to mind based on this work would be to generate the $^{15}$N labeled version of azide probe 19. That would allow for a means to follow its incorporation by NMR. In addition, if it were a substrate it would provide a means for ligation or click reaction and could also be used for NMR studies of the affected RNA. Generating the $^{15}$N labeled azide probe 18 would provide a direct means to a labeled preQ₁ Staudinger reduction, which could be utilized in preQ₁ riboswitch experiments to further elucidate the function and mechanism of the binding domain. While generating $^{15}$N labeled 19 would be straightforward as it utilizes sodium azide as the source of the azide, the situation for 18 is more complicated. It is likely that the DPPA reagent functions through a substitution in which azide is released to form the phosphoryl intermediate of the hydroxyl, in which case addition of labeled sodium azide would allow for partial incorporation of the $^{15}$N label. This reaction would also help validate the reaction mechanism and determine whether or not the conversion to the azide is concerted or stepwise.

This project has accomplished many of its objectives and has created the opportunity for alternate projects through the molecules generated as part of the study. The prevalence of queuine is still not fully elucidated, but the picture is becoming clearer. The in vitro modifications observed by us and others do not correlate to widespread prevalence in vivo, which presents some interesting questions as to how the enzyme responsible for the modification is controlled. In addition, our work has served to support some previously held ideas, e.g. that preQ₁ is absorbed and used as a nutrient. Also, we have verified some previous findings with our in vivo studies, i.e. the
preQ$_1$ riboswitch will halt synthesis of preQ$_1$ and use the provided preQ$_3$. While my work did not achieve every goal set forth at the beginning of my research, we remain optimistic that many of these can still be achieved, chiefly incorporation of the azide probes. In summary, we have gained a better understanding of queuine modification of RNAs and have developed tools that will aide in future studies.
Appendix I

Spectral and Other Characterization Data for Chapter II and IV

This appendix consists of characterization data for molecules not synthesized previous to this dissertation work and all final products. The NMR spectra were recorded on a Bruker DRX instrument at 500 MHz for $^1$H and 125 MHz for $^{13}$C spectra. Chemical shift values are recorded in δ units (ppm). Mass spectra were recorded on a Micromass ToFSpec-2E Matrix-Assisted, Laser-Desorption, Time-of-Flight Mass Spectrometer in positive ESI mode. Characterization data for tritium labeled compounds was performed by Moravek as described on provided data sheets.
Product Data Sheet

MT-1002243

7-Aminomethyl-7-deazaguanine, [methylene-^3^H(N)]-

Specific Activity: 7.0 Ci/mmol
Concentration: 1.0 mCi/ml; 25.7 µg/ml
Packaged in: Ethanol : water (1 : 1) solution
Date of Analysis: March 16, 2010
Radiochemical Purity: 96.1%
Column: Supelco Discovery C18 4.6 x 250mm
Flow Rate: 1 ml/min.
Mobile Phase: A: Water with 0.1% TFA
B: Acetonitrile with 0.1% TFA
0-20min. 0-100% B

Storage Recommendation: Store at -20°C.

Product Warranty: Stated on the reverse side of this Product Data Sheet.

Caution: Not For Use In Humans Or Clinical Diagnosis. This product is intended for investigational or manufacturing use only. It is pharmaceutically unrefined and is not intended for use in humans. Responsibility for its use in humans, as a diagnostic reagent, and compliance with federal laws rests solely with the purchaser.
Product Data Sheet

MT-1002217

7-Dihydroxycyclopentenylaminomethyl-7-deazaguanine, [methylene-3H]-


Specific Activity: 7.3 Ci/mmole

Concentration: 1.0 mCi/ml; 38.1 pg/ml

Packaged in: Ethanol : water (1 : 1) solution

Date of Analysis: October 22, 2009

Radiochemical Purity: 99.0%

Column: Supelco Discovery C18 4.6 x 250mm

Flow Rate: 1 ml/min.

Mobile Phase: A: Water with 0.1% TFA
B: Acetonitrile
0-5min. 0% B; 5-35min. 0-100% B
Hold to 40min.

Storage Recommendation: Store at -20°C.

Product Warranty: Stated on the reverse side of this Product Data Sheet.

Caution: Not For Use In Humans Or Clinical Diagnoses. This product is intended for investigational or manufacturing use only. It is pharmaceutically unrefined and is not intended for use in humans. Responsibility for its use in humans, as a diagnostic reagent, and compliance with federal laws rests solely with the purchaser.
Allen Brooks   AFB-III-84    Electrospray with Na+ added

\[ \text{m/z} \]

227 228 229 230
%
0
100

AFB-III-84_Na_HR 29 (1.499) Cn (Cen, 50.00, Ht); Sb (15, 40.00); Sm (SG, 2x7.00); Cm (1:105)

Voltage ES+

228.0611

227.0754

227.6014

229.0556

228.8841

230.0958

230.8914

230.2377

---measured m/z of [M+Na]+ --- predicted value is 228.0610

AFB-III-84_Na_HR 29 (1.499) Cn (Cen, 50.00, Ht); Sb (15, 40.00); Sm (SG, 2x7.00); Cm (1:105)

Allen Brooks   AFB-III-84    Electrospray with Na+ added
Allen Brooks   AFB-IV-99    Electrospray with formic acid added

m/z
219 220 221 222 223

% 0 100

AFB-IV-99_FA_HR  6 (0.310) Cn (Cen,7, 50.00, Ht); Sb (15,40.00 ); Sm (SG, 2x7.00); Cm (1:111)

Voltage ES+ 1.70e3

220.0949
219.1012
221.0972
222.0998 223.0835

<==measured m/z of [M+H]+
predicted value is 220.0947
Preliminary evaluation of aldehyde 6 as an inhibitor of E. coli TGT was performed. In addition, to the azide probes, aldehyde 6 was also examined as an inhibitor or inactivator of TGT. In trying to generate the halogen analogues of preQ₁ and in our synthesis of aldehyde 6 we came to realize that it was likely that Hoops had generated a C-6 regioisomer of the aldehyde 6 (see Chapter II for synthesis) and the fluoride (attempted synthesis described in Chapter IV) analogue. This possibility arose when we found we could not generate our desired fluoro analogue, and that the NMR line listing for the aldehyde Hoops reported did not match that for aldehyde 6. We had not considered testing aldehyde 6 as Hoops had reported biological data earlier on his compound⁴²a. But becoming aware that Hoops’ and our own aldehydes were not the same suggested that a reexamination was warranted. Given the binding pocket of TGT, the aldehyde analogue of preQ₁ could represent an excellent inhibitor if stable. In addition, the difference in eubacterial and eukaryotic TGT, particularly the cysteine residue that interacts with the preQ₁ primary amine that is not present in the eukaryotic version, might represent a cause for a selectivity difference between the two forms of the enzyme.

First, a “decade search” (e.g., inhibition experiments with the aldehyde at a series of ten-fold concentration dilutions) was performed to find the appropriate concentration range for the aldehyde in subsequent experiments. The data from that
experiment is shown in Figure 5-5. The reactions at a concentration of aldehyde 6 at or below 0.1 µM are identical to the no inhibitor control. The reaction at 1 µM shows a decrease in incorporation of labeled guanine. The reaction at 100 µM shows a complete inhibition of guanine incorporation. This is inconsistent with the data Hoops reported\(^{42b}\). This data warranted further investigation of the aldehyde. Given that the aldehyde can readably react with amines, as determined in our synthesis of queuine and preQ\(_1\) as well as those of others, the aldehyde could be an inactivator.

![Aldehyde 6, 1.5 µM Guanine](image)

**Figure II-1:** Preliminary evaluation of aldehyde 6 at substrate concentration of 1.5 µM at 5 concentrations with a control.

An inactivation study with aldehyde 6 was run with concentration of aldehyde in the pre-incubation at 200 µM, as seen in Figure 5-6. In all the pre-incubation intervals the similar curves were observed, which demonstrated a decrease in activity when compared to control reactions although not as expected for inactivation. It would be
expected for the rate to decrease for longer pre-incubation times if the molecule were an inactivator. At a starting concentration of 200 µM for the pre-incubation, a concentration of 4 µM would still be present when the TGT was added. This indicates that what is being seen could be the result of competitive inhibition and not inactivation of TGT. This analysis is preliminary and more experiments at different concentrations of aldehyde 6 and substrate should be performed before any conclusions are drawn. It is likely that the aldehyde 6 could be a competitive substrate, which would yield identical kinetics to those for a competitive inhibitor. Further experiments should be conducted to determine the apparent $K_i$ (possibly $K_m$) of aldehyde 6 and its mode of interaction with the enzyme, as it could be an important probe to study differences between eubacterial and eukaryotic TGT. This work fell outside the focus of my project and was not pursued further due to time constraints.
Figure II-2: Inactivation study of aldehyde 6; incubated with 200 µM of azide with 5 µM TGT in the presence of tRNA at 20 µM; guanine exchange assays, 1.5 µM guanine, initiated with enzyme from pre-incubation at 15 min, 1 h and 2 h; Control reaction from alternate day, same conditions utilized.

TGT $[^{14}C]$ guanine exchange assay of aldehyde 6. Assays were performed with aldehyde 6 under the following conditions: 0.1 µM TGT; 10 µM tRNA$^{Tyr}$; 1.50 µM $^{14}$C guanine at six concentrations of aldehyde 6: control (no aldehyde 6), 0.01, 0.1, 1.0, 10, 100 µM. Experiments were performed in duplicate in bicine buffer. Data was obtained at time points for 2, 4, 6, 8, 10 min by quenching and precipitating 70 µL of reaction mixture in 5% TCA. The resulting precipitate was collected by filtration. Liquid scintillation counting provided raw data for analysis.

Pre-incubation study of aldehyde 6 with TGT $[^{14}C]$ guanine exchange assay. Aldehyde 6 at 200 µM was incubated with 5 µM TGT in the presence of tRNA at 20 µM in bicine
buffer for 15, 30, 60, 120 min. A control pre-incubation of TGT without any of aldehyde 6 was also performed. Following incubation, TGT was diluted into a $[^{14}\text{C}]$ guanine exchange assay with the following conditions in a bicine buffer: 20 µM tRNA$^{\text{byr}}$, 0.1 µM TGT (from pre-incubation), 1.5 µM $[^{14}\text{C}]$ guanine. Experiments were run in duplicate and time points were taken at 2, 4, 6, 8, 10 min.
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