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**Synthesis of Sulfamoyl-Aminoacyl Adenylate
Analogues for use in Protein-RNA Structure
Determination**

by

Emil Mikael Nilsson

**An undergraduate honors thesis submitted in partial fulfillment of the
requirements for the degree of Bachelor of Science**

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Thesis Adviser

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Portland State University

2013

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Synthesis of Sulfamoyl-Aminoacyl Adenylate

Analogues for use in Protein-RNA Structure

Determination

Emil Nilsson, John Perona Lab and Robert Strongin Lab, PSU, Portland, Oregon

CH 403 Portland State University Honors Thesis

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Abstract:

Substrate analogs are small molecules that aid in the understanding of enzyme structure-function relationships. By careful design they can be utilized to probe the nature of the transition state in catalysis, and to assist in the process of rational drug design. Aminoacyl-sulfamoyl adenylate transition state analogs (aa-AMS) are analogs of the aminoacyl adenylate reaction intermediate in aminoacylation by aminoacyl-tRNA synthetases (aaRS) [16]. To better understand the chemical mechanism of aaRS, aa-AMS are used in crystallography experiments where x-ray diffraction helps reveal interactions crucial to catalysis. Here we report the complete synthesis and purification of Ile-AMS. The four-step process proceeds via a sulfamoyl-adenylate precursor, from which a library of aa-AMS can then be generated by the addition of particular amino acids. After addition of the amino acid

the product is put through an acid work up and purification step, giving an 85% experimental yield [17].

Because commercially produced aa-AMS are no longer available, assembly of a library of aa-AMS would be beneficial for future research both at PSU and through collaboration with other research teams worldwide.

Introduction:

The correct assembly of amino acids in mature proteins is essential for specific translation of the genetic code. The intrinsic ability of aminoacyl-tRNA synthetases (aaRS) to selectively discriminate amongst a vast variety of amino acids, some differing by a single atom, plays a crucial role in ensuring the fidelity of the genetic code.

aaRS have evolved to selectively recognize their cognate substrates (ATP, amino acid and, tRNA^{aa}); substrate binding is then followed by catalysis of aminoacylation and release of aminoacyl-tRNA (aa-tRNA^{aa}). The aaRS have evolved separate distinct pockets in which selective aminoacylation of substrate occurs. These active sites are formed through the tertiary structure of the protein and contain important amino acids whose side chains protrude in the active site and provide the necessary chemical environment for aminoacylation to occur [15].

The specific spatial orientation of the amino acids and their side chains throughout the enzyme provides aaRS with a chemical and sterical method for discrimination amongst amino acids [2, 10, 12, 15, 16, 20, 22]. This feature of the enzyme architecture is present throughout the aaRSs structure. The orientation of amino acid side chains in the aaRS allows for specific chemical interactions with its cognate substrates. It provides the cell with a way to distinguish between structural and chemical differences in possible substrates. Structural rearrangements occurring solely when aaRS is allosterically affected by its cognate substrate provide a mechanism by which selective aminoacylation of substrate is achieved, and the fidelity to the genetic code is increased. The attachment of an amino acid to its cognate tRNA by aaRS represents the true step of coding, rather than the consequent incorporation of the amino acid into protein on the ribosome.

To achieve specificity, the aaRS will have to overcome the challenge of rejecting non-cognate substrates. Class I tRNA synthetases (IleRS, ValRS and LeuRS) have evolved a second site of catalytic activity for post amino acyl transfer editing, to increase its fidelity for faithful translation [8, 15]. Post transfer editing recognizes misacylated species of tRNA through both chemical and sterical properties not present on canonical products of aminoacylation. Isoleucyl-tRNA synthetase (IleRS) has to be able to reject leucyl-AMP (Leu-AMP), which differs structurally from isoleucyl-AMP (Ile-AMP) by the placement of one methyl group. Furthermore, IleRS has to be able to recognize the subtle structural differences of valyl-AMP (Val-AMP) whose side chain structure only deviates from Ile by the absence of one methyl

group. IleRS uses a pre amino acyl transfer editing mechanism [7, 14, 21, 5] to ensure correct association of Ile to tRNA^{ile}. Preceding the transfer of the amino acid to tRNA^{ile} an editing event takes place, in which interactions within the active site of IleRS leads to the recognition and breakdown of noncanonical aa-AMP substrates. Non-cognate Val-AMP gets hydrolyzed during pretransfer editing to avoid non-canonical association with tRNA^{ile} [5, 7, 14, 21].

Investigations into the basis of this remarkable display of selectivity depend heavily on the use of small molecules designed to inhibit aminoacylation [11, 16, 18, 21]. Small molecules such as these inhibitors can be designed to probe the nature of specific interactions, during important distinct intermediate steps of catalysis, within the enzyme•substrate complex (Figure 1A). The trapped complex can be crystallized and through X-ray diffraction experiments elucidate structures from which important protein-nucleic acid interactions can be studied. Due to the selective nature of aaRS, competitive inhibitors have to be carefully designed to avoid rejection by the enzyme. Competitive inhibitors such as these can also be used in rational drug design [11, 13, 14, 18], by targeting bacterial translation. An example of such a drug is the antibiotic mupirocin that targets IleRS and leads to cell death [13, 14]. Mupirocin has also been used to solve crystal structures of IleRS•tRNA^{ile}•Mupirocin (Figure 1A). Due to the structural nature of mupirocin the available crystal structures have tRNA 3'-end (-CCA) protruding into the separate editing pocket of IleRS, [21].

Figure 1. (A) crystal structure of IleRS•tRNA^{ile}•Mupirocin. The black arrow indicates the 3'-end in the editing domain of the enzyme. (B) active domain of IleRS. The dark blue arrow and compound indicates location for binding interactions of Ile-AMS in the active site. Figure 1A provided by [21] and Figure 1B provided by [14].

The aa-AMS are a class of inhibitors used as thermodynamic traps in crystallography experiments. The use of sulfamoyl-aminoacyl adenylate analogs (aa-AMS) may allow for the crystallization of the reaction intermediate complex with the tRNA 3'-end in the active site [16]. These small molecules avoid rejection by their target aaRS by being sterically very similar to the cognate aminoacyl adenylate substrate. The inhibitor differs from the cognate substrate by the substitution of sulfur for the canonical phosphorus in the linkage between amino acid and the adenosine nucleoside (Figure 2a,b) [6, 16, 25]. The sulfamoyl group no longer provides a good leaving group during the transfer of the amino acid from AMP to

tRNA^{aa} due to the inability of the sulfamoyl group to form resonance structures. aa-AMS traps the enzyme•substrate complex by reducing the entropy in the active site through strong conformation to an active intermediate structure of the enzyme, preventing the enzyme from progressing and/or regressing in its reaction pathway.

Figure 2. Structural differences between cognate substrate aa-AMP and aa-AMS. **(a)** aa-AMP; **(b)** aa-AMS. **(c)** interactions between IleRS and Ile-AS with in the active site of IleRS, where the green represents the substituted sulfamoyl group. Figure 2(c) provided by [14]

Here we report the complete synthesis of 5'-O-[N-(L-isoleucyl)-sulfamoyl]adenylate analog (Ile-AMS) to be used in crystallization experiments aiming to determine required structural interactions within the intermediate complex of Ile-AMS•tRNA^{ile}•IleRS for progression during aminoacylation reactions. Using 2',3'-O-isopropylideneadenosine as a precursor allows for easy attachment of

standard amino acid derivatives and could be used to establish a library of aa-AMS inhibitors.

Methods and Materials:

Synthesis of Sulfamoyl chloride:

Scheme 1. Reaction pathway for the formation of sulfamoyl chloride.

The synthesis of the sulfamoyl chloride intermediate followed methods adapted from [1, 9], where a mixture of formic acid and a catalytic amount of dimethylformamide (DMF) is added drop wise to solution of chlorosulfonyl-isocyanate in dichloromethane (DCM) and allowed to reflux for 15 min at 42 °C to yield sulfamoyl chloride (Scheme 1).

Synthesis of 2',3'-O-isopropylidene-5'-O-sulfamoyladenosine:

Scheme 2. Synthesis of 2',3'-*O*-isopropylidene-5'-*O*-sulfamoyladenosine.

The synthesis of 2',3'-*O*-isopropylidene-5'-*O*-sulfamoyladenosine follows a procedure reported by Heacock [10]. During which, a solution of 2',3'-*O*-isopropylideneadenosine in dimethoxyethane (DME) is mixed with NaH and stirred for 30 min at 0 °C followed by addition of sulfamoyl chloride. The solution is left to reflux for 16 hours under argon at room temperature. Subsequent quenching of the reaction using methanol and concentration under vacuum yielded 2',3'-*O*-isopropylidene-5'-*O*-sulfamoyladenosine (Scheme 2).

Synthesis of 2',3'-*O*-isopropylidene-5'-*O*-[*N*-(Boc-isoleucyl)sulfamoyl] adenosine:

Scheme 3. Reaction pathway for the formation of 2',3'-*O*-isopropylidene-5'-*O*-[*N*-(Boc-isoleucyl)sulfamoyl] adenosine, where R = -CH(CH₃)-CH₂-CH₃.

The procedure for the synthesis of 2',3'-*O*-isopropylidene-5'-*O*-[*N*-(Boc-isoleucyl)sulfamoyl] adenosine was also adapted from Heacock [10]. A stock solution of 2',3'-*O*-isopropylidene-5'-*O*-sulfamoyladenosine was prepared in DMF. The coupling reaction was scaled to use 0.6 mmol of precursor in solution with 1.2 molar excess of *N*-t-Boc leucyl *N*-succinimide ester and DMF, 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) was added as a catalyst for the reaction.

Protective groups (isopropylidene and Boc) were designed to prevent undesired side reactions at sites of chemical activity on the molecule. *N*-succinimide provides a good leaving group for the formation of sulfamoyl linkage between AMS and Ile (Scheme 3).

Synthesis of 5'-O-[*N*-(*L*-isoleucyl)-sulfamoyl]adenosine:

Scheme 4. Final deprotection of 2',3'-*O*-isopropylidene-5'-*O*-[*N*-(Boc-isoleucyl)sulfamoyl] adenosine to yield final product Ile-AMS.

The procedure for deprotection and formation of Ile-AMS adapted from an article by Castro-Pichel [4], the ratio of TFA:H₂O was changed to 6:2 instead of literature reported 5:2. This allows for a more efficient conversion from protected to deprotected state. The reaction proceeded at room temperature for 2 hours (Scheme 4).

Purification of intermediates:

Products from each one of the above reactions, with the exception of the final deprotected product, were purified in a uniform fashion using silica gel size-

exclusion chromatography (Figure 3**b**). Where 60 mL silica powder was initially dissolved in a mixture of MeOH:CHCl₃ (1:10) to give a gel, and subsequently loaded onto a column. Products from preceding reaction were dissolved using MeOH:CHCl₃ (1:10), loaded onto the column and allowed to equilibrate. Elution of product was achieved by washing with MeOH:CHCl₃ (1:10) and 2 mL column fractions were collected and monitored by cough spotting on silica thin layer chromatography (TLC) plates to determine retardation factor (RF) of product (Figure 3**a**). The fractions containing the product with correct retardation factor were pooled and concentrated through rotary evaporation and used in ensuing synthesis step. RF corresponding to desired product reported in literature (1, 4, 9, 10).

Figure 3. (a) TLC plates under UV light. The grey line indicates the location of low RF product. (b) setup for collection of fractions during elution of silica column.

Purification of 5'-O-[N-(L-isoleucyl)-sulfamoyl]adenosine:

Procedure for purification of final deprotected product was adapted from Heacock [10], using size-exclusion chromatography. Dry Sephadex™ LH-20 was suspended in 70% MeOH/H₂O (v/v) and allowed to swell before loading into a column. The product from the deprotection reaction was dissolved in 0.1 M ammonium formate and loaded onto the Sephadex™ column. Silica TLC (MeOH:CHCl₃ (1:10)) was used to determine which collected fraction contained the final product. The selected fractions containing the desired product were pooled and concentrated using rotary evaporation.

¹H-NMR analysis:

Proton nuclear magnetic resonance spectrums were acquired in deuterated dimethyl sulfoxide (DMSO-*d*₆), at 400 MHz. ¹H-NMR spectra were collected from the final product and compared to a predicted spectra generated using ACD/HNMR predictor (version 5.12, Advanced Chemistry Development Inc.).

Activity assay:

Methods used for labeling of tRNA and aminoacylation activity assay adapted from (3, 19, 23, 24). Where tRNA^{ile} transcripts (received from Hari Bhaskaran) were radioactively labeled in the 3'-terminal internucleotide linkage using the activity of tRNA nucleotidyltransferase to incorporate a ³²P isotope. Upon exchange the tRNA transcript was heated to 80°C and allowed to refold in the presence of MgCl₂ (10mM) to its native state by slow cooling to ambient temperature. Labeled tRNA

was then incubated with isoleucine, ATP, IleRS and Ile-AMS reaction aliquots from time points were quenched with P1 nuclease and subsequently spotted and separated on cellulose TLC. Fraction of Ile-AMS bound IleRS compared to free IleRS was determined through PhosphorImager and plotted vs time to yield Figure 9.

Results:

Ile-AMS collection and quantification:

The final product was transferred to a pre-weighed vial (16.78996 g); after removal of the solvent by rotatory vacuum evaporation, the vial was weighed (17.02625 g) demonstrating an addition of 0.23629 g attributed to the mass of final product. Ile-AMS molecular weight was estimated to be 463.16 g/mole, the reaction thus produced 0.51 mmols of final product giving a %yield= $(0.51/0.6)*100= 85\%$.

Ile-AMS ¹H-NMR:

Comparison between collected and theoretical spectra for Ile-AMS indicated structural similarities between the synthesized and theoretical compound (Figure 4). Furthermore, the comparison between collected and predicted spectrums also revealed the presence of impurities within the synthesized compound due to the absence of corresponding peaks in the theoretical spectra. In the range of chemical shifts of 0.5-1.6 ppm the predicted and collected spectra integrates 9 protons and the peaks are very similar. The multiplets in the range 3.8-5.2 ppm integrate for 6 protons agreeing with the simulated spectrum. The peaks at 8.0-8.3 and 5.8 ppm

also correlate with the simulation. The peaks at 3.5 and 2.5 are characteristic of the solvent (DMSO- d_6).

Figure 4: ¹H NMR spectrum of Ile-AMS (top) and simulated spectrum (bottom).

Activity assay:

The activity assay demonstrates inhibition activity of Ile-AMS towards its cognate substrate IleRS (Figure 5). Inhibition of IleRS is also achieved by its noncognate substrate analog Val-AMS, and the positive control establishes normal enzyme function under experimental conditions in the absence of aa-AMP analog inhibitors.

Figure 5. Acylation assay demonstrating inhibition of IleRS by both Ile-AMP and Val-AMP sulfamoyl analogs.

Discussion:

The presence of Ile-AMS in the final product is elucidated in part by structural information deduced by comparison of collected and predicted ¹H-NMR spectrum (Figure 4). Combined with the canonical activity demonstrated during aminoacylation assay (Figure 5) we can confirm a successful synthesis of Ile-AMS. During the comparison of collected and predicted ¹H-NMR we see strong correlations between the two within the aromatic region. This region corresponds to ¹H attached to aromatic portions of the Ile-AMS; this suggests the presence of an adenosine like structure. However, we do see some differences within the region of exchangeable protons when comparing the collected spectrum to the predicted one.

This is to be expected due to the nature of these exchangeable protons. The exaggeration of the peak at 3.5 ppm in the collected spectrum is due to impurities in the sample associated with H₂O, and the peak at 2.5 ppm is dedicated to DMSO-*d*₆. The DMSO peak can stem from the sample being too dilute, this would further explain the level of noise observed in the region between 3.5-8 ppm. We must also take in to account the innate level of errors in the predicted ¹H-NMR spectra. The presence of H₂O in our sample can be due to insufficient rotary evaporation following final purification on the Sephadex™ column. H₂O is notoriously difficult to separate by evaporation due to its hydrogen bonding potential; increased assiduousness during coevaporation with ethanol can overcome problems associated with H₂O evaporation. The presence of Ile-AMS species in collected sample is made evident by the conformation of canonical inhibition of IleRS during activity assay (Figure 5). The intrinsic ability to selectively discriminate amongst substrates is essential for all enzymes. Inhibition of IleRS by the final product during acylation assay could therefore only ensue in the presence of Ile-AMS. Thus aminoacylation assay constitutes the final conformation of successful synthesis of Ile-AMS.

The achieved %-yield of the designed pathway is slightly elevated compared with %-yields reported in literature for analogous reaction pathways. This can be explained by the presence of impurities in the sample leading to overestimation in the mass from our product and consequently an inflated %-yield. Further research is required to increase experimental yields and efficiency of the designed pathway.

Future steps to increase our understanding for the basis of selective discrimination and communication within the nucleic acid-protein (Ile-AMS•tRNA^{ile}•IleRS) complexes would include the set up of crystal screens. This could allow for successful crystallization of the pretransfer active intermediate with tRNA^{ile} 3'-end bound in the active site. This could allow for determination of crucial interactions between the enzyme and substrate essential for successful recognition of cognate substrate. Furthermore, due to the demonstrated inhibition of IleRS by Val-AMS, during aminoacylation assay, we could foresee an investigation focusing on the active intermediate preceding hydrolysis of the noncanonical substrate Val-AMP. Unlike the existing crystal structure (Figure 1A) these investigations would focus on the pathway intermediate where the tRNA's 3'-end (-CCA) is interacting with the enzyme and substrate within the confines of the active site of IleRS. Allowing for conclusions to be made pertaining to the nature of the tRNA and its role in selective discrimination of noncognate substrates.

For the reason that commercially produced aa-AMSs are no longer available the establishment of a library of aa-AMSs might be beneficial for future research both locally within Portland State University as well as worldwide through collaborations with international research teams.

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