*(Example of Proposal for Independent Research)*
**Title: Design and Analysis of Novel Peptidyl Transferase Inhibitors**
by Scott Strobel

## Hypothesis

What is the chirality of the tetrahedral intermediate that occurs during ribosome catalyzed peptide bond formation? How does the ribosome provide transition state stabilization of the tetrahedral intermediate ?

## Background Information

Ribosomes are the macromolecular machines responsible for synthesizing proteins in all living cells (reviewed in (*1*)). The large ribosomal subunit (50S in prokaryotes) contains the site of catalysis, termed the peptidyl transferase center (PTC). The reaction substrates include a peptidyl-tRNA, charged with the growing peptide chain bound to a tRNA binding site on the ribosome, termed the P-site, and an aminoacyl-tRNA, charged with a single amino acid bound to a second location on the ribosome, termed the A-site. Peptide bond formation involves aminolysis of the P-site ester by the A-site $\alpha$-amino group (Fig. 1a). The reaction requires no additional energy source beyond the equivalent of ATP used to form the high energy ester linkage during aminoacylation of the tRNA by its cognate synthetase. The reaction is expected to proceed through a transition state intermediate whose features include a tetrahedral geometry about the carbonyl carbon and a negatively charged oxyanion. The collapse of the transition state intermediate results in a deacylated P-site tRNA and an elongated peptide chain coupled to the A-site tRNA. The recent high resolution crystal structure of the 50S subunit revealed that the PTC is comprised exclusively of RNA, and therefore, that the ribosome is a ribozyme (*2*). However, the specific catalytic mechanism of peptide bond formation within the ribosome remains uncertain.

The location of the PTC within the 50S structure was identified using the transition state inhibitor CCdApPmn (Fig. 1b) (*2, 3*). This compound simulated simultaneous binding of both A-site and P-site charged tRNA fragments. The three nucleotide CCdA portion bound to the P site and the single nucleotide puromycin (Pmn) bound to the A site. The segments were covalently joined by a phosphoramidate linkage with tetrahedral geometry, which geometry is proposed to mimic the tetrahedral intermediate of the transition state. The spatial location of this chemical group has served as the structural basis for models of catalysis by the PTC (*2*).

Despite its clear utility in defining the placement of A-site and P-site substrates within the PTC, CCdApPmn is not an ideal transition state analogue, and it is becoming increasingly clear that the differences may be functionally important (*4, 5*). Of particular concern is the 2'-deoxy adenosine (dA) substitution on the P site side of the inhibitor. tRNAs containing a dA substitution at the terminal residue are reported to be inactive as P-site substrates (*6*). Deletion of the 2'-OH removes a potentially important functional group that in the transition state may participate in hydrogen bonding, charge neutralization or proton transfer. Furthermore, the deoxy substitution results in a different sugar pucker in the ribose ring, which could distort the conformation of the residues closest to the site of chemistry. Consistent with this possibility, one of the non-bridging phosphate oxygens is within 2.8Å of the dA C2' within the CCdApPmn 50S ribosomal complex (*2*). Such a geometry would create steric clash if the 2'-OH were present at this position. An additional concern is the identification of the transition state chirality. The phosphoramidate is achiral because the two non-bridging oxygens are chemically identical. This is not the case for the tetrahedral intermediate, where the transition state is chiral and is likely to be specific to one diastereomer. The result is stereochemical ambiguity as to which oxygen of the phosphoramidate corresponds to the oxyanion and which corresponds to the C$\alpha$ carbon of the nascent peptide chain. Thus, there are

significant chemical and conformational issues to address in developing improved transition state mimics of the peptidyl transferase reaction.

**Specific Aims**
*1. Develop a general method for inhibitor synthesis using solid phase oligonucleotide chemistry.*
      The exclusion of the adenosine 2'-OH was necessitated by the solution phase synthetic approach used to prepare the inhibitor (*3*). We will reintroduce the 2'-OH and add additional functionality by developing a general solid phase synthetic approach for this series of molecules. The method will be adopted from the 2'-ACE chemistry reported by Scaringe et al. (*7*). The α-amino group of puromycin will be substituted with a hydroxyl for efficient phosphoramidite coupling. The α-hydroxyl will be protected with Fmoc, and the 2'-position protected with acetate. The derivative will be coupled to solid support via a succinyl moiety at the 5'-position. Following Fmoc deprotection, CCA will be added to the α-position of puromycin by standard phosphoramidite based solid phase chemistry.

*2. Prepare a series of inhibitors, including an all ribose inhibitor and an inhibitor with a chiral phosphate.*
      We have two primary synthetic targets. The first is an all ribose variant of the original transition state inhibitor, CCApPmn. This molecule will make it possible to determine how the binding affinity and geometry of the active site are affected by the additional 2'-OH group. The second target is a transition state inhibitor with a chiral phosphate. Using the general scheme outlined in the first specific aim, one of the nonbridging oxygens of the phosphoramidate will be replaced with a cyclopentane ring joined to the inhibitor by a carbon-phosphorous linkage. Cyclopentane serves as a mimic of the α-linked amino acid proline. It is a bulky moiety that will make it possible to biochemically define the orientation of the first amino acid in the growing peptide chain and the location of the oxyanion. Our hypothesis is that one of the chiral inhibitors will have a substantially higher binding affinity than the other. One diastereomer should create a steric clash as the cyclopentane ring is jammed against the active site, while the cyclopentane in the other inhibitor will be readily accommodated. This will make it possible to test the crystallographic predictions for the active site stereochemistry.

*3. Determine the binding affinity of the transition state analogues for the ribosomal PTC by chemical footprinting and/or enzymatic inhibition.*
      CCdApPmn binding protects U2585, a residue in the PTC, from modification by 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (CMCT) (*8*). This modification produces a stop when the RNA is reverse transcribed. This appears as a specific band on a polyacrylamide gel when the reverse transcription products are separated by electrophoresis. We will measure the extent of modification at differing inhibitor concentrations to determine the $K_d$ of inhibitor binding as was described previously (*5*). Alternatively, the affinity of CCdApPmn for the ribosome active site could be determined by measuring an inhibition constant ($K_i$). This is done by measuring the extent of peptidyl transferase inhibition at various concentrations of analogue (*5*).

*4. Obtain the structures of the inhibitors bound to the 50S subunit.*
      The all ribose form of the inhibitor and the diastereomer that binds with highest affinity to the ribosome will be provided to the Steitz/Moore laboratories for structural analysis (*2*). This complex will establish the path that the growing peptide travels between its site of synthesis and its escape out the exit tunnel. The location of this pathway and the stereochemistry of inhibitors are essential for the design and synthesis of novel antibiotics and chemotherapeutic agents that target the ribosomal active site.

**Bibliography**

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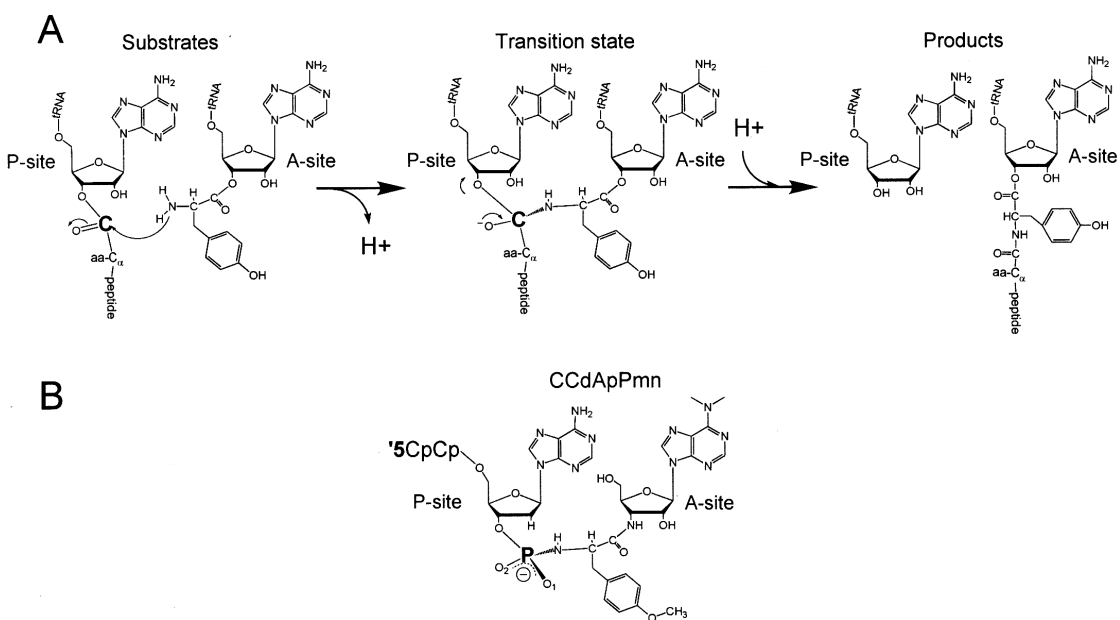
**Figure 1.** Peptidyl transferase reaction substrates, products, intermediates and inhibitors. A. Reaction of P-site and A-site tRNAs. The α-amino group of an A-site tRNA nucleophillically attacks the carbonyl carbon of the P-site tRNA (left). The reaction proceeds via a tetrahedral intermediate (center) and results in the transfer of the nascent peptide onto the A-site tRNA (right). B. Structure of CCdApPmn. The phosphoramidate mimics the tetrahedral geometry of the transition state during peptide bond formation. Chemical variations of this inhibitor that include a 2'-OH on the P-site adenosine and a chiral phosphorous center will be investigated in this study.