UNIVERSITY OF CALIFORNIA, SAN DIEGO

Targeting RNA with Small Molecules: Dihydropyrimidinone Derivatives as Novel RNA-Binding Ligands

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Chemistry

by

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2011

The Dissertation of Emily Satkiewicz is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego
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DEDICATION

I dedicate this dissertation to all those who helped teach me to believe in myself and go after my dreams, and who have overwhelmed me with support throughout this process. In particular I would like to especially dedicate this to my wonderful fiancé Adrian Garcia Sega, who carried out more than his fair share of the cooking, cleaning, and reassuring throughout the writing process, even going so far as to give up his first class airline seat so I could have the room and power to finish writing within my deadline. Also for believing in my dreams enough to give up the warmth and sunshine of San Diego to allow me to pursue my career ambitions in cold, snowy upstate New York. ILUM

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ABSTRACT OF THE DISSERTATION

Targeting RNA with Small Molecules: Dihydropyrimidinone Derivatives as Novel RNA-Binding Ligands

by

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Professor Thomas Hermann, Chair

The high functionality and complex three dimensional structure of RNA offers promise for the development of a wide range of RNA-targeting therapeutics. Previous studies of known RNA-friendly molecules reveal properties which promote RNA-small molecule interactions, such as distinct

edges containing multiple hydrogen-bonding donors and acceptors, positively charged amino groups, opportunities for pi-stacking, and molecule rigidity.

TAN 1057 is a natural dipeptide antibiotic thought to inhibit translation through interactions with ribosomal RNA. Modeling studies of the dihydropyrimidinone core of TAN 1057 show multiple potential interactions with each of the RNA bases. The synthesis of potential “RNA-friendly” small molecules was carried out using derivatives of the natural core of TAN 1057. Coupling of these derivative cores with various side groups containing “RNA-friendly” properties sought to promote further RNA-small molecule interactions.

Three cores in addition to the natural core were synthesized and derivatized when possible, creating a small library of compounds to be tested against functional regions of RNA. Specifically, this work sought to target functional RNA within the hepatitis C virus and thymidylate synthase RNA, and structure activity relationships were explored.

CHAPTER 1: INTRODUCTION. RNA as a Therapeutic Target

Central Dogma of Molecular Biology

The central dogma of molecular biology provides a framework for the flow of genetic information within biological macromolecules. In 1958, just five years after the elucidation of the structure of DNA, Francis Crick proposed that transfer of genetic information follows the path of DNA to RNA to protein (Figure 1.1).[1,2] Within this process, DNA contains the genetic material, proteins are responsible for the expression of a phenotype, and RNA was designated as a template for protein synthesis.[3] However, RNA is now recognized as a versatile biomolecule with many important functions.

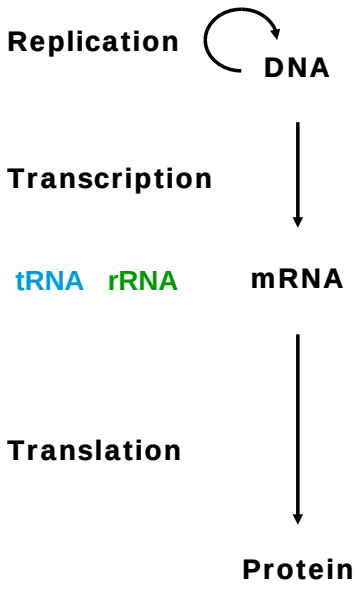

Figure 1.1: Framework of the central dogma of molecular biology.

RNA as a Therapeutic Target: Functional RNA

Since the 1980s RNA has been found to perform many functions outside of its traditional roles within the central dogma of molecular biology. RNA is an essential regulator for both transcription and translation, can act as a catalyst, and has been determined to be responsible for many other biological functions. Some examples of functional RNAs include ribozymes, riboswitches, microRNAs, internal ribosomal entry site (IRES), and siRNAs.[4-6] Key events within bacterial and viral reproduction are mediated by RNA and RNA-protein interactions, leading to intense investigations regarding RNA as a potential therapeutic target.[7]

The nucleotide building blocks of RNA are made up of three main components: one of four possible nucleobases, and a backbone formed by a ribose sugar and a phosphate group. These nucleotides allow RNA to adopt a regular helix with major and minor grooves similar to DNA. Unlike DNA grooves, the 2'-hydroxyl group on RNA results in alternative puckering of the ribose sugars.[4,8] Also unlike DNA, the A-helix of RNA is often disrupted by regions of mismatched or unpaired bases. These regions allow RNA to adopt a more complex three-dimensional structure and give rise to defined pockets for interaction or binding, analogous to those of proteins (Figure 1.2).

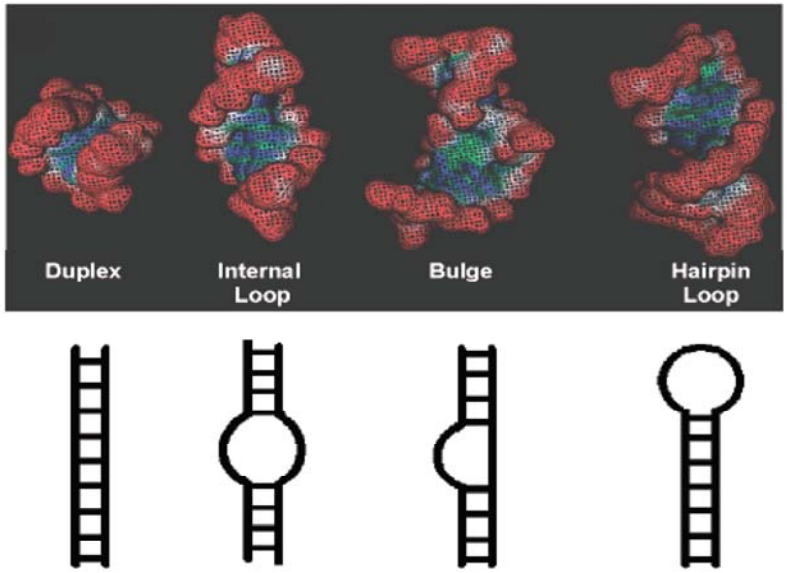

Figure 1.2: Perturbations of A-form helix found in RNA[4].

The broad range of important functions played by RNA paired with its complex three-dimensional structure presents RNA as an attractive therapeutic target. When compared structurally to protein targets, RNA has limited chemical variation found within its components. This may provide greater potential for targeting distinct binding pockets containing less chemical diversity. RNA's recently discovered functions include regulatory mechanisms essential for the survival of viral and bacterial pathogens. Through targeting the appropriate structural sequence of the RNA associated with an essential function of these perpetrators, progression of the invader may be halted. The principle of providing a moiety which recognizes structural RNA perturbations to convey binding specificity and another moiety which supports binding

strength through other non-specific interactions is frequently exploited by RNA-binding molecules[9]. This idea will be discussed further in future sections.

Although RNA presents itself as a therapeutic target, it is not without potential problems. It is a difficult target, but viable where other options are scarce, such as the case of viruses, or where there is good precedent in nature, such as the bacterial ribosome. Some problems that occur when targeting RNA include a lack of deep solvent-excluded clefts, and the delicate interplay between overall charge, affinity, and selectivity due to the overwhelming electrostatic interactions.[5,10] RNA is a largely unexploited category of potential targets, and a lack of structure activity relationship (SAR) information regarding known RNA binders also presents itself as a hurdle to RNA therapeutics. There are currently relatively few therapeutic agents known to target RNA or RNA-protein interactions. Of these, by far the most well studied RNA target is the ribosome.

Overview of the Ribosome

The ribosome is a lead player within the central dogma of genetic information. As discussed in the central dogma section above, the ribosome is the cellular machinery responsible for the translation of mRNA into protein. The ribosome contains three binding sites for tRNA which play essential roles in peptide formation: the aminoacyl (A) site, the peptidyl (P) site, and the exit (E) site. The A-site is the decoding site where aminoacyl tRNA binds,

providing the introduction of amino acids for use in peptides. The P site is responsible for peptide bond formation between the peptidyl tRNA introduced in the A site to the peptide being synthesized. The E site is the exit site where the now uncharged tRNA exits the ribosome after delivering an amino acid to the peptide. Although the ribosome consists of RNA subunits associated with initiation factor proteins, crystal structures have shown an absence of the proteins in close proximity to the reaction sites for peptide synthesis[8]. This serves to illustrate the ribosomal RNA (rRNA)'s ability to synthesize proteins without the use of other proteins as the direct catalyst. Therefore, the ribosome is a ribozyme.[11,12] The subunits of the ribosome each play a distinct role in the transcription process. Although the ribosome has been found to be a highly conserved structure present in all kingdoms, there are distinct differences between prokaryotes and eukaryotes with respect to the number, sequence, and structure of the components involved in translation.[13]

The ribosomes of prokaryotes and eukaryotes are fairly similar, and both keep the basic structural and mechanistic themes of protein synthesis practiced in all forms of life. However, differences within protein synthesis and its machinery can be observed. In prokaryotes a small 30S subunit binds to mRNA and is responsible for monitoring base pairing through decoding. The large 50S subunit binds tRNA and is responsible for accomplishing the peptidyl transferase reaction.[14,15] These two subunits combine to form a 70S particle with a mass of 2700 kd and facilitates protein synthesis. In eukaryotes, the functions of the small and large subunits remain the same, but

the eukaryotes contain more proteins and the densities of the subunits are larger, with the small subunit being 40S and the large subunit being 60S. These come together to form a 4200 kd 80S particle to facilitate protein synthesis.[16]

Another difference between the translation process of eukaryotes and prokaryotes is observed in the initiation of protein synthesis. Although both utilize a start codon found on mRNA, the mRNA of prokaryotes contains a purine-rich sequence called the Shine-Delgarno sequence which directs the ribosome to the start codon. This sequence also serves to distinguish the start AUG codon from internal AUG codons. Eukaryotic mRNA contains a 5' cap, a modified nucleotide on the 5' end of a precursor mRNA which is essential in directing the ribosome and initiating scanning of the mRNA for the start codon. In most cases, the mRNA contains only one start site and is therefore a template for a single protein. Prokaryotes, however, can have multiple Shine-Delgarno sequences and start codons on a single mRNA and therefore can serve as a template for multiple proteins. Throughout translation, eukaryotes have been found to utilize many more initiation factors, making their protein synthesis more intricate than in prokaryotes. Finally, termination of protein synthesis is carried out by a single release factor for eukaryotes compared with two in prokaryotes.

These differences between prokaryotic and eukaryotic translation can be exploited to allow for disruption of protein synthesis as a mechanism for defense against invading bacteria or viruses. A large variety of natural or

synthetic antibiotics inhibit the proliferation of pathogenic bacteria by binding to their ribosome and interfering with translation.[17]

Ribosome-binding compounds: Tetracyclines, Macrolides, and Aminoglycosides

Many compounds used in clinical medicine for treatment of infectious diseases interfere with bacterial protein synthesis by targeting the ribosome.[18] In general, antibiotics target the ribosome at locations of functional relevance, e.g., decoding, translocation, and peptidyl transfer.[19] Among the most prominent of these are classes of compounds mainly used as antibiotics including tetracyclines, macrolides, and aminoglycosides. The macrolides and the aminoglycosides in particular have provided insight and inspiration for the design of RNA-friendly small molecules synthesized in the Hermann laboratory.

Tetracyclines

Tetracyclines (Tcs) are a class of antibiotics discovered in the 1940s useful against both Gram negative and Gram positive strains of bacteria. This class of antibiotics is named for their four hydrocarbon rings (Figure 1.3), and is considered the first “broad spectrum” antibiotic. These Tcs were widely used in humans and animals for treatment of bacterial infections within the respiratory tract, sinuses, middle ear, urinary tract, and intestines.

Unfortunately, in the 1990s, a widespread microbial resistance to Tcs resulted in strongly decreased use.

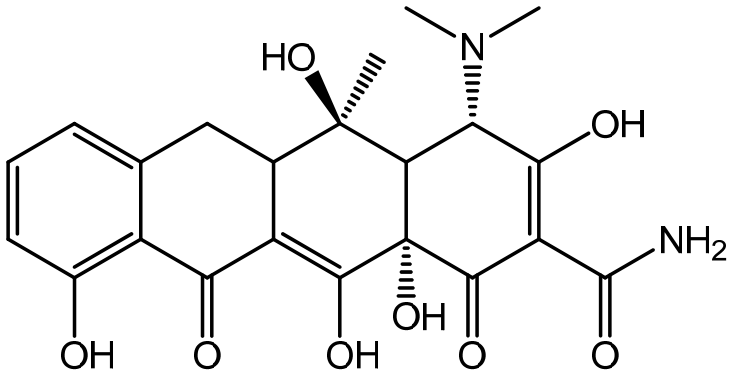

Figure 1.3: Structure of Tetracycline illustrating the four hydrocarbon ring system of the Tcs class of antibiotics.

The mechanism of action of the Tcs occurs through binding within the 30S ribosomal subunit and inhibiting protein synthesis through blocking the binding of aminoacylated tRNAs to the A-site. It has been suggested that Tcs may bind to A892 near the decoding site, as it protects this residue from chemical attack.[20] Studies have also been performed which establish the importance of several major 30S proteins in helping Tcs to achieve high-affinity binding at its high affinity site.[21] Consistent with affecting the binding at the A-site, Tcs have also been shown to block the binding of release factors during termination.[20]

Crystal studies have shown Tcs have one primary binding site with multiple secondary sites with the relevance of the second sites remaining unclear. Interestingly, resistance to Tcs have not been observed through changes within the ribosome itself, but instead through changes in the export

of Tcs across cell membranes or through chemical modifications of Tcs within the cell to render them inactive.[22] These changes help to exhibit the stability and importance of maintaining a highly conserved ribosomal structure in order to maintain precise functioning within each cell.

Macrolides

The macrolides are a class of compounds known to act on the 50S ribosomal subunit. Discovered in the 1950s, the macrolides mostly serve as antibiotics against gram positive strains of bacteria. This class of compounds are named for their large (14-16 member) macrocyclic lactone ring. This ring is attached to one or more deoxy sugar (Figure 1.4). The macrolides are popularly used for the treatment of pneumonia and strep throat. This class of compounds has a slightly wider spectrum for treating microbial infections, and for this reason it is commonly used by those who are allergic to penicillin. Of significant interest is the selectivity of the macrolides, with very little toxicity observed in humans.

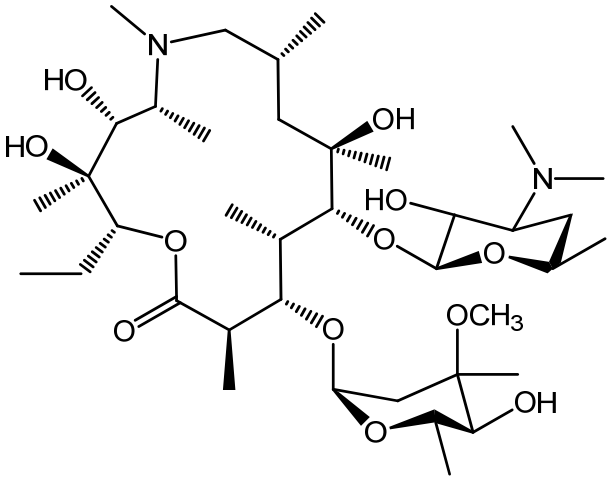

Figure 1.4: Structure of erythromycin illustrating the characteristically large macrocyclic lactone ring and attached sugars of macrolides.

The mechanism of action of the macrolides is somewhat dependent on the size of the lactone ring: 14-membered macrolides have been shown to inhibit translocation through blockage of the peptide exit site, whereas 16-membered rings inhibit peptidyl transferase reactions by inducing premature dissociation of peptidyl tRNAs.[23] Recent crystal structures have served to provide structural binding information showing the macrolides bind to the P-site of the 50S ribosomal subunit, but also interact with nascent peptides.[24,25] More structural information is needed to determine other potential binding sites of the macrolides within the ribosome, and the importance of those interactions.

A closer look reveals uniform interactions between the macrolides and rRNA. Throughout the macrolide class of antibiotics, the lactone ring has been shown to bind flat against the exit tunnel wall, while the saccharides are

seen protruding toward the peptidyl transferace center.[17] These contacts have been found to be universal amongst macrolides, with various side-chains establishing further rRNA binding interactions.

Aminoglycosides

Aminoglycosides are a class of antibiotics first introduced with streptomycin in 1944, and are clinically used for gram-negative baceterial infections.[26] Since the introduction of streptomycin, many powerful natural and semisynthetic aminoglycosides have been revealed. Aminoglycosides consist of sugars decorated with multiple amino groups (Figure 1.5). The central core of aminoglycosides, 2-deoxystreptamine, establishes the primary interaction within the ribosomal A-site and is essential to its subsequent antibacterial effects. The glycoside moieties further decorating the aminoglycosides provide secondary interactions and increase binding affinity.[6]

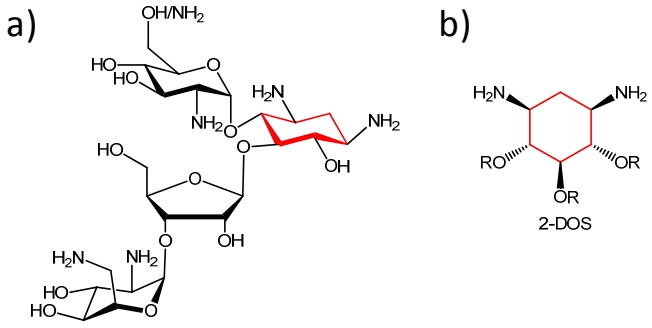

Figure 1.5: a) Structure of Paramomycin/Neomycin illustrating the hydrophilic sugar system strewn with amino groups. b) Central 2-deoxystreptamine core essential for incurring specific binding to rRNA.

The mechanism of action of the aminoglycosides is well studied. Aminoglycosides target accessible regions of polyanionic 16S rRNA on the 20 S ribosome, most notably at the A-site. Through creating a hydrogen bonding network, this class of molecules forms multiple interactions with the bases of RNA rather than the backbone.[27] During the mRNA decoding process, interactions of A site bases with the hybrid formed between the mRNA codon and the tRNA anticodon facilitates a conformational switch resulting in a decreased translational error rate.[28] Aminoglycoside antibiotics falsely induce this conformational switch resulting in acceptance of near-cognate tRNA and an increased error rate.

Aminoglycosides are well known as promiscuous RNA binders due to their charged nature and conformational flexibility.[10] This results in multiple binding sites and reduction of selectivity between prokaryotic and eukaryotic ribosomes. Unfortunately, serious side effects including renal toxicity and ototoxicity are also often observed, which limit the use of many aminoglycosides to serious infections or even as the “drug of last resort”, resorting to use only in extreme situations when all other potential treatments have failed.

Resistance to aminoglycosides has been seen through enzymatic modifications of the hydroxyl or amino group decorations. A few examples of observed modification of aminoglycosides include ATP O-phosphoryl transfers and acetyl CoA transfer of an acetyl group. Resistance have come about

through changes in gram-negative outer membranes, including both the decrease of the number of porin channels and through modifications to the lipopolysaccharide outer leaflet.[27] These changes once again help to exhibit the stability and importance of maintaining a highly conserved ribosomal structure in order to maintain precise functioning within each cell.

Learning from Known RNA-binding ligands

As observed in the examples of the macrolides and aminoglycosides, the interactions of a compound with the ribosome can be rather specific or promiscuous, depending on various characteristics. For example, one can easily observe the importance of electrostatic interactions within a drug-ribosome complex. Looking at aminoglycoside amino groups provides further explanation. These amino groups are protonated at physiological pH, and are able to function as polycations within the cellular environment. This overall charge enables increased polar and H-bonding interactions with the negatively charged RNA, resulting in promiscuity. In addition to charged electrostatic interactions, hydrogen bonding interactions also maintain an important role. Dense arrangements of H-bond donors and acceptors are necessary to confer strong interactions with RNA. It is also important for a molecule to maintain low conformational flexibility and high structural complexity in order to maintain selectivity. For example, the bulkiness of the substituents of aminoglycosides limits structural flexibility. This results in blocking rotation and subsequent

unwanted contacts with various RNA residues, making the aminoglycoside more selective toward the binding site. Therefore, in order to provide opportunities for intricate binding and stronger binding complexes the molecule must contain structural complexity.

Drug molecules can interact with both highly conserved nucleotides within functional RNA sites, and with peripheral, often less conserved RNA residues. Often, a core central to a class of antibiotics is found to be responsible for a specific binding interaction to rRNA. In addition to this specific binding interaction of the central core of a drug class with RNA, interactions between decoration around the core and RNA residues in proximity can interact. These subsequent interactions result in a strengthening of the drug-RNA interaction and can also help to increase specificity of the drug to the target RNA site. Also as exampled in aminoglycosides, specific spatial and physical characteristics found within a class of molecules can help to serve as a template for the design of new RNA-binding compounds. These models, discussed in macrolides and aminoglycosides, which take advantage of both primary and secondary interactions to a given target area and command distinguished characteristics helpful to confer binding interactions, have served as inspiration in the design of my project: dihydropyrimidinone derivatives as novel RNA-binding ligands.

TAN 1057

TAN 1057 is a natural product metabolite produced by the gram-negative soil bacterium *Flexibacter sp..*[29] TAN 1057 consists of two sets of dipeptide antibiotics active against Gram-negative and Gram-positive bacteria, including methicillin-resistant Staphylococcus aureus.[30] Excitingly, TAN 1057 exhibits *in vivo* efficacy superior to vancomycin, however, it also shows a high toxicity in mice most likely due to the nonspecific inhibitory activity between prokaryotes and eukaryotes.[31,32] Existing as diastereomers (A &B, and C&D, Figure 1.6), TAN 1057 C/D is composed of a beta-homoarginine moiety coupled to a distinct 7-membered lactam heterocycle, and TAN 1057 A/B is composed of a beta-homoarginine moiety attached to a unique heterocyclic amidinourea derivative based on diaminopropionic acid. My focus for the design and synthesis of RNA-friendly molecules has been based on TAN 1057 A/B.

The heterocyclic core of TAN 1057 A/B contains a dihydropyrimidinone (DHP) pharmacophore thought to act on the ribosome and is an essential part of TAN 1057's ability to inhibit translation. A close look at the pharmacophore offers strong potential for RNA interactions. The DHP core contains two distinct edges of hydrogen-bonding donors and acceptors, a quality helpful for interaction with RNA bases. In addition, modeling studies of DHP suggest a potential for hydrogen-bonding interactions with all four of the RNA bases (Figure 1.7).

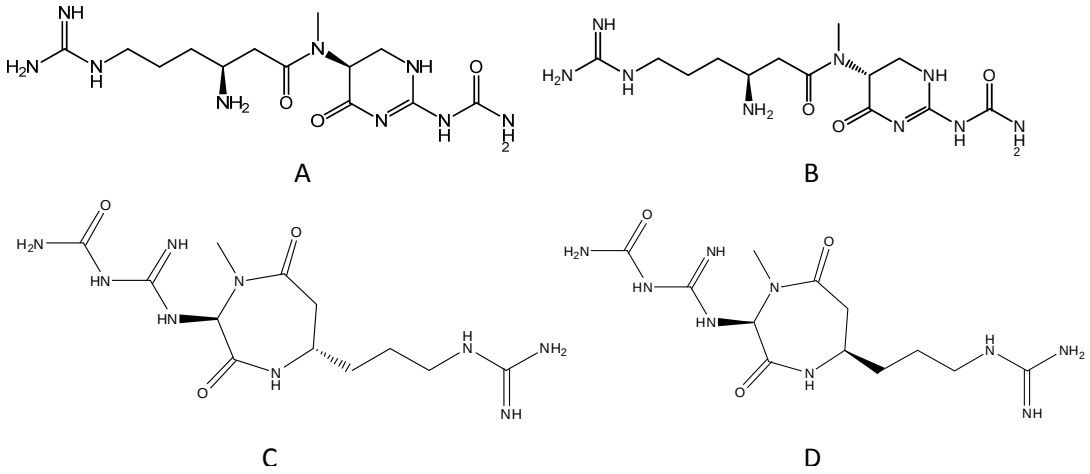

Figure1.6: Figure of the structures of TAN 1057 A-D.

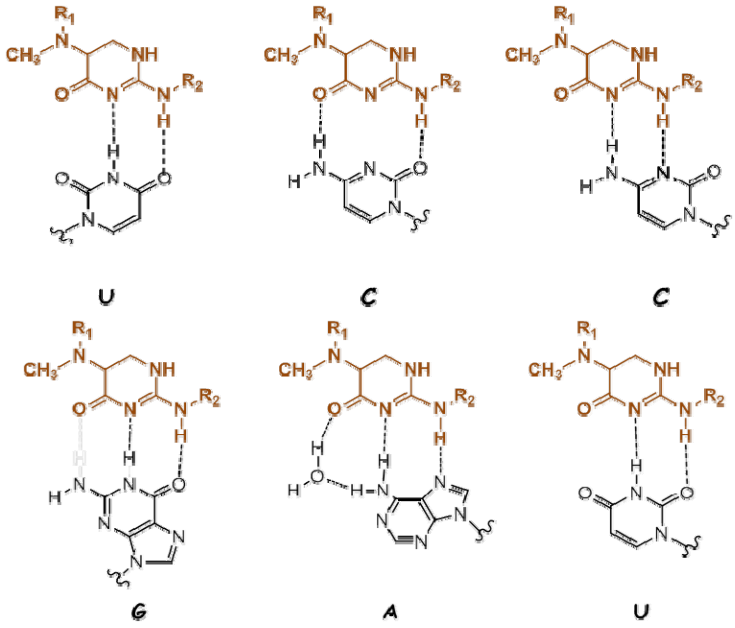

Figure1.7: Modeling studies of DHP pharmacophore showing potential hydrogen-bonding interactions between the different bases of RNA.

Many studies have been performed in an attempt to elucidate the mechanism of action of TAN 1057 A/B. While an exact mechanism of action is still unknown, studies strongly suggest the compound inhibits bacterial translation through specific interactions with RNA.[13] TAN 1057 has been illustrated as a viable target through the ability of TAN 1057 to inhibit protein

synthesis.[30] A detailed analysis of the mechanism of action was performed by a group at Gilead Sciences, Inc. through a dissection of each step of translation using isolated purified reaction components.[31] In these assays, it was discovered that TAN 1057 has no effect on mRNA binding to the ribosome, and translation initiation was only modestly reduced. However, elongation was shown to be completely inhibited. As described above in my discussions of the ribosome, the site of the central step during protein elongation has been mapped to the peptidyl transferase center located on the 50S subunit.

In an attempt to further elucidate the binding site, antibiotics known to bind at or near the peptidyl transferase site (chloramphenicol, tetracycline, and erythromycin) were radiolabeled and competitive binding studies were performed[13]. Binding studies with puromycin, a tRNA analog which binds to the A site and forms a covalent bond to the P site residue, also show no interaction of TAN 1057 with the A site or the P site. The results of these binding studies show no overlap with TAN 1057 binding, suggesting a unique binding site for TAN 1057.

Problems or obstacles to overcome associated with TAN 1057 include lack of selectivity between prokaryotic and eukaryotic translation, and that TAN 1057 requires active transport for uptake. In addition, previous studies have demonstrated that the (S) configuration of each set of diastereoisomers of TAN 1057 (A, D) are fifteen times more active than their (R) counterparts, and the component diastereoisomers of TAN 1057 A/B spontaneously

epimerize in solution.[33] The heterocyclic core has also exhibited a high sensitivity to acidic and basic media and to heat, resulting in a hydrolytic opening of the ring system and a subsequent loss of antibacterial activity. Nonetheless, the substantial antibacterial activity and strong potential for RNA binding interactions makes TAN 1057 A/B, and more importantly the central dihydropyrimidinone (DHP) core, of particular interest to the Hermann laboratory, and to my project in general.

Other RNA Targets

Besides the ribosome, there are numerous other potential RNA targets for small molecules including bacterial riboswitches, aminoacyl tRNA synthetases, ribozyme-catalytic RNAs, HIV viral Rev Response Elements (RRE), HIV Trans-Activating Response Element (TAR), Hepatitis C Virus (HCV) Internal Ribosomal Entry Site (IRES), and Thymidylate Synthase (TS) mRNA[4]. Of particular interest in the Hermann laboratory are the RNA targets within the HCV IRES and TS mRNA.

The Hepatitis C Virus

The Hepatitis C Virus is a small, 9.6 kilobase, enveloped positive-sense RNA virus from the family F*laviviridae.*[34] Identified in 1989, it has become a serious infectious disease affecting 150-200 million people worldwide.[35]

Infection occurs through blood, and the largest source of transmission is via drug injection use. The main replication site of HCV is the liver, causing inflammation, cirrhosis, and cancer of the liver. For these reasons, it is also the leading indication of liver transplantation in the United States.[36]

HCV infection begins with host cell entry via endocytosis. Next, HCV uncoats, freeing the positive-sense genomic RNA into the cytoplasm. This positive-sense RNA serves as the mRNA for the synthesis of the viral polyprotein. Initiation of translation is mediated by the IRES, found in the 5' untranslated region (UTR) of the viral genome. Translation of the single-stranded RNA genome yields a single, large polyprotein which is cleaved post-translationally into multiple structural and nonstructural (NS) peptides (Figure 1.8).[37]

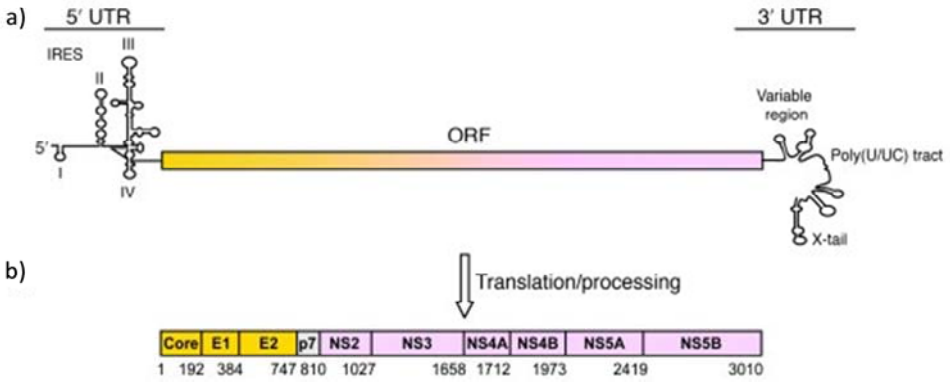

Figure1.8: a) The HCV genome consisting of a 5' and 3' UTR flanking an ORF. b) Post-translational polyprotein containing structural (yellow) and nonstructural (pink) peptides.[38]

The structural components consist of the nucleocapsid core (C), and two envelope glycoproteins (E1 and E2) responsible for cell interactions. The

NS peptides include NS2 through NS5 which have yet to be completely elucidated. NS3 is known to be involved in both helicase and protease activity, and NS5B contains RNA-dependent RNA polymerase activity essential for RNA viral replication. The enzymatic activities of NS3 and NS5B make them promising potential antiviral targets. In addition to the structural and NS parts of the genome, there are highly conserved 5' and 3' untranslated regions (UTR). The 3' UTR contains structured RNA elements essential for replication and translation of the virus. As stated above, the 5' UTR includes the IRES, which is essential for initiation of viral protein translation. An RNA binding assay developed in the Hermann Lab focuses on targeting the IRES, and will be discussed later in further detail.

Current therapies include interferon alpha, an antiviral which also boosts immune response, and interferon alpha in combination with ribavirin, a prodrug used to inhibit viral replication. Use of these drugs cause severe flu-like symptoms and only provide a resolution of the virus in less than 50 percent treated.[39] There are many challenges involved in developing new therapies for HCV, and extremely recently there have been a few successes.

Some challenges plaguing the development of new treatments include the virus' rapid rate of mutation, lack of small animal models, and the absence of efficient systems from which to study the infection system. As a result of HCV's high replication and mutation rates, the virus exists as different genotypes in nature.[40] Therefore, drug-resistant mutants may already exist in

patients. New drugs must be found which will be able to target all viral genotypes. Some current potential therapies target the HCV enzymes (protease or polymerase inhibitors), employ other immunomodulatory agents, or target the IRES RNA. Recently two drugs, Victrelis from Merck and Incivek from Vertex, have been approved for use as protease inhibitors. While not 'magic bullets' these new protease inhibitors exhibit increased efficacy in clearing the virus. More investigation is being completed on the various potential targets found within HCV, including the Hermann lab's focus on the HCV IRES.

HCV Internal Ribosomal Entry Site

The IRES is able to bypass cap-dependent initiation and the use of most other initiation factor proteins necessary for the recruitment of the ribosome to the start codon (Figure 1.9).[41] Although the HCV genome endures rapid mutation, the 5' UTR containing the IRES has been found to be highly conserved.[42] The highly conserved nature of the IRES highlights its importance within viral survival, and as a result offers promising evidence of a potential therapeutic target conceivably against any HCV mutant strain.

The IRES consists of four main structural domains of approximately 340 nucleotides (Figure 1.10). Under physiological salt conditions, tertiary folding occurs providing the structural integrity necessary for efficient protein synthesis.[11] Previous work emphasizes the importance of functional domains within the HCV IRES. Of particular interest in the Hermann lab is domain II,

particularly the subdomain IIa. It has been observed that domains II and IV contact the 40S ribosomal subunit (Figure 1.10).[43] Domain II is positioned in the head region of the 40S and occupies the E-site otherwise filled by deacylated tRNA just before leaving the ribosome. Domain II also extends into the mRNA cleft close to the P site where met tRNA must be placed to recognize the AUG start codon. Upon association with the IRES, the 40S undergoes significant conformational changes. Rotation of the head of the 40S results in movement of a latch structure which then in turn opens the mRNA entry channel. Although not confirmed, it is theorized that upon mRNA binding, this latch is thought to clamp around the incoming strand, stabilizing the mRNA on the 40S subunit.[44]

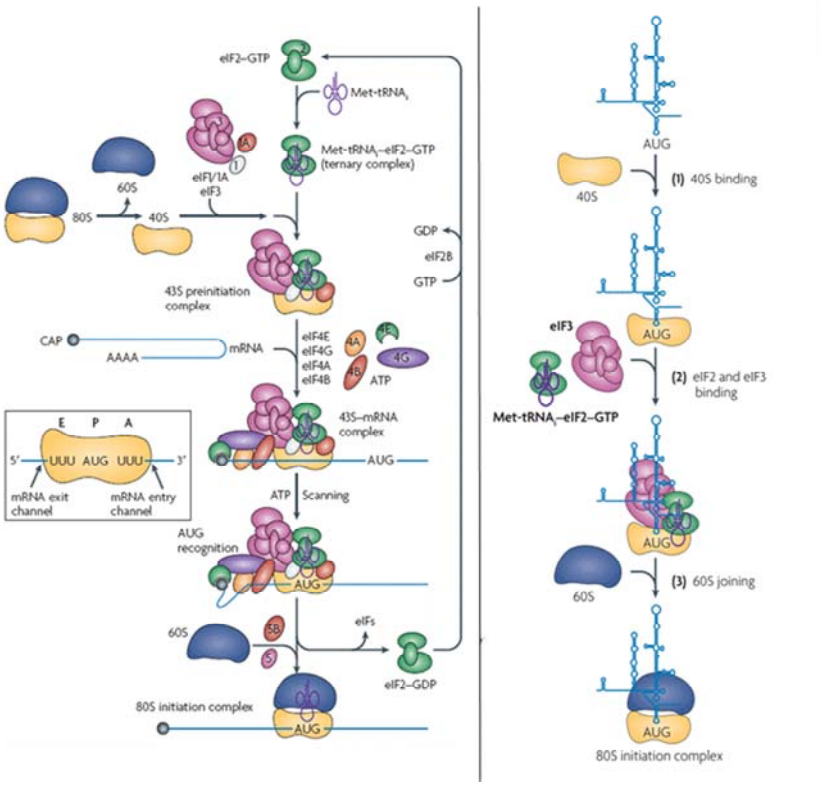

Figure 1.9: On the left illustrates eukaryotic translation, including numerous initiation factors (IF) and CAP recognition and scanning.[11] On the right is the comparable process carried out with the HCV IRES.[11] As one can observe, the IRES is able to facilitate translation without the use of most eukaryotic IFs and eliminates the need for scanning, directing the ribosome precisely to the start codon.

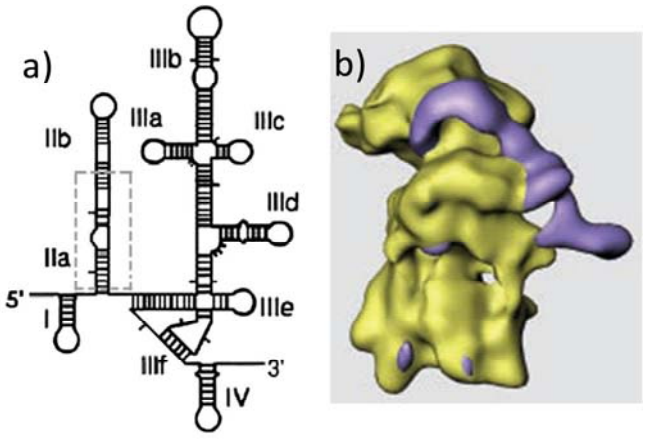

Figure 1.10: a) Designation of the domains and subdomains of the HCV IRES.[45] b) Cryo- EM image of the HCV IRES (purple) bound to the 40S subunit.[3,43] This illustrates the hinge-like character of domain IIa directing domain IIb directly to the E site and within proximity of the P site.

As illustrated in Figure 1.10, domain II interacts directly with the 40S ribosomal subunit. Formation of the IRES-eIF3-40S complex is dependent on the adoption of a cation-dependent L-shaped conformation by subdomain IIa.[44,45] The bent architecture of subdomain IIa is responsible for the positioning of the subdomain IIb hairpin, overlapping with the ribosomal E site and in close proximity to the P site. It is stabilized by a combination of not only the metal ions, but also by base-stacking and hydrogen bonding interactions. Our goal is to disrupt or alter this 90^{o} bent architecture through introduction of a small molecule.[45]

A mass spectroscopy assisted structure activity relationship (SAR) study identified a benzimidazole derivative as a 'hit' as targeting domain IIa of the HCV IRES (Figure 1.11).[46] Further exploration led to derivatives with sub-micromolar binding-affinity for the IIa RNA construct developed. SAR provided important information regarding the importance of each moiety within the structure. The benzimidazole and subsequent derivatives synthesized for SAR studies show that the dimethyl amino headgroup is critical. It also demonstrates the length of the alkyl tether is also important, that substituent additions are mostly not well tolerated, and highlights the importance of the charged nature of the compounds.

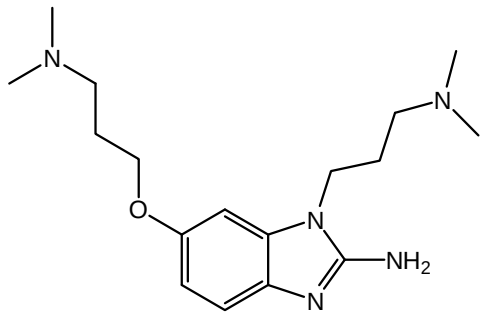

Figure 1.11: Structure of original benzimidazole 'hit' found from a high throughput screening (HTS) using a MS-based study.

Thymidylate Synthase

Thymidylate synthase (TS) is a highly conserved catalytic protein essential for the biosynthesis of the DNA building block thymidylate.[47] Active as a homodimer, TS is responsible for catalyzing the reductive methylation of 2'-deoxyuridine-5'-monophosphate (dUMP) to 2'-deoxy-thymidine-5'-monophosphate (dTMP), assisted by co-factor N,N-methylentetrahydrofolate (mTHF). Phosphorylation of dTMP affords dTDP which then continues on to the triphosphate dTTP, a direct precursor of DNA synthesis (Figure 1.12).[48] This TS catalyzed reaction serves as the only *de novo* source of dTMP, and inhibition of the reaction results in a halting of cellular proliferation and growth.

Figure 1.12: The role of TS in *de novo* DNA biosynthesis.

Besides its role as a catalyst, TS has also been found to undergo translational autoregulation through binding with high affinity to its own mRNA causing translational repression.[48] There are two TS binding sites found within the TS mRNA (Figure 1.13). The first is within the 5' UTR which includes a stem loop containing the AUG start sequence. The second binding site is found in the protein coding region corresponding to nucleotides 434 to 634. Studies have primarily focused on the 5' UTR binding site because of its known key role in placating translation initiation inhibition. This autoregulation highlights a potential mechanism by which DNA synthesis can be managed, and also offers a potential protective mechanism from cytotoxic stress.

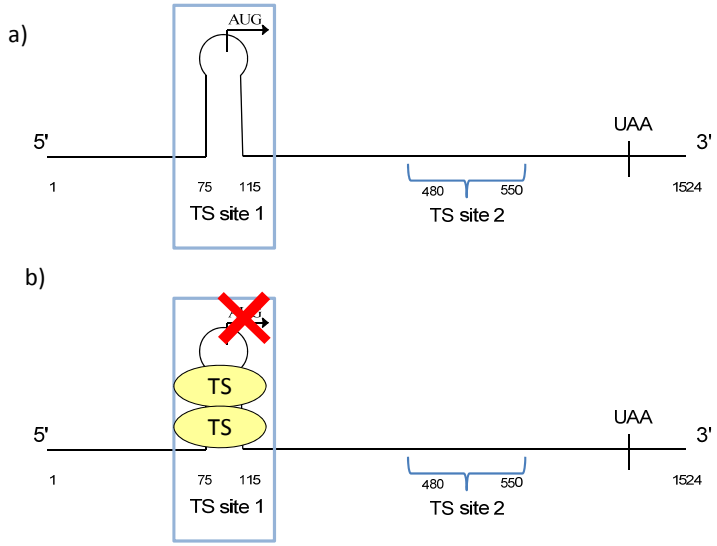

Figure 1.13: TS transcriptional autoregulation. a) When small concentrations of TS enzyme are present, the AUG start codon is available and transcription progresses. b) When excess TS protein exists, it binds to the stem loop in the 5' UTR and blocks translation initiation. Ligands can bind to the TS protein, releasing it from the binding site and restoring protein synthesis.#

Elevated TS levels found in tumors are thought to undergo more active cellular proliferation. TS protein and mRNA levels are elevated in patients with various human cancers, and an overexpression of TS results in apoptotic cell death.[48] This in turn may be associated with tumor invasiveness and metastisis.[49] This is further evidenced by the correlation between higher TS and a poor prognosis of many cancers.[49] Characterization of the TS protein-mRNA interactions may provide valuable information regarding the design of novel therapeutics. Current therapeutics, such as the dUMP analog 5-FU, target the TS protein. Another potential target is the folate cycle, targeted by antifolates. Our goal in the Hermann lab is to develop small molecules to target the TS mRNA in order to stabilize the hairpin in TS site 1 resulting in sequestration of the start codon and subsequent repression of TS protein synthesis.

Binding and Functional Assays

The Hermann lab has developed assays to test small molecule-RNA binding activity against Domain IIa of the HCV IRES and against the TS hairpin mRNA. The compounds currently being synthesized are being tested in a binding assay and a functional assay. The binding assay evaluates small molecule binding in the HCV IRES subdomain IIa using Förster Resonance Energy Transfer (FRET), and the functional assay employs a bi-cistronic

reporter to measure in translation inhibition generated via small molecule binding to the HCV IRES and TS mRNA binding.

FRET

FRET is a quantum-mechanical phenomenon that occurs when two chromophores are in molecular proximity. Through the use of a distance-dependent transfer of energy from a donor fluorophore to an acceptor molecule, it is possible to obtain information regarding molecular interactions of a given system. For this reason, its applications within bioscience are widespread and frequently used to study various cellular functions.[50] In FRET, the radiationless transfer of energy occurs when a donor chromophore, in its excited state, loses some fluorescence intensity through transfer to an acceptor, resulting in an increase in the acceptor's emission intensity. Monitoring these changes provides a means by which one can observe changes in interactions within a given system. In order for this transfer to occur the FRET donor/acceptor pair must be in close proximity of each other and the excititation spectrum must overlap the fluorescence emission spectrum of the donor (Figure 1.14).

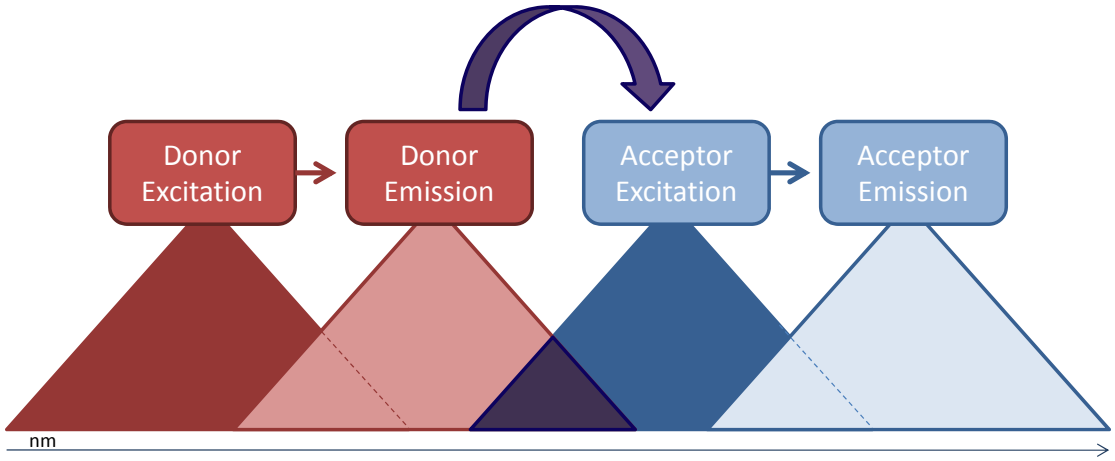

Figure 1.14: Spectral overlap necessary for a successful FRET pair.

Binding Assay: HCV IRES IIa FRET

As discussed in previous sections, subdomain IIa of the HCV IRES exhibits a sharp bend responsible for directing the hairpin loop of IIb towards the ribosomal E site and the active site. Our goal is to design and synthesize small molecules to displace the stabilizing divalent metal ions, altering the L-shaped conformation. We hypothesize that by altering the architecture of the IRES we can consequently disrupt the interactions of the IRES to the ribosome, therefore inhibiting viral translation.

In order to exploit the IIa bend as a potential therapeutic target, an assay was developed in which the angle of the construct could be monitored.[51] By labeling the 5' termini of each stem with a fluorescent dye, FRET could be used to monitor changes of the angle (Figure 1.15). Using information from a crystal structure of subdomain IIa[45], an RNA construct was designed to provide an ideal distance between the stem termini, accommodating FRET as

a means of measuring the angle. This assay allows for one to observe the changes in the angle as a result of small molecules binding to the RNA construct. Specifically, the assay allows for analysis of binding resulting in a conformational change to the native subdomain IIa. Even a small change in the conformation may lead to significant changes in translational activity. Previously, the assay has been validated through a titration with magnesium ions causing the RNA to fold into the stable bend. I have performed this previously developed assay on the compounds I synthesized and will discuss the results in future chapters.

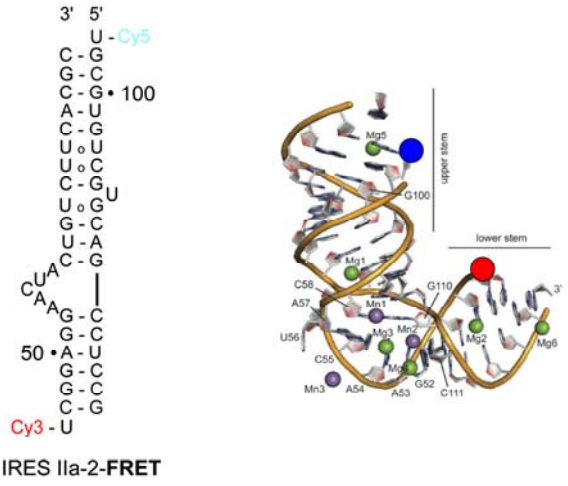

Figure 1.15: RNA construct of subdomain IIa developed for the FRET assay.[51]

Functional Assay: HCV IRES and TS mRNA

Described in previous sections, TS is responsible for the only *de novo* synthesis of dTMP, a precursor essential for DNA synthesis. TS is involved in autoregulation through interaction with its own mRNA at two specific binding

sites. Of these binding sites, TS binding site 1, which contains the start codon, has been well studied and plays essential roles in suppressing translation. Since evidence suggests a strong correlation between levels of TS expression and various types of cancer, the feedback mechanism associated with TS, specifically the TS mRNA, has great potential as a therapeutic target against cancer. Our objective is to develop small molecules to bind to the hairpin of TS binding site 1 in a similar fashion as the TS dimer in order to sequester the start codon and as a result, inhibit translation.

The functional in vitro translation (IVT) assay developed in the Hermann lab is used as a means to measure translational inhibition accredited to interactions from small molecule binding. Using a dual reporter system (renilla and firefly luciferase) in a cell-free extract translation system, we are able to measure and compare cap driven translation to IRES mediated translation. Through the development of a second scaffold containing both the hairpin found within TS binding site 1 and the IRES, we are able to acquire information regarding binding affinity of two Hermann lab RNA targets and obtain information about RNA binding promiscuity of our small molecule inhibitors (Figure 1.16). Specifically, through changes of reporter signal we can determine if a compound hits the cap-driven TS target, the IRES target, or translation in general. A reduction in firefly signal with no change in renilla signal would signify a hit of the TS target whereas a reduction of renilla signal with no change in firefly signal would signify an IRES hit. A general inhibitor of translation would result in decrease of both renilla and firefly signal.

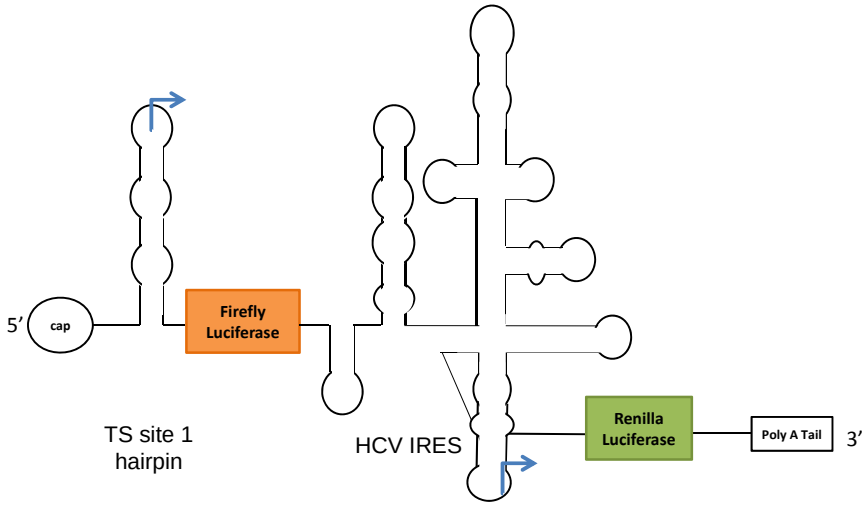

Figure 1.16 Layout of dual reporters measuring CAP driven TS translation versus IRES driven translation.

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CHAPTER 2: Derivatives of TAN 1057 A/B

Introduction

TAN 1057 A/B is a natural antibiotic which is thought to inhibit translation through specific interactions with RNA.[2] Specifically, the heterocyclic core of TAN 1057 A/B is essential for its antibacterial activity.[3] A close look at the structure of the heterocyclic core shows potential as an RNA binding small molecule (Chapter 1). Unfortunately, TAN 1057 A/B is plagued with poor solubility, toxicity,[2] and a core which can hydrolyze making it inactive. Here, we set out to design and synthesize analogs of TAN 1057 A/B with the goal of creating novel high-affinity RNA-binding small molecules with greater stability and solubility than the natural counterpart. The molecules synthesized were then tested for binding with the HCV IRES and thymidylate synthase (TS) RNA.

Design and Synthesis

TAN 1057 A/B consists of a unique dihydropyrimidinone core containing an exocyclic urea (DHP Urea) attached to a beta-homoarginine side group. Our efforts sought to investigate changes to both the core and the side group (Figure 2.1).

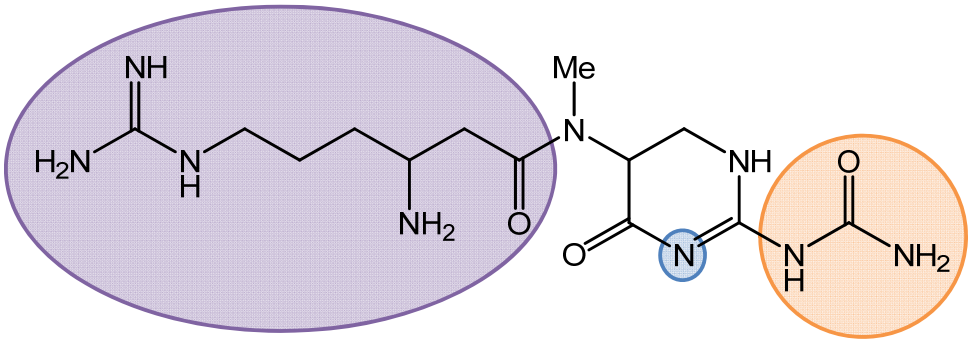


Figure 2.1: TAN1057 A/B with areas for derivatization highlighted.

Derivatization of the Heterocyclic Core

The syntheses of derivatives in respect to the heterocylclic core were designed with the intention of improving upon the liabilities of the natural heterocycle. In order to offer comparison, the natural heterocycle, here referred as the dihydropyrimidinone urea (DHP Urea), was synthesized and derivatized with various side group moieties. In an attempt to improve the solubility of the heterocyclic core, the exocyclic urea moiety was altered, providing a free amine referred to as the dihydropyrimidinone (DHP) core. Finally, we sought to improve solubility and stability through disruption of the heterocycle, substituting a carbon for one of the cyclic amines. This core also substitutes the poorly soluble urea moiety for an acetyl group, and is referred to as the carbon analog. All three cores designed conveniently utilize a common methyl ester precursor (Figure 2.2).

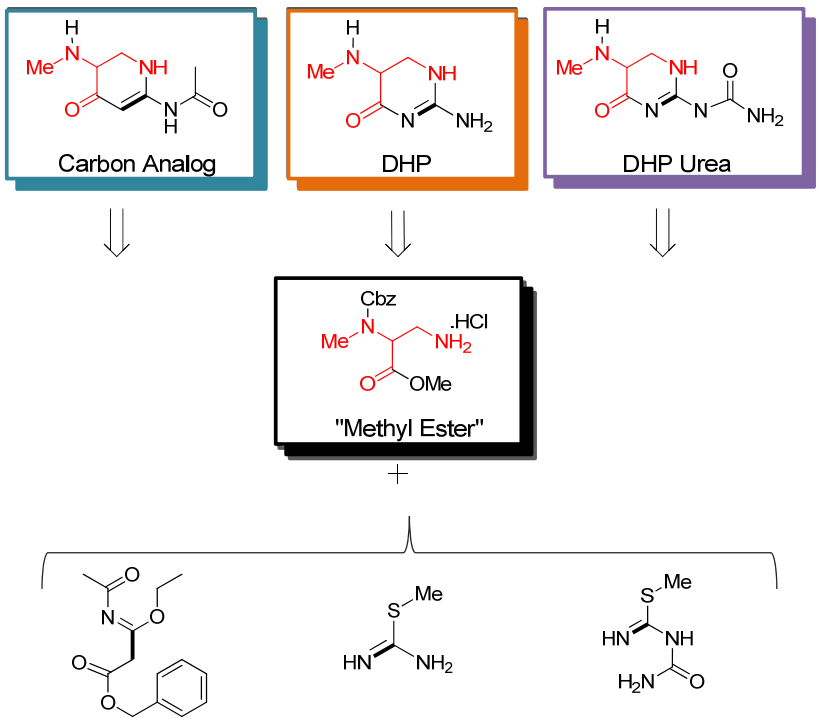
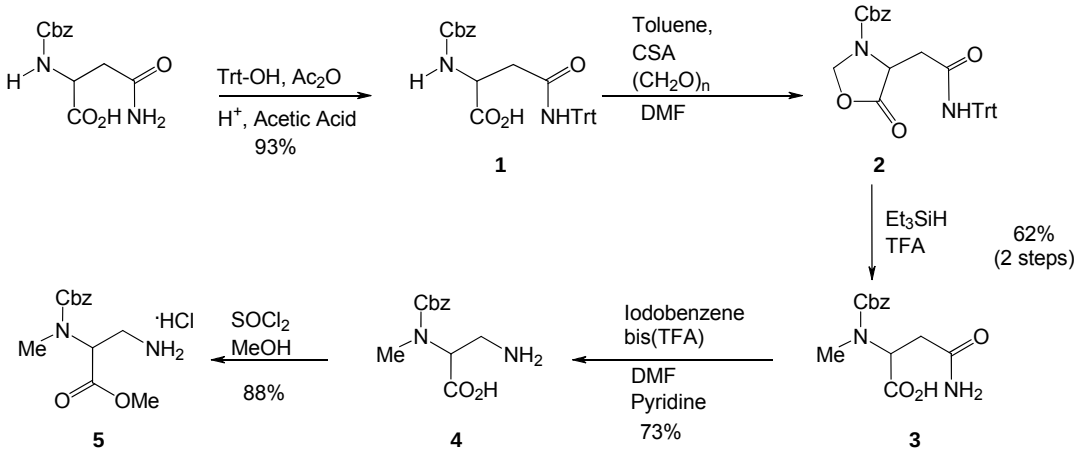


Figure 2.2: Proposed DHP analogs from a condensation with a common methyl ester precursor.

Methyl Ester Synthesis

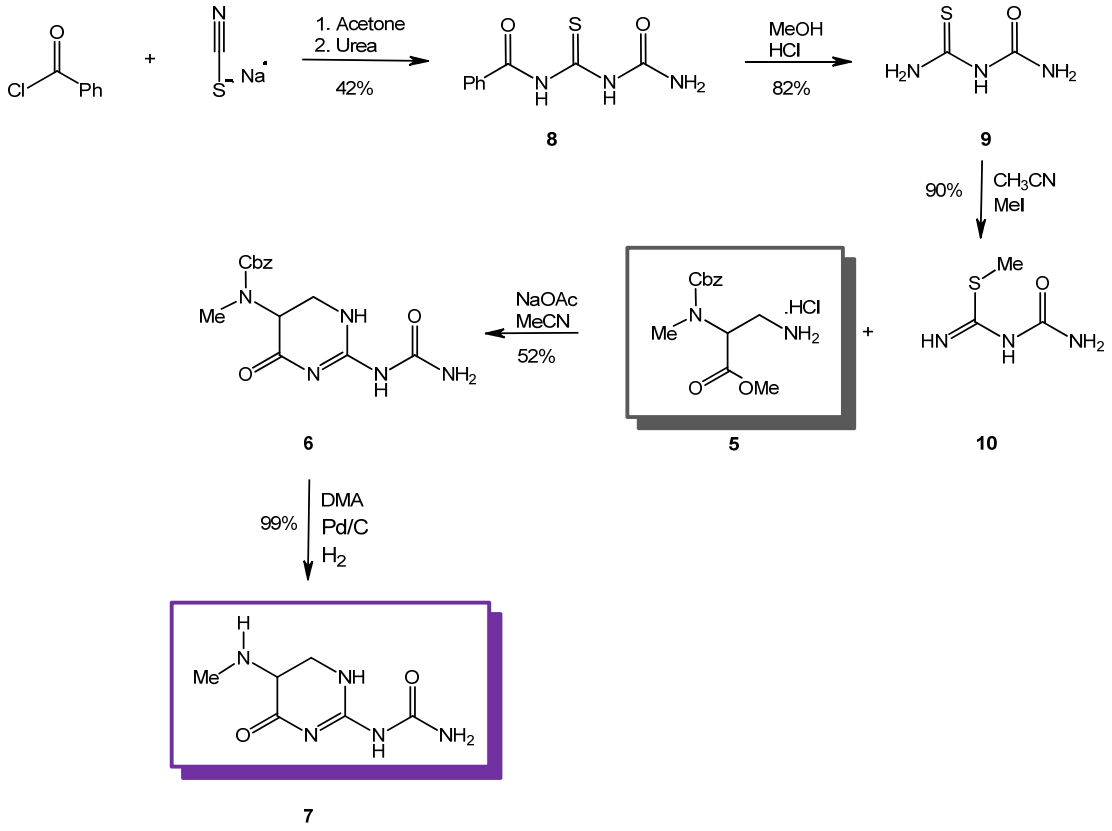


Scheme 2.1: Synthesis of the methyl ester precursor **5**.

The methyl ester precursor **5** used in the synthesis of the new cores can be made in five steps in good yield. As previously published,[4,5] the methyl ester precursor **5** was accomplished starting from commercially available benzyloxycarbonyl (Cbz)-protected asparagine (Scheme 2.1). The Cbz-asparagine first undergoes a trityl protection at the amide nitrogen in acidic conditions. Next, using the addition of paraformaldehyde and heat, the oxazolidinone **2** was formed. This cyclization was followed by a ring opening through the use of triethylsilane and subsequent trityl deprotection to form the N-methyl-Cbz-asparagine **3**. Compound **3** then undergoes oxidation of the amide nitrogen by iodobenzene bis-trifluoroacetic acid accompanied by Hofmann degredation yielding propionic acid **4**. This was then esterified to yield the methyl ester precursor **5**.

A large scale-up yielding 30 grams of the methyl ester was performed, and the overall yield was improved from that of the published procedure. In addition to an improvement in yield, the procedure of the oxazolidination was altered to provide a slow addition of paraformaldehyde in order to avoid the buildup of polymerized formaldehyde within the reflux condenser, and proved to make the reaction safer and more efficient.

Dihydropyrimidinone Urea



Scheme 2.2: Synthesis of DHP Urea **7**.

This synthesis of DHP Urea starts with the condensation of commercially available benzoyl chloride with sodium thiocyanate (Scheme 2.2). Upon addition of urea, benzoyl thiourea **8** is formed. The benzoyl group is then removed by methanolysis to yield N-(aminothioxomethyl)-urea **9**. Methylation using methyl iodide yields compound **10**. Using the methyl ester precursor **5** condensed with compound **10** gave Cbz-DHP Urea **6**, which then undergoes hydrogenation to yield the natural DHP Urea product **7**.

The synthesis of this core has been previously published, [4,6] however problems occurred when Cbz-deprotection was attempted. Various attempts were made to rectify the problems with deprotection (Table 2.1).

Table 2.1: Conditions explored to resolve unsuccessful deprotection of the DHP Urea core.

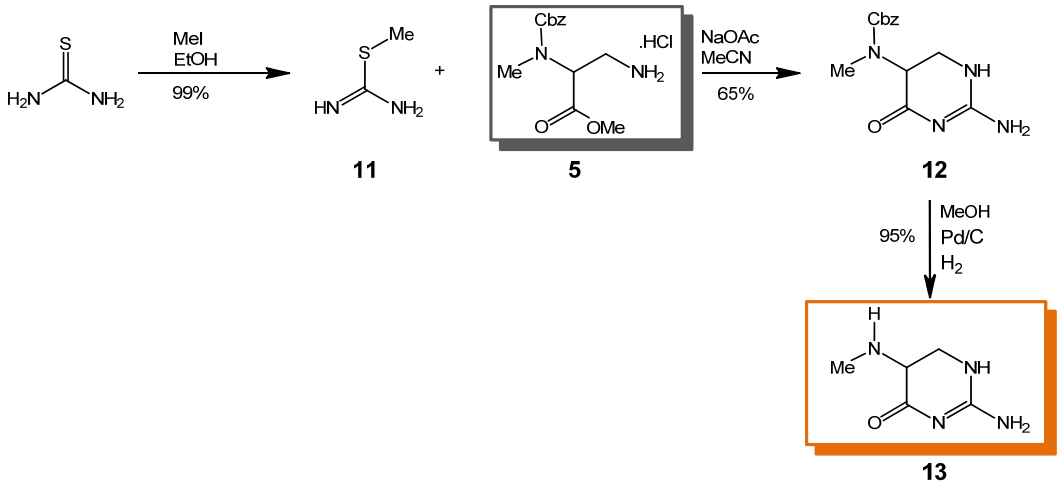
Z-DHP Quality	Water Disruption	Solvent	Catalyst	Reaction Conc.	Pressure	Sulfur Poisoning	I^- Poisoning
^{1}H NMR ^{13}C NMR MS	dry sm under P_2O_5 in vaccuum	anhydrous acetic acid DMF	test on Z-Ser and Z-Arg in MeOH	vary from [0.05] to [0.25]	used parr shaker 40 psi	use excess catalyst (stoichiometric)	use 10% silver nitrate solution to produce AgI

Attempts to both determine and resolve the problem with the deprotection are displayed in Table 2.1. In case of iodine poisoning from the previous methylation step to yield compound **10**, a 10% silver nitrate solution was employed. In order to eliminate potential sulfur poisoning from the condensation reaction of compounds **10** and **5**, stoichiometric amounts of catalyst was used. Additional steps to resolve the issue included testing the catalyst in a test reaction, trial reactions at different concentrations and pressures in multiple solvents, removal of water by P_2O_5, and re-verifying the quality of the precursor, compound **6**. While no sufficient result was yielded from these efforts, it was ultimately determined that a combination of low solubility, presence of sulfur residue from previous reaction steps, and the slow reactivity were the reasons for the Cbz-deprotection failure. This issue was resolved by rinsing of the Cbz-DHP Urea with ample isopropanol, and

performing the deprotection immediately following Z-DHP Urea formation, allowing the reaction to run for 24 hours under H_2 gas.

Coupling of the DHP Urea core with various side chains was achieved through the use of standard peptide coupling. In our trials, we found the best yield and the cleanest reaction through the use of 1-Hydroxy-7-azabenzotriazole (HOAt) and 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU) with triethylamine. Using this method a library of eleven compounds was made, and will be discussed in later sections.

Dihydropyrimidinone (DHP)



Scheme 2.3: Synthesis of DHP.

The DHP scaffold is made in a very similar fashion to the DHP Urea in 3 steps beginning with commercially available thiourea. Methylation with iodomethane yields the S-Methyl thiopseudourea **11**.[7] Condensation of

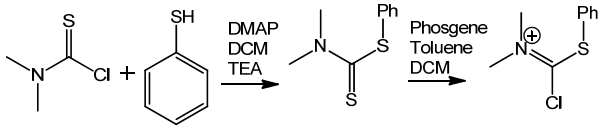
compound **11** with the common methyl ester precursor **5** results in the Cbz-protected DHP core **12**. Hydrogenation was then used to remove the Cbz group to yield the unprotected DHP core **13**. The solubility of this core, which lacks the exocyclic urea, was found to be improved, and deprotection readily occurred without incident. However, this deprotected core proved more volatile towards hydrolysis (Table 2.2).

Table 2.2: DHP and Cbz-DHP degradation.

Condition	Cbz-DHP	H-DHP
Air	No degradation	Some degradation
Methanol	No degradation	No degradation
Water	No degradation	Hydrolysis
Acetic Acid	Some degradation	Complete degradation
HCl	No degradation	Some degradation
Triethylamine	Complete degradation	Complete degradation
Sodium Bicarbonate	Some degradation	Some degradation
DIPEA	Complete degradation	-

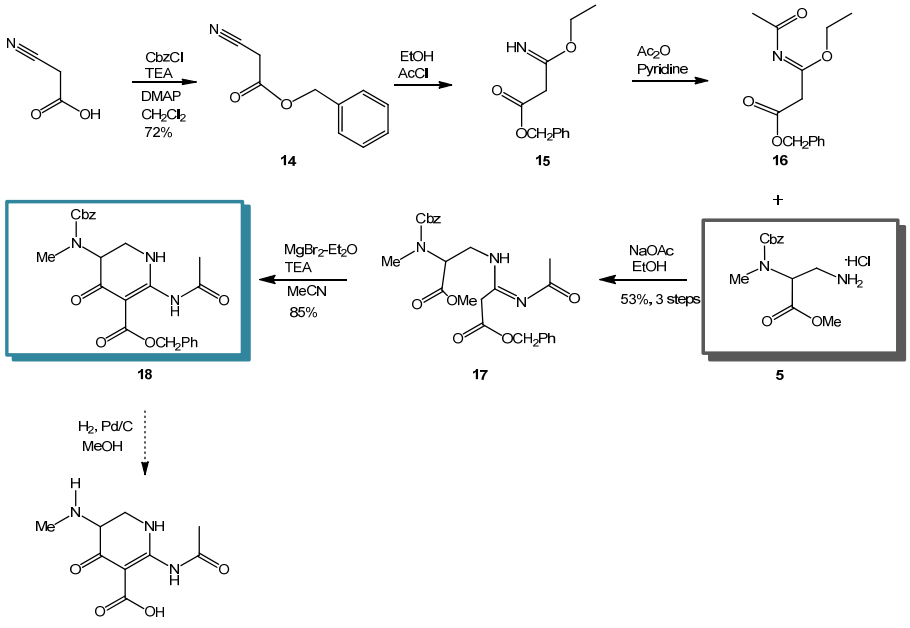
The instability of the DHP core lead to problems with coupling the core to various side-chains. Standard peptide coupling, as used in the DHP Urea core requires the use of base. However, we observed degradation of the core in the presence of base, making our current peptide coupling method unusable. Instead, (chlorophenylthiomethylene)dimethylammonium chloride (CPMA) and polymer supported isobutyl 1,2-dihydro-2-isobutoxy-1-quinoline-carboxylate (PS-IIDQ) were investigated which do not require basic conditions. The synthesis of CPMA was achieved in two steps based on a previously

published synthesis (Scheme 2.4).[8] A coupling of dimethylthiocarbamoyl chloride with thiophenol yields the dithiocarbamate. This then undergoes a reduction with phosgene to yield CPMA. Unfortunately, use of this coupling reagent requires dichloromethane as the solvent. Solubility of the core was therefore a problem, and the use of CPMA was abandoned. Use of PS-IIDQ was then investigated. While still concerned about solubility, I was able to accomplish coupling of the DHP core with Cbz-protected phenylalanine with the use of DMSO as a co-solvent.



Scheme 2.4: Synthesis of CPMA.

Carbon Analog



Scheme 2.5: Synthesis of Carbon Analog core **18**.

The carbon analog was synthesized as published starting from commercially available cyanoacetic acid (Scheme 2.3).[9] First, the cyanoacetic acid was Cbz protected at the carboxylic acid. The protected compound **14** is then exposed to HCl to provide imidate formation and ethyl esterification. Compound **15** was carried directly forward without purification. Acetylation of the imine nitrogen is achieved using acetic anhydride and pyridine to give the acetylated imidate **16**. Condensation with the methyl ester precursor **5** leads to an open-ring form of the carbon analog. With the help of the chelation activator magnesium bromate di-etherylate, the ring undergoes a successful closing. The final step to deprotect and decarboxylate the core was unsuccessful. We speculate the deprotection leads to degredation of our product, since no recognizable fragments were found by TLC or mass spectrometry. This paired with the instability of the intermediate products lead to the termination of our attempts at synthesizing the carbon-substituted core.

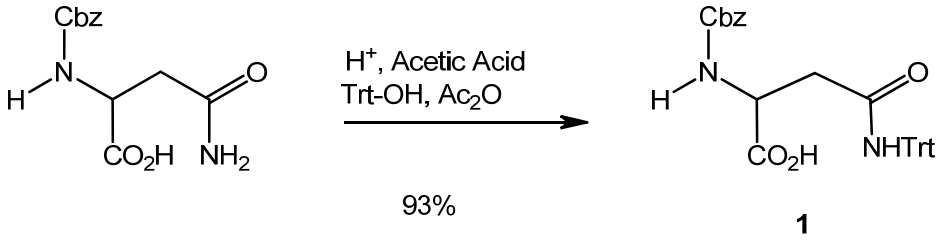
Side Group Derivatization

In an attempt to explore the effects of side group interaction and confer additional RNA-binding interactions, various carboxylic acids were coupled to the derivative cores. Side group addition was carried out by employing various peptide coupling conditions as discussed in previous sections. Chosen side groups consisted of amine-protected amino acids and commercially available carboxylic acids. Of these, we sought to include “RNA-

friendly" moieties such as free amines, to allow for additional H-bonding and ionic interactions, and phenyl groups, to allow for pi stacking.

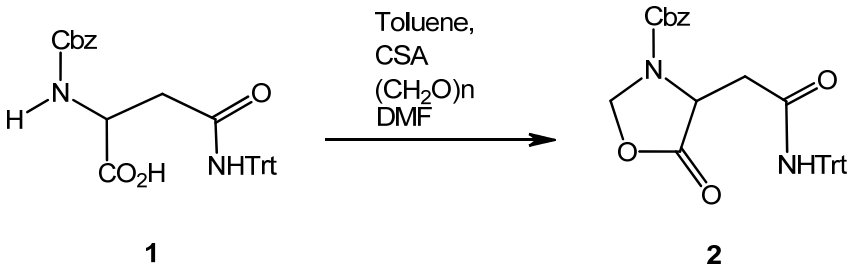
Materials and Methods

Compound Synthesis



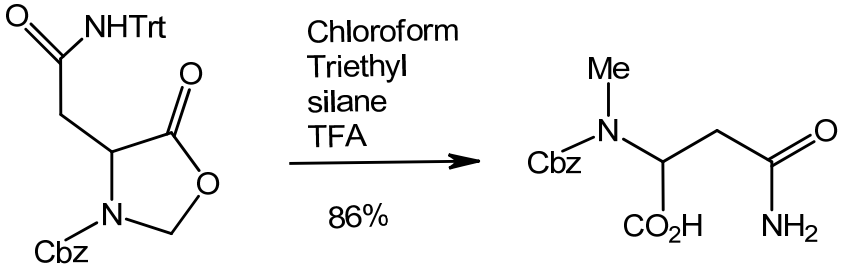
Scheme 2.6: Trt Protection

Z-protected asparagine (1 eq.), triphenyl methanol (2 eq.), acetic anhydride (2 eq.), and sulfuric acid (0.1 eq.) were suspended in acetic acid (0.33M). The reaction mixture was heated to 50°C for 2.5 hours. The reaction mixture was then slowly added to cold water. The precipitate which formed was filtered off and re-dissolved in ethyl acetate. Next, the ethyl acetate layer was washed with water, dried over magnesium sulfate, and concentrated in vacuo. The residue was recrystallized from ethyl acetate/hexanes to yield compound **1** as a white solid.



Scheme 2.7: Cyclization

Compound **1** was dissolved in a minimum amount of DMF (2.5M), and then combined with camphorsulfonic acid (0.04 eq) and paraformaldehyde (35 eq. added over the length of the reaction). The reaction mixture was suspended in anhydrous toluene (0.04 M), and was heated to reflux. Monitored by TLC for depletion of starting material **1**. Upon completion, the reaction mixture is filtered in order to remove excess paraformaldehyde, and the filtrate is concentrated in vacuo. The residue was then re-dissolved in ethyl acetate and washed with saturated sodium bicarbonate. The organic layer was then dried using magnesium sulfate, filtered, and concentrated in vacuo. The residue was purified by column chromatography using 40% ethyl acetate in hexanes to yield compound **2** as a yellow oil.

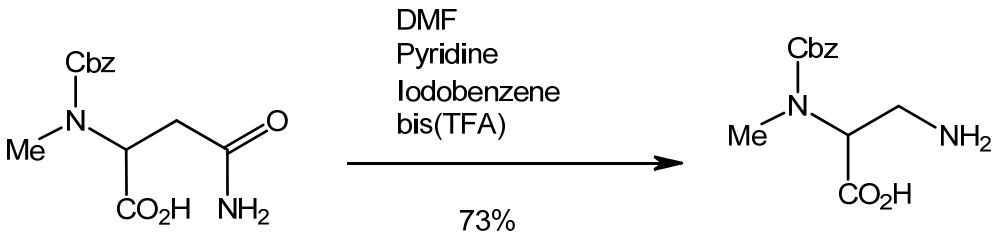


2 **3**

Scheme 2.8: Ring opening and Trt deprotection

Anhydrous chloroform (0.15M) was added to compound **2**. Next, triethylsilane (3.9 eq.) was added, followed by addition of trifluoroacetic acid

(0.15M). The reaction mixture was left to stir for 48 hours, monitoring by TLC. Upon completion the reaction was stopped and was concentrated in vacuo. Purification via column chromatography was carried out using 90:10:0.5:3 chloroform: methanol: water: acetic acid as the solvent system to yield compound **3** as a solid.

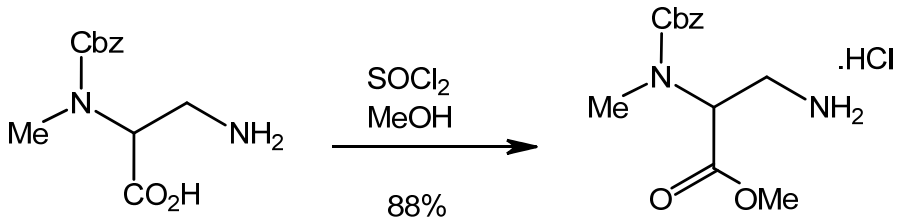


3 **4**

Scheme 2.9: Oxidation and Hofmann Degredation

To compound **3** was added iodobenzene bis(trifluoroacetic acid) (1.5 eq.) and the mixture was suspended in 50% (v/v) DMF/H_2O. The reaction was stirred at room temperature for 15 minutes, followed by the addition of pyridine (2 eq.). The reaction was then stirred for an additional 5 hours, followed by a quick concentration in vacuo at 60^{o}C. The oil residue obtained was next dissolved in water and washed with ether. The aqueous layer was concentrated in vacuo, followed by re-dissolving the sample in ethanol. The pH of the sample was adjusted to 7 using 15M aqueous ammonia, placed in an ice bath and carefully precipitated with ether. After allowing for

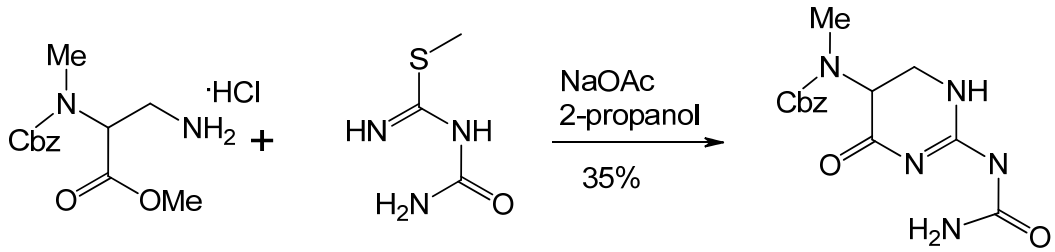
precipitation for 2 hours, the solid was collected and dried to yield pure compound **4**.



4 **5**

Scheme 2.10: Methyl esterification

Thionyl chloride (3.5 eq.) was added to anhydrous methanol (0.33M) and was cooled to -15°C for 10 minutes. To this solution was added propionic acid **4** and the mixture was allowed to warm to room temperature. After 24 hours, the reaction mixture was concentrated in vacuo. The residue was recrystallized from methanol and ether at -20°C yielding compound **5** as a white precipitate.

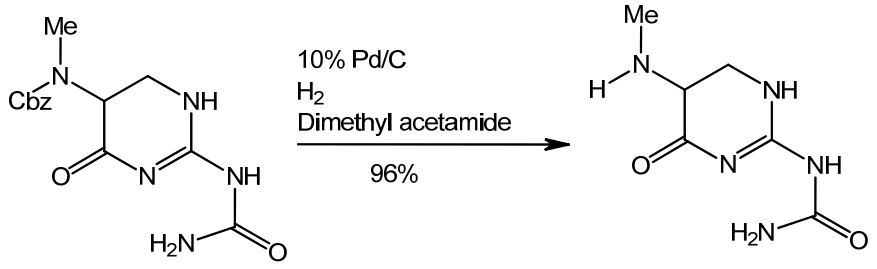


5 **3a** **6**

Scheme 2.11: Cbz-DHP Condensation

A suspension of compound **5**, compound **10**, and sodium acetate (2.2 eq.) in acetonitrile (0.125M) was stirred at 55°C. After 2 days, the reaction

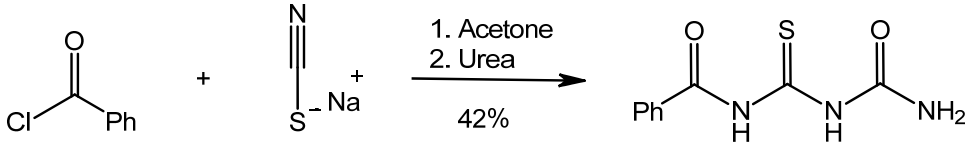
was concentrated and the residue was washed with 3% aqueous sodium bicarbonate. The reaction solution was then filtered with suction, and the solid was washed with water, methanol, isopropanol, and ether to yield compound **6** as an off white solid.



6 **7**

Scheme 2.12: Cbz deprotection of DHP Urea

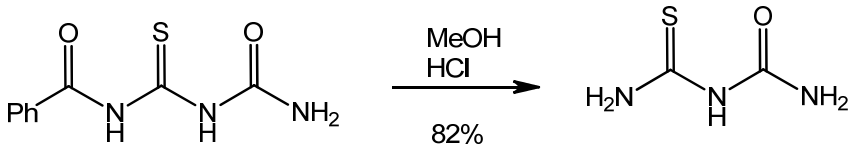
To compound **6** was added anhydrous dimethylacetamide (0.03M). The reaction mixture was purged with argon followed by addition of 10% Pd/C (0.5 eq.). The mixture was purged a second time with argon, followed by purging for 15 minutes with hydrogen. The reaction was left to stir at room temperature under hydrogen overnight, and was monitored by TLC. Upon completion, the reaction was purged with argon, filtered through a pad of celite, and concentrated in vacuo to yield compound **7** as a colorless solid.



Benzoyl Chloride Sodium Thiocyanate **8**

Scheme 2.13: Benzyl thiobuiruet

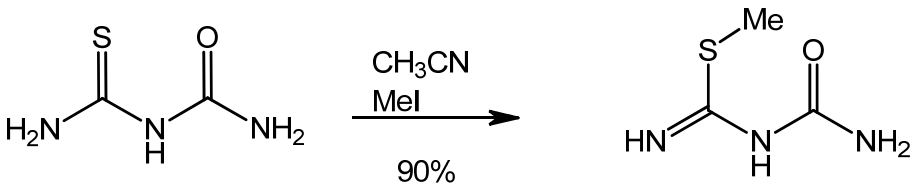
Sodium thiocyanate (1.2 eq.) was dissolved in acetone (1.4M) and warmed to 50°C. Benzoyl chloride (1 eq.) was then slowly added and the reaction remained heating for 15 minutes. The reaction mixture was then cooled to room temperature and the filtrate was removed. The filtrate was next heated to 50°C with urea (1 eq.) for 1.5 hours. The mixture was removed from heat and allowed to cool, followed by filtration. Purification was carried out by recrystallization in acetonitrile to yield compound **8** as yellow.



8 **9**

Scheme 2.14: Thiobiuret

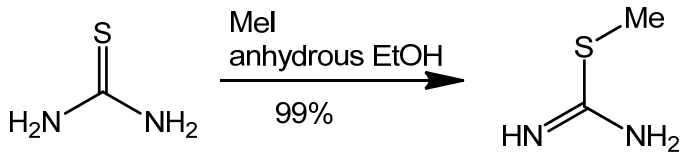
Methanol (2.2M) and hydrochloric acid (0.5 eq.) were added to compound **8**. The solution was then refluxed at 70°C, monitoring the reaction progress by TLC. The reaction was stopped after 3 days, and was concentrated in vacuo. The residue was washed with hexanes and recrystallized from water/charcoal. The crystals collected from the water recrystallization were then recrystallized from acetonitrile to yield compound **9** as pure crystals.



9 **10**

Scheme 2.15: S-methylation of Thiobiuret

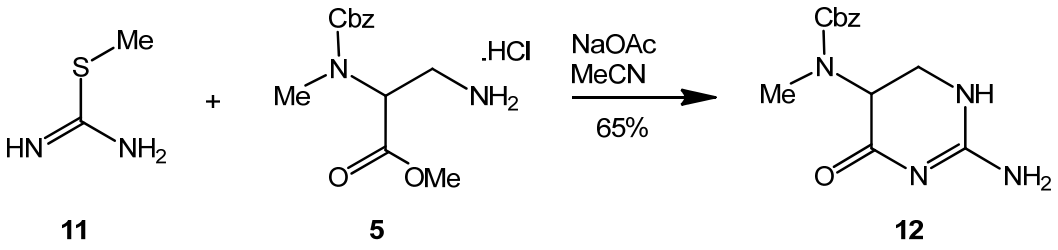
Thiobiuret **9** was suspended in acetonitrile (1M). Next, methyl iodide (1.2 eq.) was added and the mixture was heated to reflux for half an hour. After the reaction was stopped and allowed to cool a white precipitate formed. The filtrate was filtered off and allowed to dry overnight in the vacuum to yield compound **10** as a shiny white solid.



Thiourea **11**

Scheme 2.16: S-Methylation of Thiourea

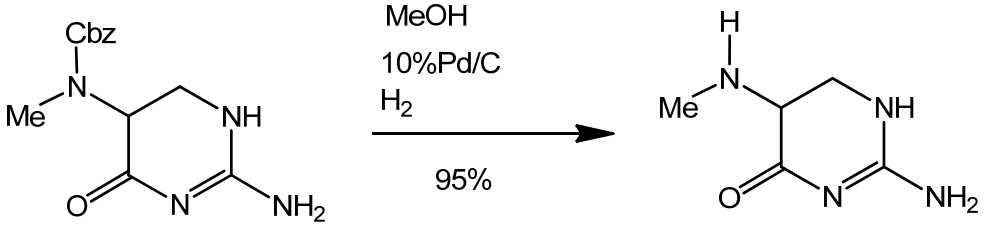
Thiourea (1 eq.) was dissolved in anhydrous ethanol (1.3M). The mixture was cooled in an ice bath to 0^{o}C. Next, methyl iodide (1.4 eq.) was slowly added to the cooled mixture. The reaction was then refluxed at 85^{o}C for 90 minutes. The solvent was removed and the product was recrystallized from ethanol yielding compound **11** as yellow crystals.



11 **5** **12**

Scheme 2.17: Cbz-DHP Condensation

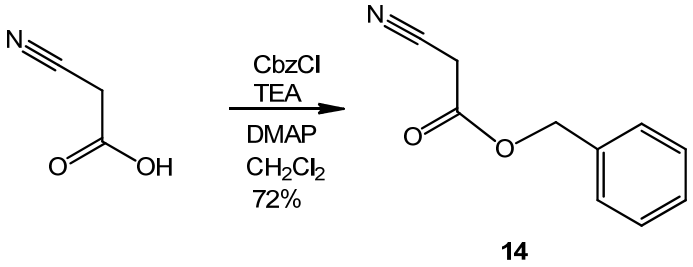
A suspension of compound **5**, compound **11**, and sodium acetate (2.2 eq.) in acetonitrile (0.125M) was stirred at 55°C. After 2 days, the reaction was concentrated and the residue was washed with 3% aqueous sodium bicarbonate. The reaction was concentrated in vacuo, and the residue was washed with small amounts of dichloromethane, isopropanol, and ether. The solid was then dissolved in methanol, filtered and concentrated in vacuo. Finally, the residue was washed again with isopropanol and ether and dried to yield compound **12** as a colorless solid.



12 **13**

Scheme 2.18: Cbz deprotection of DHP

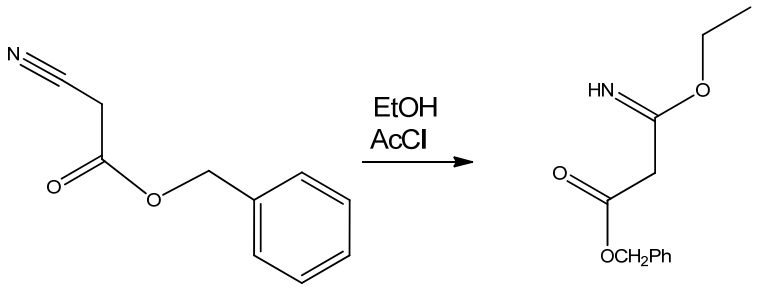
To compound **12** was added anhydrous methanol (0.03M). The reaction mixture was purged with argon followed by addition of 10% Pd/C (0.5 eq.). This mixture was purged again with argon, followed by purging for 15 minutes with hydrogen. The reaction was stirred at room temperature under hydrogen overnight, and was monitored by TLC. Upon completion, the reaction was purged with argon, filtered through celite, and concentrated in vacuo to yield compound **13** as a colorless solid.



14

Scheme 2.19: Cyanoacetic benzyl ester

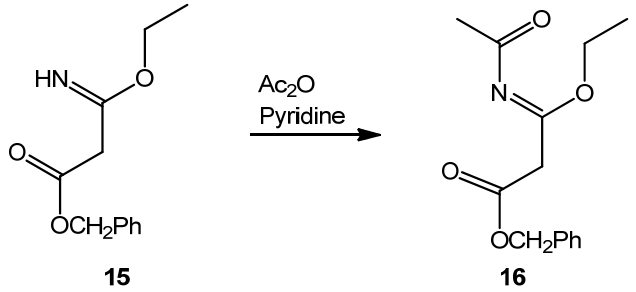
Cyanoacetic acid (1 eq.) was mixed with triethylamine (1.1 eq.) in dichloromethane (0.3M) and cooled to 0^{o}C. Benzyl chloroformate (1 eq.) was then added to this mixture. After cooling for 5 minutes, dimethylaminopyridine (DMAP) (1 eq.) was added and the reaction remained stirring at 0^{o}C for 30 minutes. The reaction was stopped by diluting with dichloromethane and the reaction mixture was washed with saturated sodium bicarbonate, 0.1M hydrochloric acid, and saturated aqueous sodium chloride. The organic layer was dried over magnesium sulfate, and concentrated under reduced pressure to yield compound **14** as an orange oil.



14 **15**

Scheme 2.20: Imine Formation

Cyanobenzyl ester **14** was dissolved in anhydrous ethanol (1.4M) followed by addition of acetyl chloride (8 eq.). The reaction was sealed and stirred at room temperature for 1 day. The solvent was removed in vacuo and the sample was carried directly onto the next step without further characterization (Scheme 2.21).

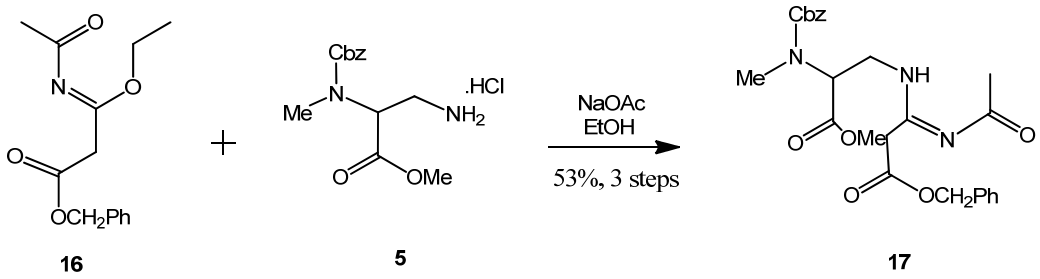


15 **16**

Scheme 2.21: Imine Acetylation

Directly from previous step (Scheme 2.20), the residue was mixed with pyridine (75 eq.) and placed in an ice bath. After stirring for 5 minutes, acetic anhydride (75 eq.) was added. The reaction was then removed from the ice

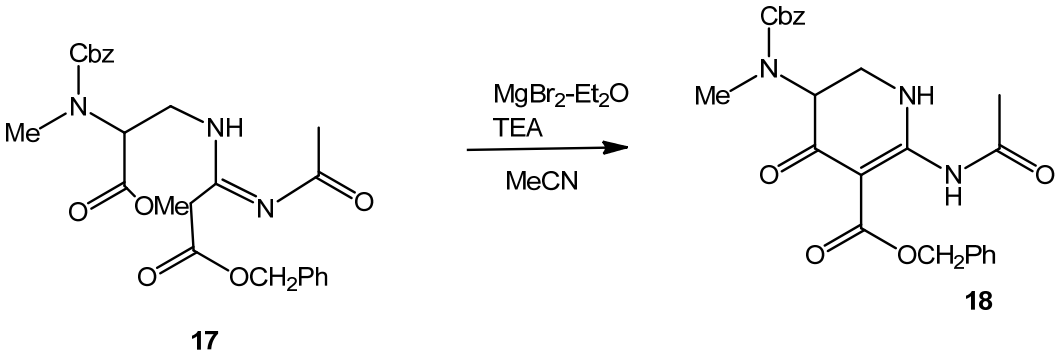
bath and allowed to warm to room temperature while stirring under argon overnight. The reaction mixture was concentrated in vacuo and carried directly onto the next step (Scheme 2.22).



16 5 17

Scheme 2.22: Condensation to form open C-analog

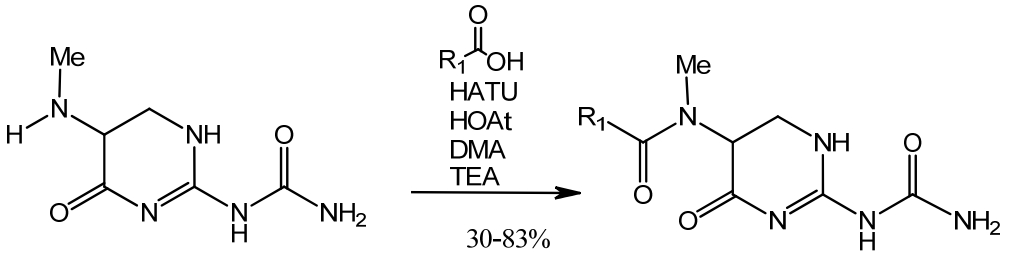
Directly from the previous step (Scheme 2.21), the acetylation product **16** was combined with methyl ester **5** and sodium acetate (2.2 eq.) in acetonitrile (0.125M). The reaction was stirred at 55°C for 48 hours, followed by concentrating in vacuo. The sample was purified using column chromatography in 2 to 1 ethyl acetate to hexanes to yield **17** as an oil.



17 18

Scheme 2.23: C-analog ring formation

Compound **17** in acetonitrile (0.13M) was combined with magnesium bromate diethyl etherate (3 eq.) followed by slow addition of triethylamine (4 eq.). The reaction was stirred at room temperature overnight. Next, the reaction was diluted with ethyl acetate and washed with 0.2M hydrochloric acid, water, and saturated sodium bicarbonate. The extraction was carried out multiple times until a single spot is seen in the organic layer. The organic layer was dried with magnesium sulfate, filtered, and concentrated. Compound **18** was obtained as a colorless solid.

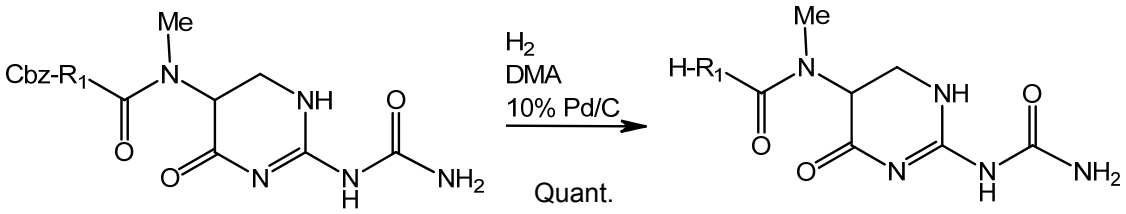


7 **19,21,23,25,27,29,30,32,34,37,38**

Scheme 2.24: Peptide coupling of DHP Urea

Compound **7** (1 eq.) was combined with a substituted carboxylic acid (2.9 eq.), 1-Hydroxy-7-azabenzotriazole (HOAt) (2.9 eq.), 2-(1H-7-Azabenzotriazol-1-yl)--1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU) (2.9 eq.), and triethylamine (7 eq.) in DMA (0.07M). The reaction mixture was stirred under argon at room temperature, while following by TLC. Once compound **7** is no longer seen in TLC, the reaction

mixture was concentrated, and the residue was re-dissolved in ethyl acetate. The sample was then washed with 1M hydrochloric acid, saturated sodium bicarbonate, and saturated sodium chloride. The ethyl acetate layer was then dried under magnesium sulfate and concentrated in vacuo. Finally, the sample was purified by column in 2 to 1 hexanes to ethyl acetate, or when necessary by HPLC (**29**, **32**).

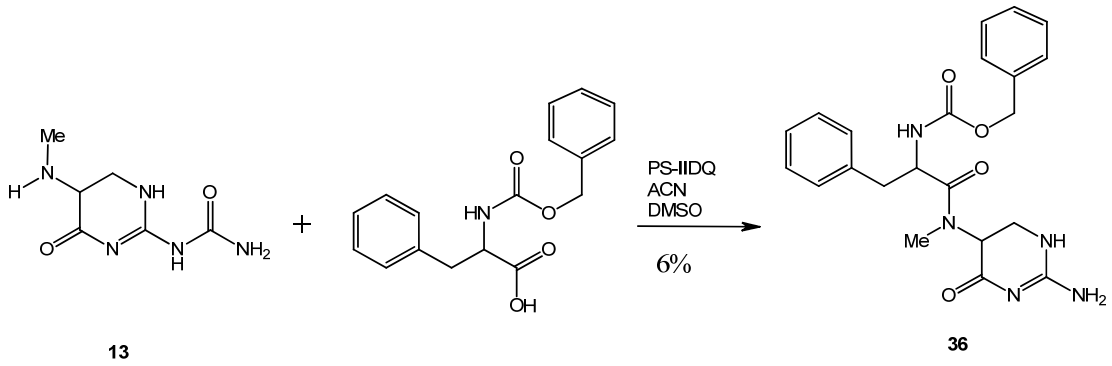


19,21,23,25,27,30,32,34

20, 22, 24, 26, 28, 31, 33, 35

Scheme 2.25: Deprotection of Cbz protected side group moieties

To the Cbz-protected compound (**19,21,23,25,27,30,32,34**) was added anhydrous methanol (0.03M). The reaction mixture was purged with argon followed by the addition of 10% Pd/C (0.5 eq.). The mixture was purged again with argon, followed by purging for 15 minutes with hydrogen. Next, the reaction was stirred at room temperature under hydrogen overnight while monitoring by TLC. Upon reaction completion, the reaction was purged with argon, filtered through celite, and concentrated in vacuo to yield the deprotected compound (**20,22,24,26,28,31,33,35**).



13 36

Scheme 2.26: Base-free Peptide coupling of DHP

A round bottom flask containing poly-styrene supported isobutyl 1,2-dihydro-2-isobutoxy-1-quinoline-carboxylate (PS-IIDQ) (2.05 eq) was stirred with acetonitrile for 10 minutes, allowing the PS-IIDQ beads to swell. A second round bottom flask containing compound **13** and Cbz-protected phenylalanine (1 eq.) was dissolved in acetonitrile (0.03M) and DMSO (0.09M). After PS-IIDQ beads were allowed to swell, the mixture of compound **13** and Cbz-protected phenylalanine were added to the PS-IIDQ mixture. After 1 day, the reaction was filtered through a fritted disk vacuum filter, and the solid was washed three times with methanol and dichloromethane. The filtrate was then concentrated in vacuum, and dried on the manifold overnight. The sample was purified by column in 90% dichloromethane in methanol to yield **36** as a film.

FRET Binding Assay for the HCV IRES domain IIa RNA

All compounds were dissolved in DMSO at a concentration of 10 mM. When preparing for testing, compounds were diluted to 10 mM, 1 mM, and 100 uM concentrations to provide a sequence of final concentrations ranging from 416 uM to 1.7 uM (416 uM, 167 uM, 83.3 uM, 41.7 uM, 16.7 uM, 8.3 uM, 4.2 uM, 1.7 uM). A compound known to be active against the HCV IRES, benzimidazole (Figure 2.3) was used as the positive control.[1,10] The negative control was RNA (100 nM) and buffer in the absence of any compound. The RNA strand was terminally labeled with cyanine dyes Cy 3 and Cy 5, as described previously.[1] The RNA was dissolved to a final concentration of 100 nM in 10 mM HEPES buffer containing 2.0 mM $MgCl_2$ at a pH of 7.0. The RNA/buffer mixture was then delivered into PCR tubes, adjusting volume so each tube has a final volume of 120 uL. The compounds were then added to the PCR tubes, and the samples were votexed and spun down. Samples were next moved to a thermocycler where they were heated to 65 ^{o}C for 5 minutes and snap cooled to 4 ^{o}C. The samples were spun down again, and loaded onto a 96-well plate. Emission of the Cy3 and Cy5 labels (570 and 670 nm, respectively) was read while exciting Cy3 (520 nm) or Cy5 (620 nm) in order to obtain measures for the direct fluorescence and the transferred fluorescence (FRET). Correction terms for direct excitation (DE) (0.03878 * Cy 5), cross talk (CT) (0.03613 * Cy 3) were determined using single labeled RNA strands. Results for the DE, CT, EE (FRET reading – DE-CT), and

FRET efficiency (eFRET) (EE/Cy5) were graphed for analysis. The data was also visualized relative to the negative control for comparison.

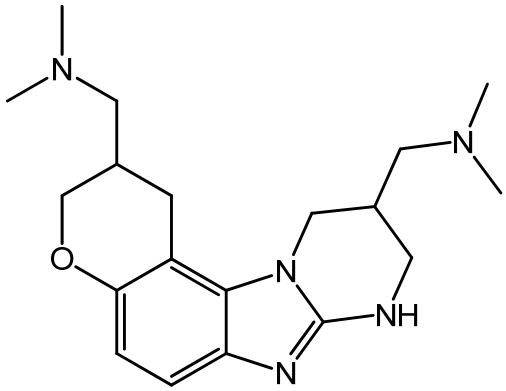


Figure 2.3: Benzimidazole compound with EC_{50} =5.4uM used as the positive control for the FRET assay.[1]

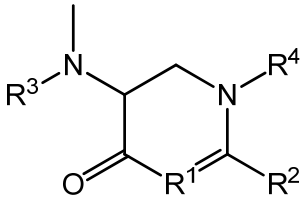
Testing for Translation Inhibition in an In Vitro Translation Assay

Using a DNA template, the in-vitro transcription-translation assay was carried out according to the recommendations of the cell extract's manufacturer (Promega). Dual Glo reagent was used to determine the level of translation of each reporter. The luminescence from the Firefly reporter was read, followed by quenching of the Firefly signal and addition of the Renilla substrate. The luminescence from Renilla luciferase was read. The Firefly activity was normalized to the Renilla activity, and the ratios of normalized Renilla/Firefly signal was calculated and plotted.

Activity Testing

Overall, sixteen compounds (Table 2.3) were tested in the FRET and IVT assays described above.

Table 2.3: Results of TAN 1057 derivatives in the FRET binding assay for the HCV IRES domain IIa RNA and as translation inhibitors in the in vitro translation assay. n.a. stands for no activity. IVT activity is at 100 uM of compound.



Cmpd	R_1	R_2	R_3	R_4	FRET % decrease at 416.6 uM	IVT Relative Firefly Luc.	IVT Relative Renila Luc.	IVT Ren. Luc. /FF Luc.
5	OMe	-	Cbz	H_2	32	0.765	0.92	1.203
6	N	Urea	Cbz	H	n.a.	1.064	1.012	0.952
7	N	Urea	H	H	n.a.	1.041	1.071	0.952
12	N	NH_2	Cbz	H	n.a.	1.011	1.098	1.085
13	N	NH_2	H	H	n.a.	0.828	0.966	1.167
18	C-CO_2H	acetyl	Cbz	H	n.a.	1.076	1.11	1.032
20	N	Urea	Arg	H	n.a.	n.d.	n.d.	n.d.
22	N	Urea	H	Tyr	n.a.	1.162	1.408	1.212
24	N	Urea	Tyr	H	n.a.	1.096	1.363	1.244
26	N	Urea	Trp	H	n.a.	0.997	1.348	1.352
28	N	Urea	Phe	H	n.a.	0.941	1.231	1.308
29	N	Urea	4-OH-B.A.	H	n.a.	1.129	1.448	1.293
31	N	Urea	Ser	Ser	n.a.	1.008	1.441	1.43
33	N	Urea	Orn	H	n.a.	0.979	1.086	1.11
35	N	Urea	Ser	H	n.a.	1.111	1.149	1.033
36	N	NH_2	N-Cbz-Phe	H	8.5	1.082	1.195	1.104

Results Testing Binding at the HCV IRES IIa Target

A representative set of FRET data is shown below for compound **33** (Figure 2.3). The lack of change in eFRET as observed in Figure 2.3 leads us to the conclusion that the compounds do not affect the angle of domain IIa of the HCV IRES. This lack of interference was seen with all compounds except **5** and **36**. Compound **36**, Cbz-Phe-DHP, showed an 8.5% decrease in FRET signal, and compound **5**, the methyl ester precursor, showed a 32% decrease.

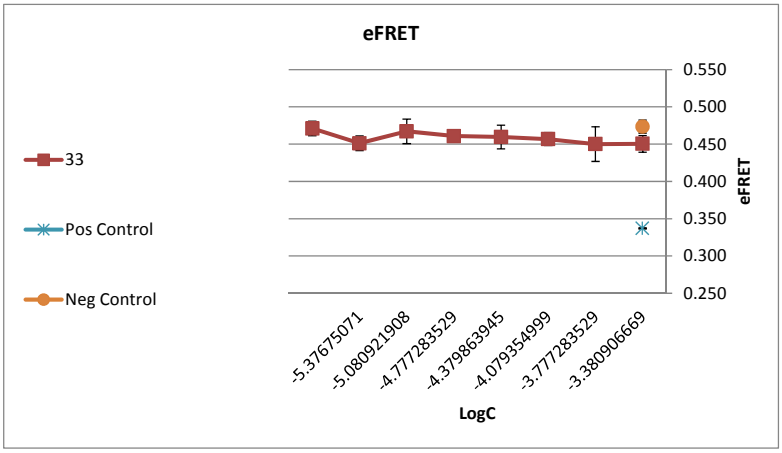


Figure 2.4: eFRET of representative compound **33**.

As a means of verifying the change observed in eFRET, the CT, DE, and EE are plotted against concentration (Figures 2.4-2.6). This allows one to determine if a change in eFRET is due to the compound interacting with the IIa angle, changing the distance between the FRET pair, or if the compound may be interfering with one of the dyes. If this were the case, we would observe a change in signal with concentration. This interaction was fortunately not

observed with any of the compounds tested, and a representative result is shown in Figures 2.4-2.6 for compound **33**.

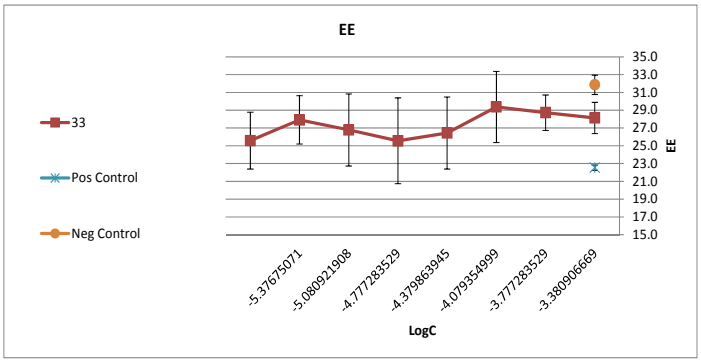


Figure 2.5: EE measurements of compound **33** as a representative compound.

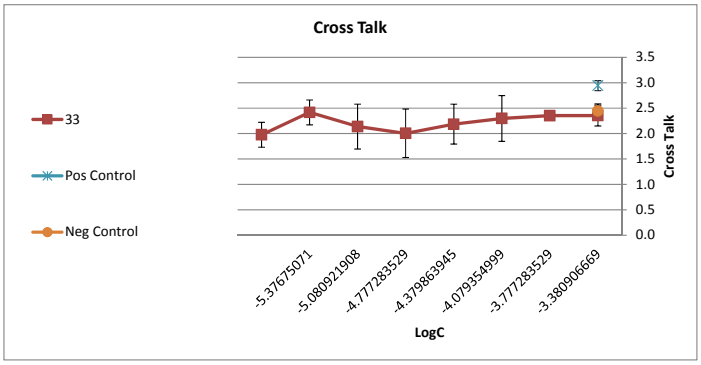


Figure 2.6: Cross Talk of compound **33**.

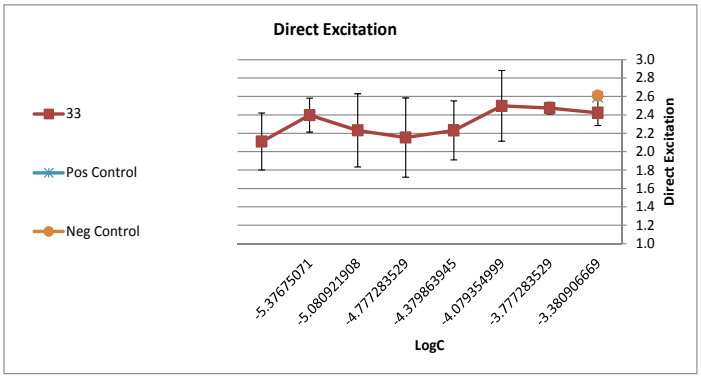


Figure 2.7: Direct Excitation of compound 33.

Unfortunately, because none of the compounds were active, no structure activity relationships (SAR) can be realized. However, both compound **5** and **36** contain Cbz groups and free amines which may provide pi stacking and H-bonding opportunities respectively.

Results of Translation Inhibition from the IVT Assay

For compounds tested in the IVT assay, the results are shown as normalized IRES/CAP ratios (Figures 2.7-2.10). I was interested in looking at compounds which fell outside 3σ from the control. In particular, an overall increase in normalized IRES/CAP ratios may suggest either an increase in firefly luciferase signal caused by small molecule interactions with the TS hairpin, or a decrease in renilla luciferase signal resulting from small molecule interactions with the HCV IRES. An increase in firefly luciferase may be possible via a destabilization of the hairpin structure meant to sequester the start codon resulting in an increase in translation. An increase in normalized IRES/CAP ratios may suggest a decrease in firefly luciferase signal caused by small molecule interactions with TS via hairpin stabilization causing an inhibition of translation, or an increase in renilla luciferase signal resulting from small molecule interactions with the renilla luciferase. In order to determine which of the above circumstances may be occurring it is necessary to look at the individual relative activities of the renilla and firefly signals (representative results in Figures 2.11-2.12). In addition, comparing the IRES hits from the IVT assay with the results from the HCV FRET assay can provide further

information on whether a given compound may bind in domain IIa of the HCV IRES, our desired target, or affect translation through interactions elsewhere.

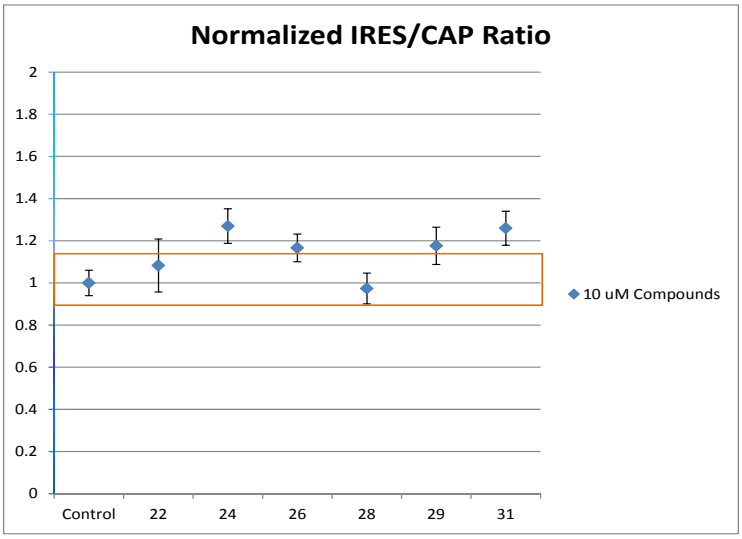


Figure 2.8: Normalized IRES/CAP Ratio for compounds **22-31** at 10 uM compound, where the orange box signifies 3σ standard deviation of the control.

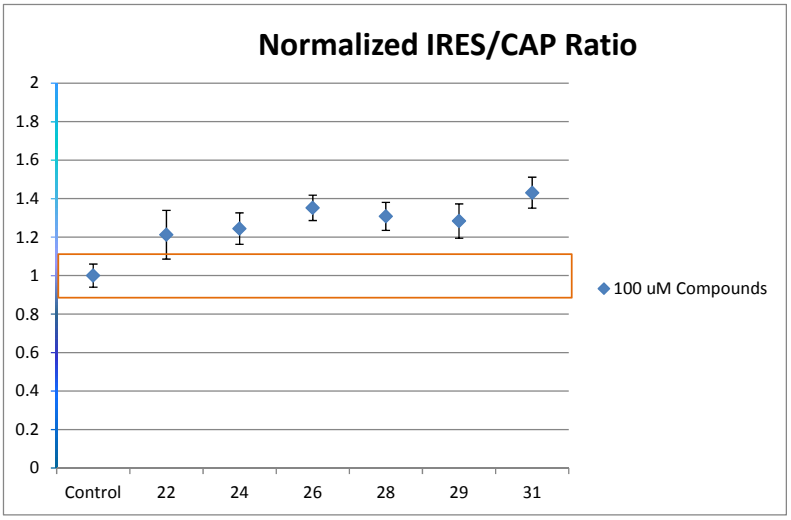


Figure 2.9: Normalized IRES/CAP Ratio for compounds **22-31** at 100 uM compound, where the orange box signifies 3σ standard deviation of the control.

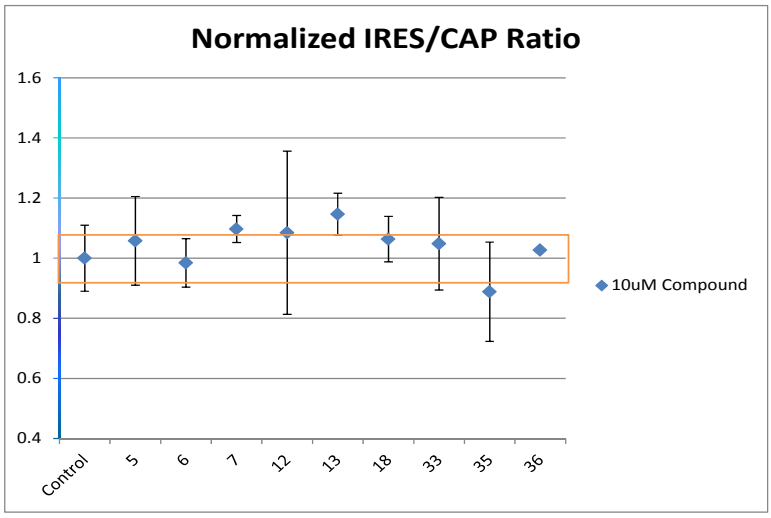


Figure 2.10: Normalized IRES/CAP Ratio for compounds **5**-**18** and **33**-**36** at 10uM compound, where the orange box signifies 3σ standard deviation of the control.

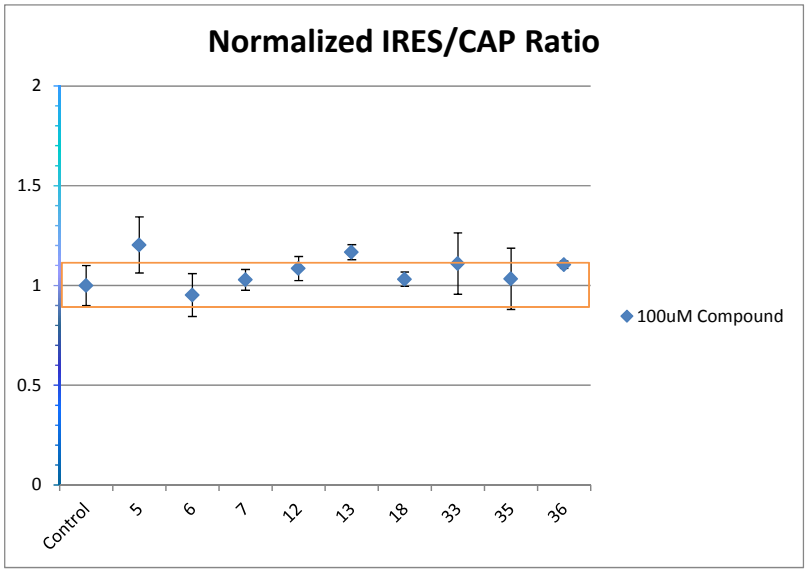


Figure 2.11: Normalized IRES/CAP Ratio for compounds **5**-**18** and **33**-**36** at 100 uM compound, where the orange box signifies 3σ standard deviation of the control.

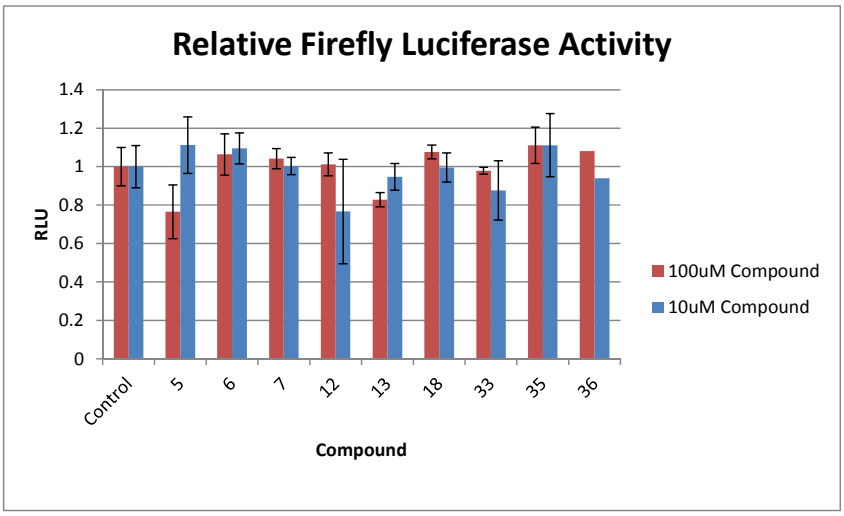


Figure 2.12: Relative Firefly Luciferase Activity of Compounds **5-18** and **33-36**.

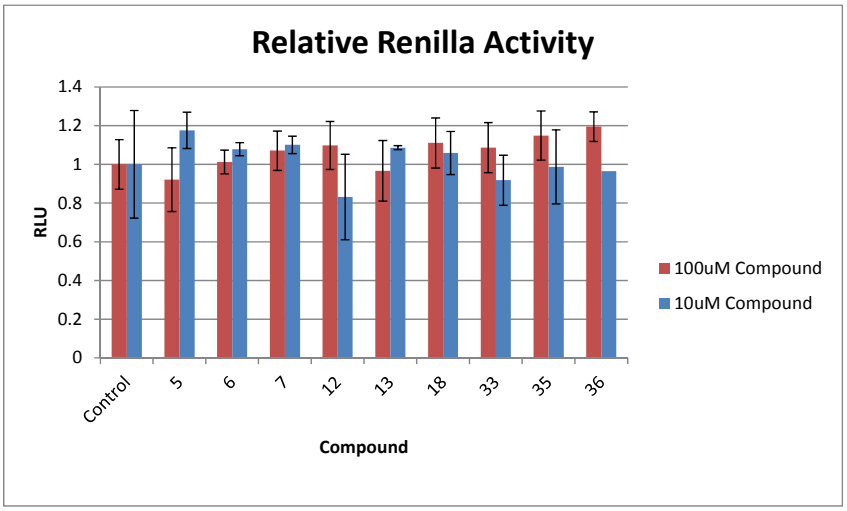


Figure 2.13: Relative Renilla Activity of Compounds **5-18** and **33-36**.

Based on the FRET assay results above, I was particularly interested to see if the activity of compounds from the FRET assay resulted in an effect on IRES translation (Figures 2.10-2.11), specifically with compounds **5**, the methyl ester precursor, and **36**, Cbz-Phe-DHP. Compound **5** does not show

activity against the IRES in the IVT assay, rather it appears to specifically decrease the TS hairpin mediated translation, as is indicated by compound **5**'s value (Figure 2.11). This result, coupled with the net-unchanged value for the renilla luciferase (Figure 2.13) suggests that the binding of compound **5** to the TS hairpin can affect translation, likely resulting from the stabilization of the hairpin structure. Compound **36** however, shows consistent results between the two assays.

Looking at the data in Figures 2.8 and 2.9, compounds **26,**Trp-DHP Urea, **28**, Phe-DHP Urea, and **29**, 4-hydroxybenzoic acid-DHP Urea, show an increase in normalized IRES/CAP ratios, and upon further investigation of the individual signals, we observe all three result in an increase in IRES driven translation. However, all three of these compounds do not exhibit activity at 10uM, but only at 100uM. As a result, I feel this warrants further investigation to determine the effect of the compound concentration on translation inhibition, and therefore propose future work to include further testing at smaller intervals in order to determine EC_{50} values for these compounds.

Compounds **24**, Tyr-DHP Urea, and **31**, Ser-DHP urea-Ser from figures 2.8 and 2.9, and compound **13**, H-DHP, from figures 2.10 and 2.11 also fall outside of the 3σ range. Compounds **24** and **13** do not show a corresponding increase in activity with an increase in concentration. Looking at the individual luciferase signals compound **13** shows a slight increase in renilla signal at 10uM compound (Figure 2.13) which interferes with the ratio caused by firefly signal. Looking solely at the firefly signal (Figure 2.12), it is consistent with a

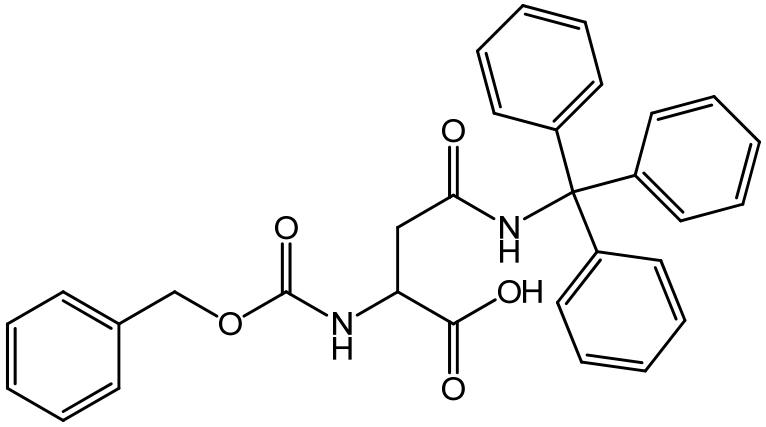
decrease in activity as compound concentration increases. Therefore, compound **13** is presented as a potential inhibitor of TS, and may stabilize the TS site 1 hairpin in order to block translation. However, because of the inconsistencies with renilla signal, in order to be confident in these results, retesting of compound **13** is necessary. Compounds **24** and **31** show consistent trends of increasing renilla signal coinciding with increasing concentration. This indicates their potential to act on the IRES and effect translation. However, these compounds did not show any activity in the HCV FRET assay, leading to the conclusion that the effect of IRES translation is caused by small molecule-IRES interaction outside of domain IIa. Although these compounds may be interesting to look at in the future, our current focus is targeting domain IIa of the HCV IRES and the TS site 1 hairpin structure.

Overall, the IVT assay identified six potential hits against the IRES, and one potential hit against the mRNA TS site 1 hairpin. Because only one hit was identified for the TS, not much structural inference can be made, except that maybe a peptide bond at the N-methyl position may result in too much steric hindrance. Future work using smaller side chain moieties may produce interesting results against TS. Although six compounds were identified as potential hits for the IRES, only one, compound **36**, also showed activity against domain IIa via the HCV FRET assay. Even compound **36** only showed minor disruptions against translation. However, with the exception of compound **31**, all of the IRES hits incorporate aromatic rings providing the opportunity for pi stacking interactions with their target. Based on these

results, I thought it would be interesting and necessary to further explore derivatives of the DHP core, specifically with the goal of keeping the non-amide exocyclic free amine and increasing stability of the core.

Spectral Data

Compound 1 (ES-008A)



Chemical Formula: $C_{31}H_{28}N_2O_5$

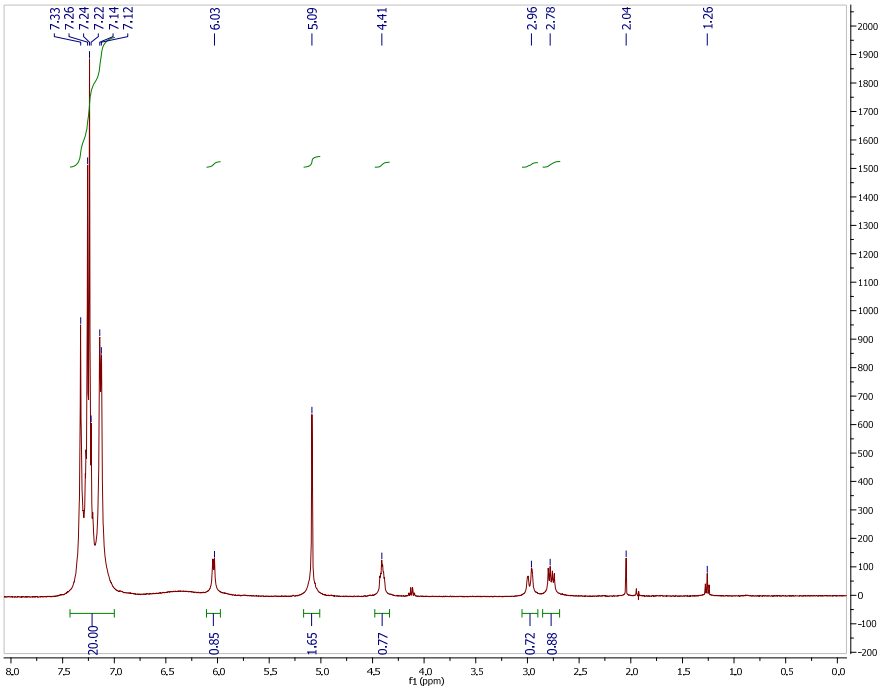
Exact Mass: 508.20. Found $[M+H]^+$ 508.97 (ESI Positive Ion Mode).

NMR Assignments

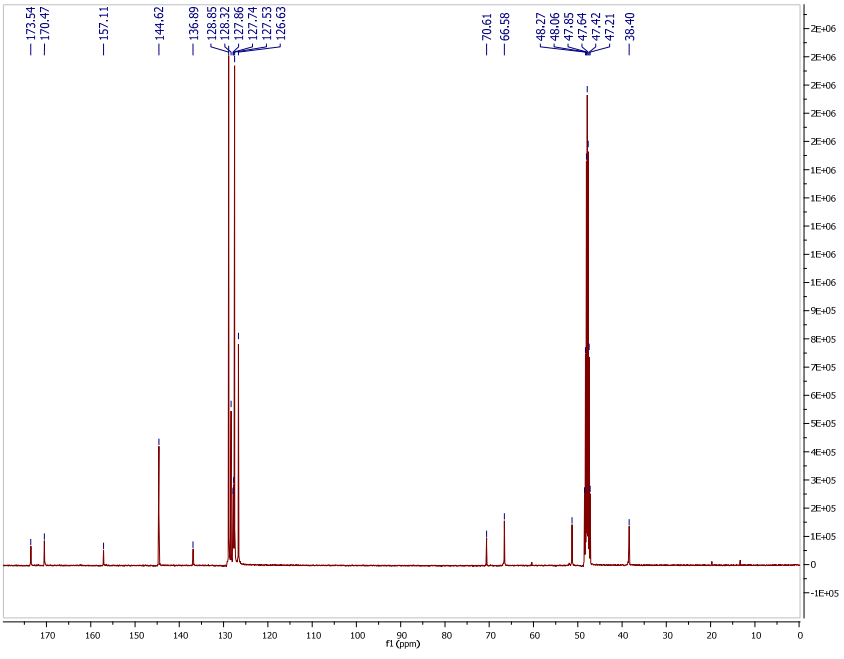
^{1}H NMR: δ 7.33-7.12 (m, 20H), 5.09 (s, 2H), 4.41 (m, 1H), 2.96 (J_1=2.62Hz, J_2=8.42Hz, dd, 1H), 2.78 (J_1=7.44, J_2=8.30, dd, 1H).

^{13}C NMR: δ 173.55, 170.49, 144.63, 136.91, 128.86, 128.62, 128.54, 128.34, 127.88, 127.76, 127.54, 126.82, 126.64, 70.62, 66.58, 51.30, 38.40.

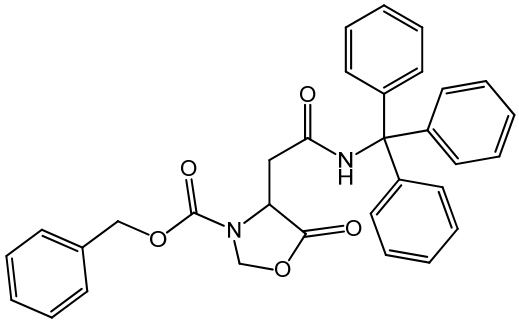
Spectrum 2.1: Compound **1** (ES-008A) ^{1}H NMR, $CDCl_3$, 400 MHz



Spectrum 2.2: Compound **1** (ES-008A) ^{13}C NMR, CD_3OD, 400 MHz



Compound **2** (ES-060A)



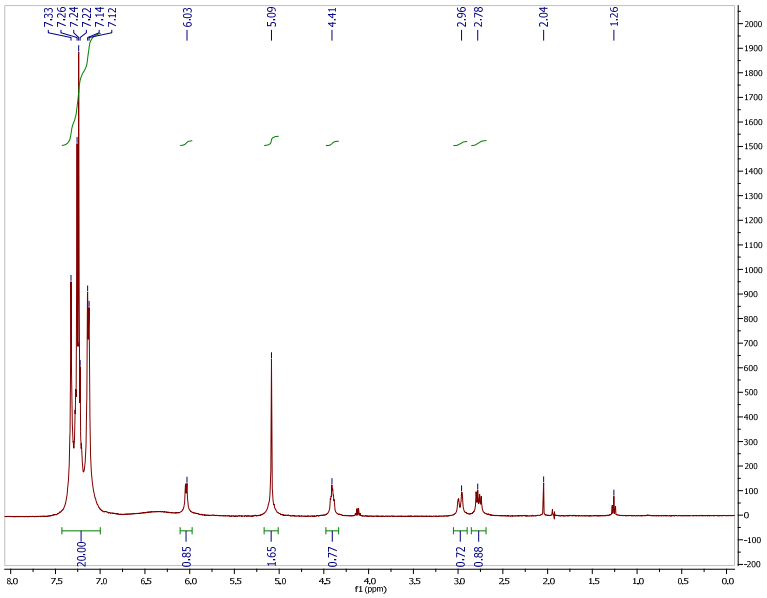
Chemical Formula: $C_{32}H_{28}N_2O_5$.

Exact Mass: 520.20. Found $[M+H]^+$ 520.80 (ESI Positive Ion Mode).

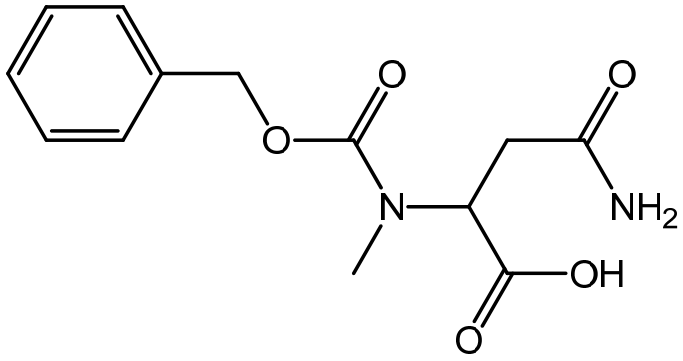
NMR Assignment

^{1}H NMR: δ 7.35-7.04 (m, 20H), 5.26 (s, 2H), 5.18 (J=7.24 Hz, d, 2H), 4.63 (s, 1H), 4.23 (s, 1H), 3.12 (m, 2H).

Spectrum 2.3: Compound **2** (ES-AP-81) ^{1}H NMR, $CDCl_3$, 400 MHz



Compound **3** (ES-013A)



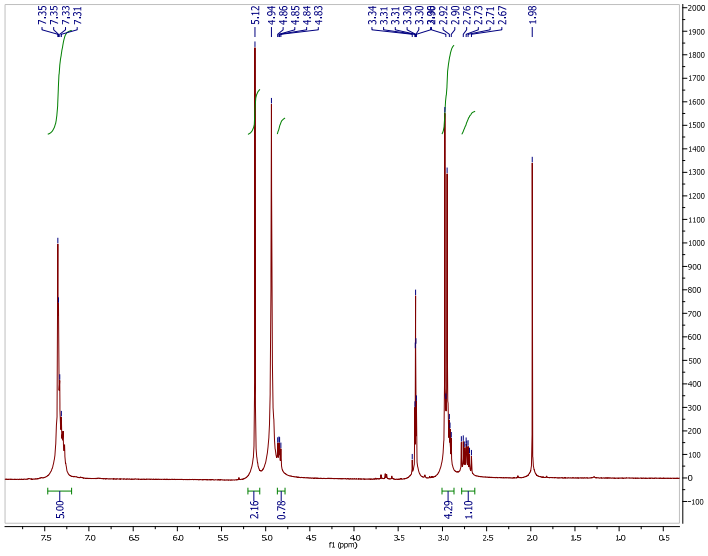
Chemical Formula: $C_{13}H_{16}N_2O_5$

Exact Mass: 280.11. Found $[M-H]^-$ 279.03 (ESI Negative Ion Mode).

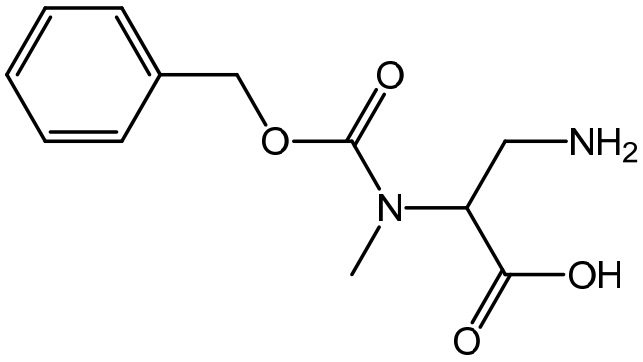
NMR Assignments

^{1}H NMR: δ 7.35-7.31 (m, 5H), 5.12 (s, 2H), 4.86-4.83 (m, 1H), 2.97-2.92 (m, 4H), 2.73-2.67 (m, 1H).

Spectrum 2.4: Compound **3** (ES-013A) ^{1}H NMR, CD_3OD, 400 MHz



Compound **4** (ES-020B)



Chemical Formula: $C_{12}H_{16}N_2O_4$

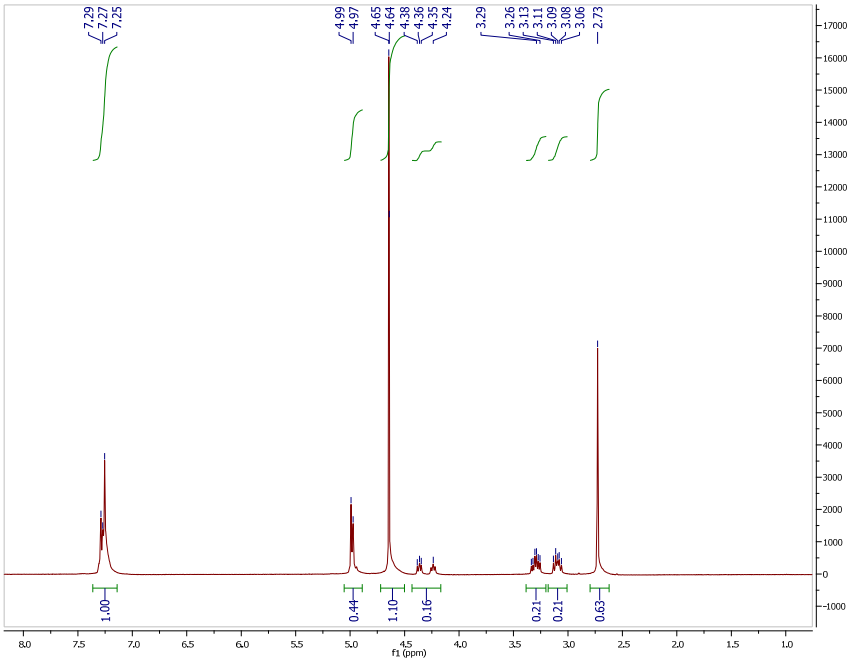
Exact Mass: 252.11. Found $[M+H]^+$ 253.1184 (HR-ESI-TOFMS)

NMR Assignments

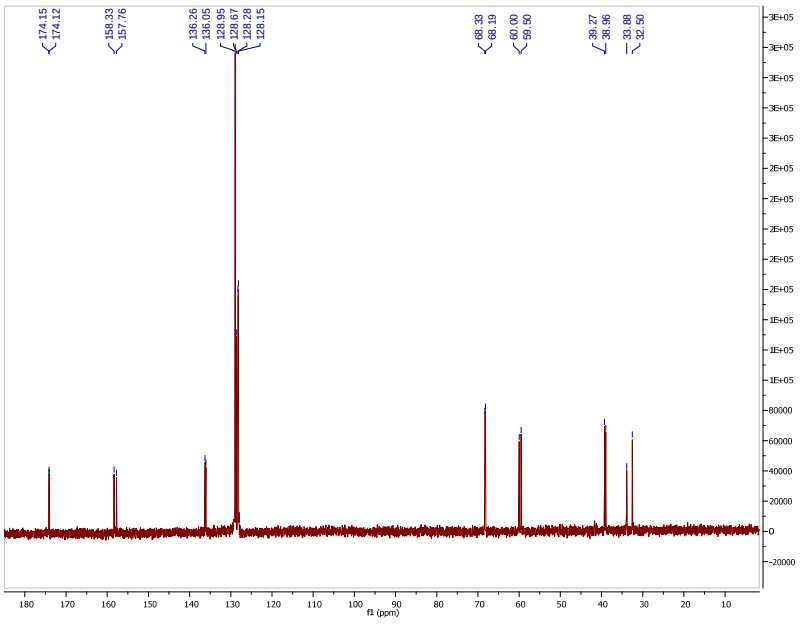
^{1}H NMR: δ 7.29-7.25 (m, 5H), 4.99, 4.97 (J=7.35Hz, d, 2H), 4.38-4.22 (m, 1H), 3.34-3.26 (m, 1H), 3.13-3.06 (m, 1H) 2.73 (s, 3H).

^{13}C NMR (mix of rotamers)**:** δ 174.15, 174.12, 158.33, 157.76, 136.26, 136.05, 128.96, 128.68, 128.29, 128.17, 68.34, 68.20, 60.01, 59.51, 39.96, 38.96, 33.88, 32.51.

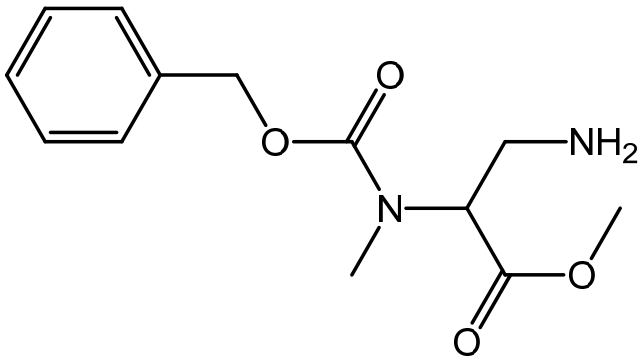
Spectrum 2.5: Compound **4** (ES-020B) ^{1}H NMR, D_2O, 400 MHz



Spectrum 2.6: Compound **4** (ES-079A) ^{13}C NMR, CD_3OD, 400 MHz



Compound **5** (ES-021A)



Chemical Formula: $C_{13}H_{18}N_2O_4$

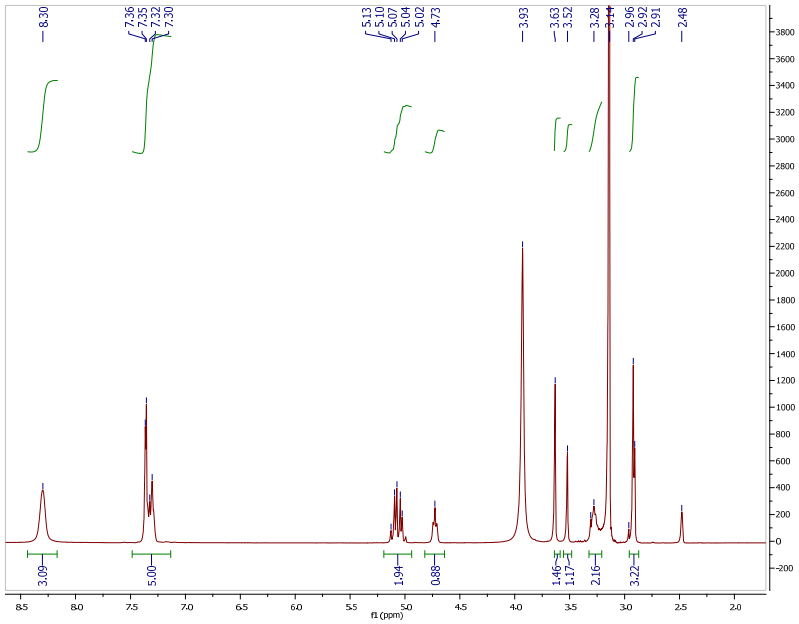
Exact Mass: 266.13. Found $[M+H]^+$ 267.09 (ESI Positive Ion Mode).

NMR Assignments

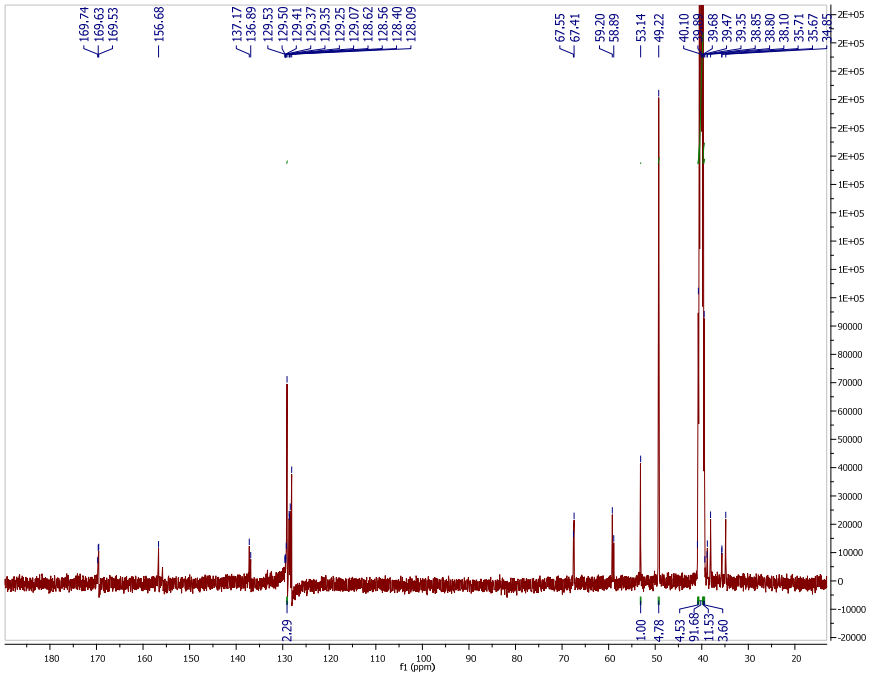
^{1}H NMR: δ 8.30 (bs, 2H), 7.35-7.29 (m, 5H), 5.10-5.03 (m, 2H), 4.73 (J=6.89Hz, t, 1H), 3.63 (s, 3H), 3.32-3.26 (m, 2H), 2.91 (s, 3H).

^{13}C NMR: δ 169.74, 156.68, 137.17, 129.09, 128.41, 128.10, 67.55, 59.22, 53.15, 38.11, 34.85.

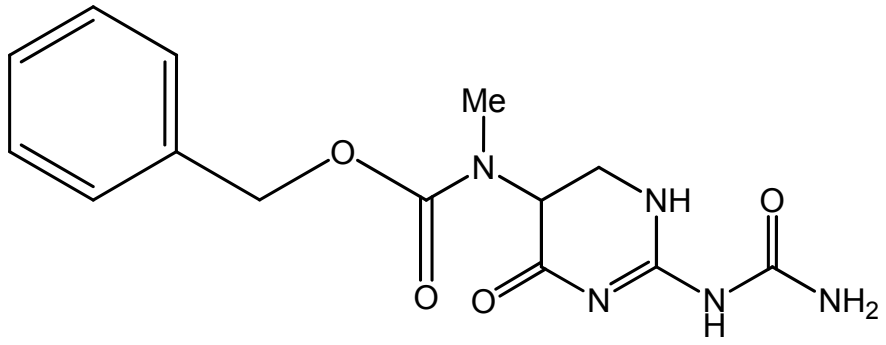
Spectrum 2.7: Compound **6** (ES-081B) ^{1}H NMR, DMSO, 400 MHz



Spectrum 2.8: Compound **5** (ES-081B) ^{13}C NMR, DMSO, 400 MHz



Compound **6** (ES-027B)



Chemical Formula: $C_{14}H_{17}N_5O_4$

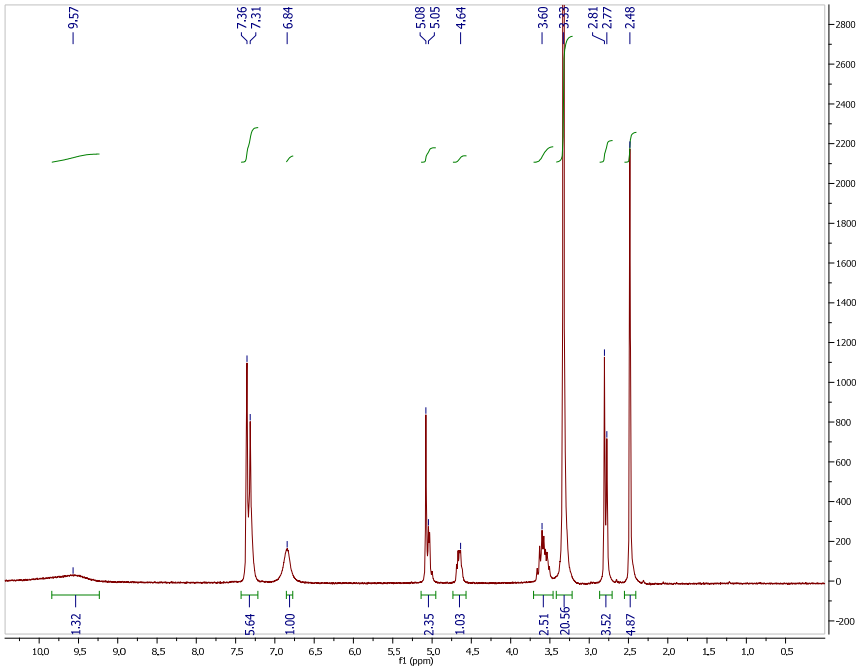
Exact Mass: 319.13. Found $[M+H]^+$ 320.1352, Delta ppm=-0.3 (HR-ESI-TOFMS).

NMR Assignments

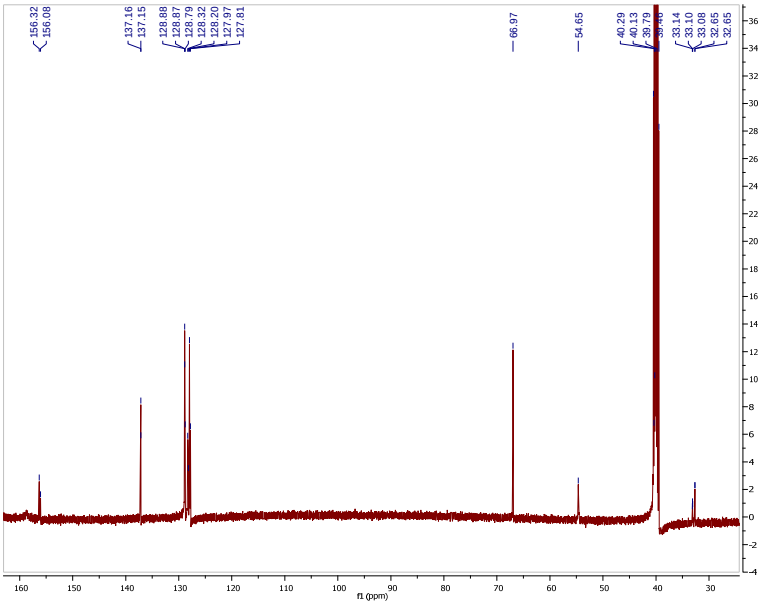
^{1}H NMR: δ 9.57 (bs, 2H), 7.36-7.314 (m, 5H), 5.08 (m, 2H), 4.64 (m, 1H), 3.67-3.50 (m, 2H), 2.81 (J=12.82Hz, d, 3H).

^{13}C NMR: δ 156.32, 156.08, 137.19, 137.17, 128.92, 128.89, 128.01, 127.98, 6.98, 54.66, 33.14, 32.66.

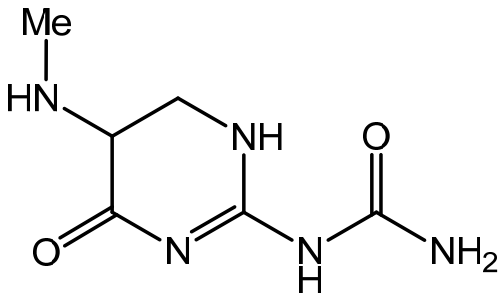
Spectrum 2.9: Compound **6** (ES-027B) ^{1}H NMR, DMSO, 400 MHz



Spectrum 2.10: Compound **6** (ES-130B) ^{13}C NMR, DMSO, 500 MHz



Compound **7** (ES-027B)



Chemical Formula: $C_6H_{11}N_5O_2$

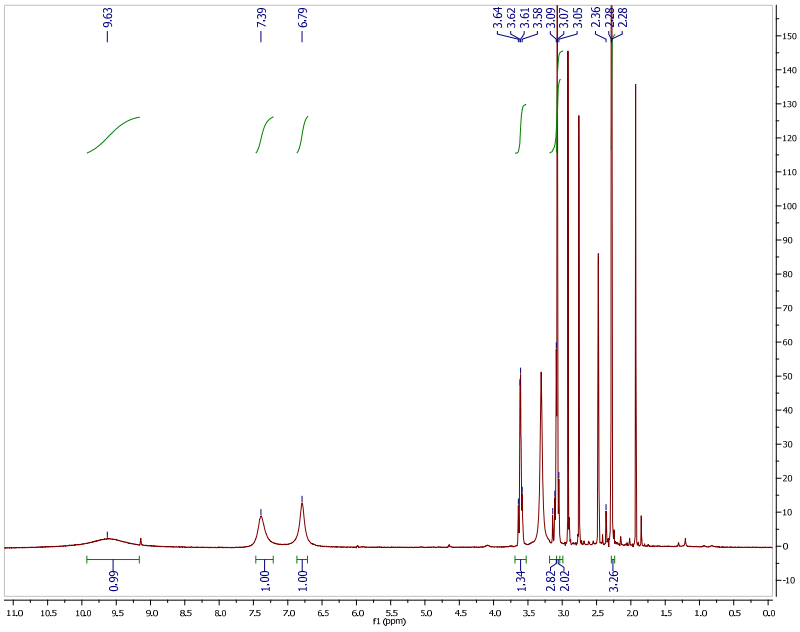
Exact Mass: 185.09. Found $[M+H]^+$ 186.0985, Delta ppm=-0.3 (HR-ESI-TOFMS).

NMR Assignments

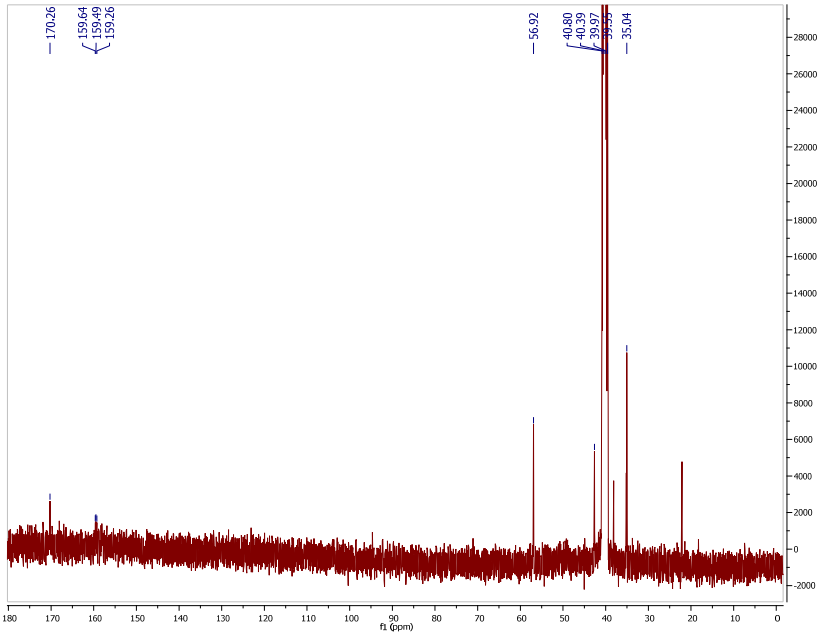
^{1}H NMR: δ 9.63 (bs, 1H), 7.39 (bs, 1H), 6.79 (bs, 1H), 3.64-3.58 (m, 1H), 3.12-3.07 (m, 2H), 2.76 (J=1.0Hz, d, 3H).

^{13}C NMR: δ 170.26, 159.64, 159.26, 56.92, 42.64, 35.04.

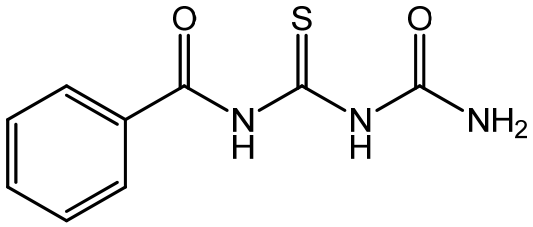
Spectrum 2.11: Compound **7** (ES-139A) ^{1}H NMR, DMSO, 500 MHz



Spectrum 2.12: Compound **7** (ES-139B) ^{13}C NMR, DMSO, 500 MHz



Compound **8** (ES-012B)



Chemical Formula: $C_9H_9N_3O_2S$

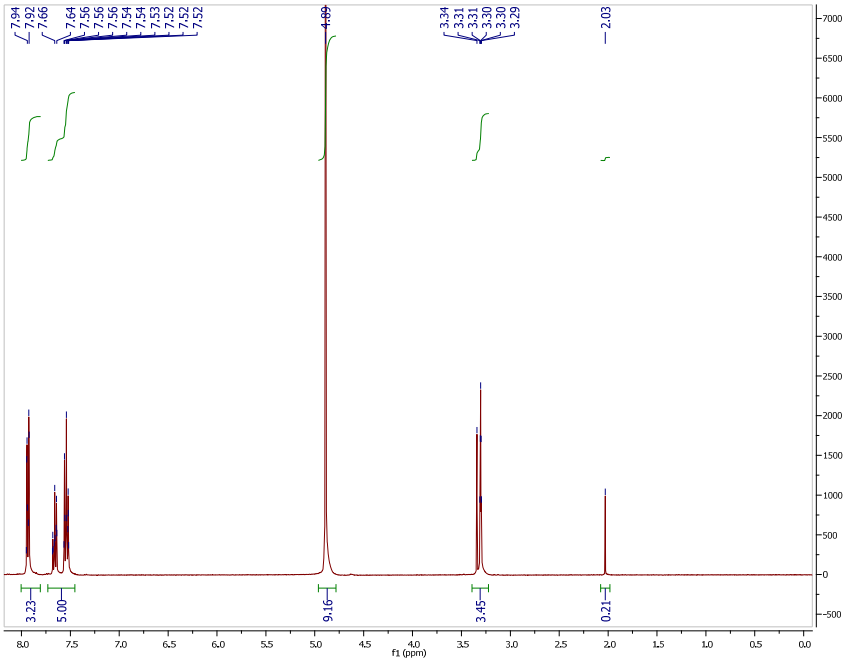
Exact Mass: 223.04. Found $[M+H]^+$ 223.99 (ESI Positive Ion Mode).

NMR Assignments

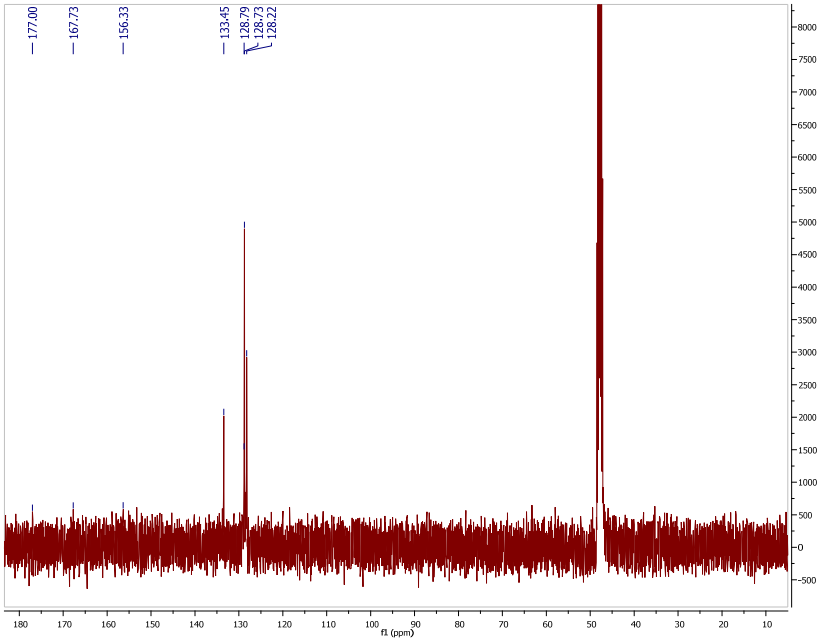
^{1}H NMR: δ 7.94-7.92 (m, 2H), 7.66-7.52 (m, 5H).

^{13}C NMR: δ 177.00, 167.73, 156.33, 133.45, 128.79, 128.73, 128.22.

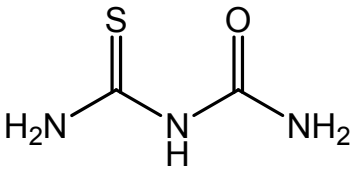
Spectrum 2.13: Compound **8** (ES-012B) ^{1}H NMR, CD_3OD, 400 MHz



Spectrum 2.14: Compound **8** (ES-012B) ^{13}C NMR, CD_3OD, 400 MHz



Compound **9** (ES-021B)



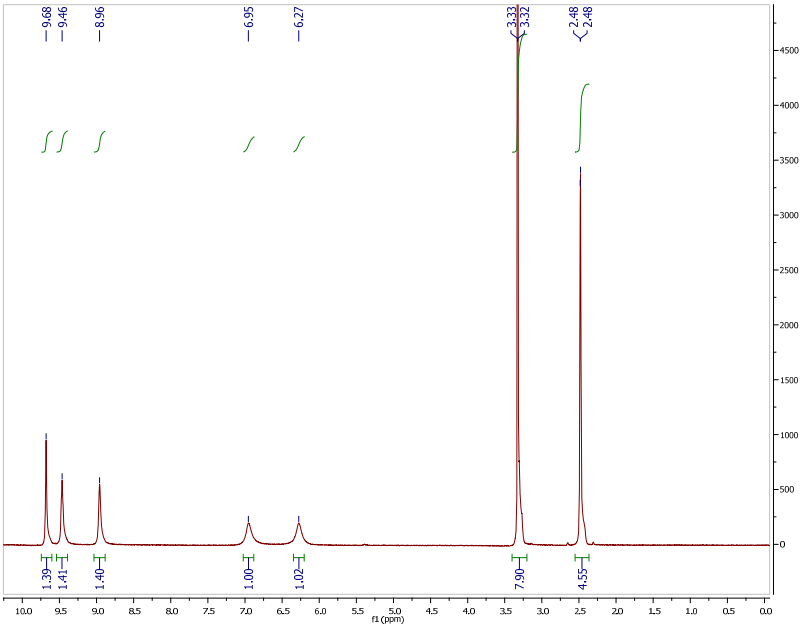
Chemical Formula: $C_2H_5N_3OS$

Exact Mass: 119.02. Found $[M+H]^+$ 120.04 (ESI Positive Ion Mode).

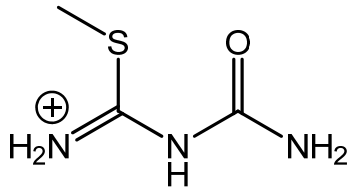
NMR Assignments

^{1}H NMR: δ 9.68 (bs, 1H), 9.46 (bs, 1H), 8.96 (bs, 1H), 6.95 (bs, 1H), 6.27 (bs, 1H).

Spectrum 2.15: Compound **9** (ES-021B) ^{1}H NMR, DMSO, 400 MHz



Compound **10** (ES-024A)



Chemical Formula: $C_3H_8N_3OS^+$

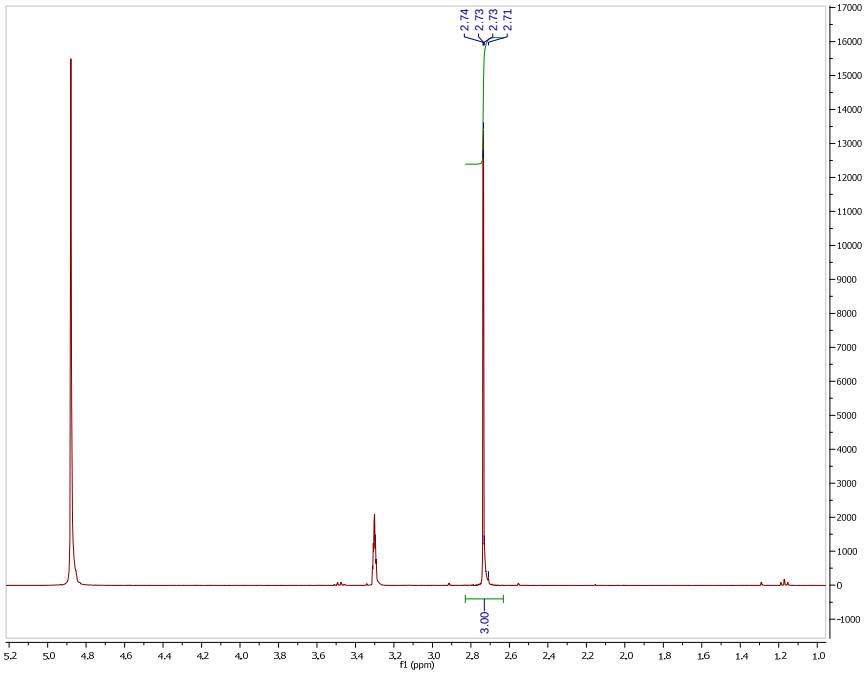
Exact Mass: 134.04. Found $[M]^+$ 134.05 (ESI Positive Ion Mode).

NMR Assignments

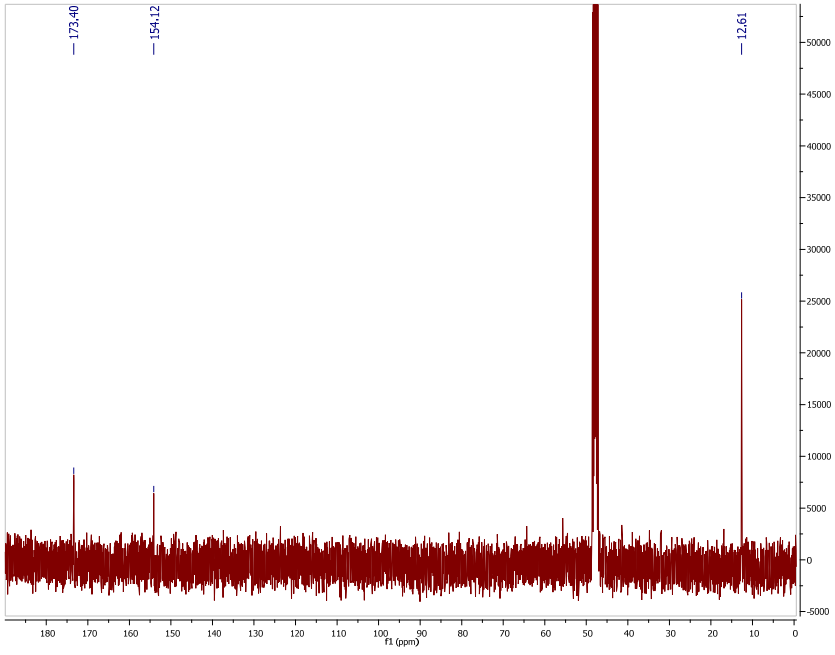
^{1}H NMR: δ 2.74 (s, 3H).

^{13}C NMR: δ 173.40, 154.12, 12.61.

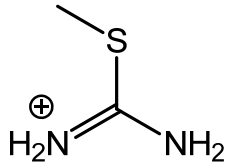
Spectrum 2.16: Compound **10** (ES-024A) ^{1}H NMR, CD_3OD, 400 MHz



Spectrum 2.17: Compound **10** (ES-024A) ^{13}C NMR, CD_3OD, 400 MHz



Compound **11** (ES-027B)



Chemical Formula: $C_2H_7N_2S^+$

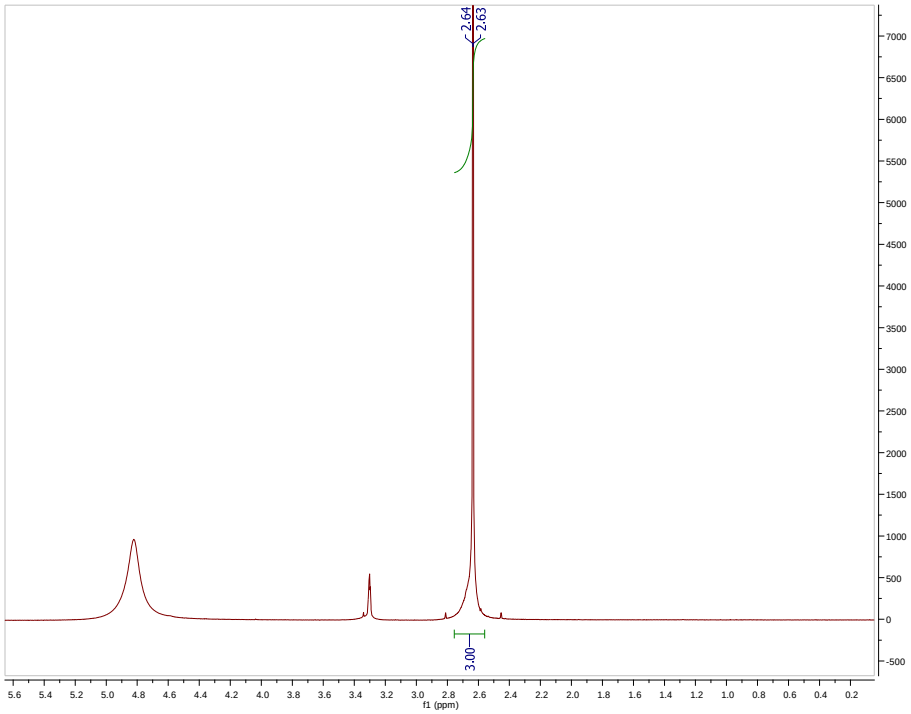
Exact Mass: 91.03. Found $[M]^+$ 91.08 (ESI Positive Ion Mode).

NMR Assignments

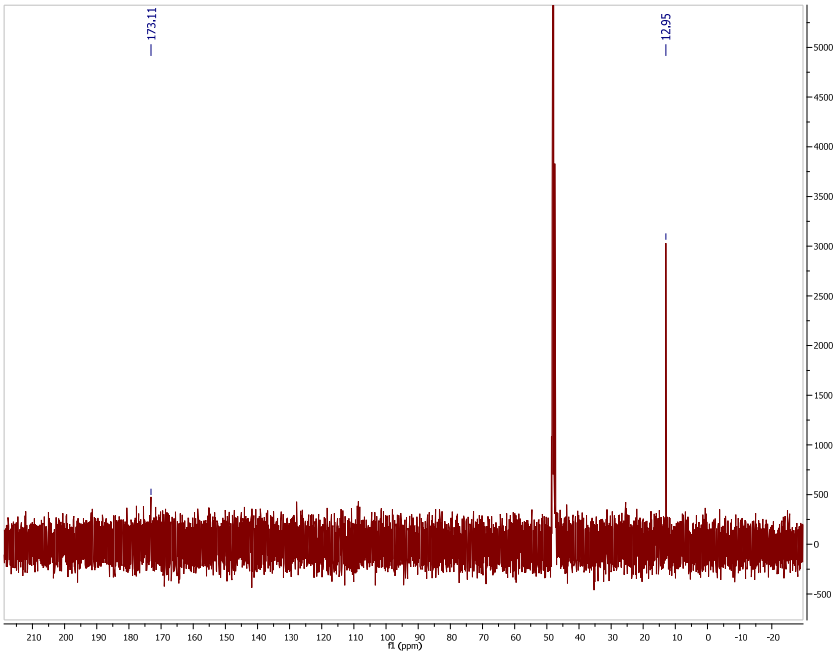
^{1}H NMR: δ 2.64 (s, 3H).

^{13}C NMR: δ 173.11, 12.95.

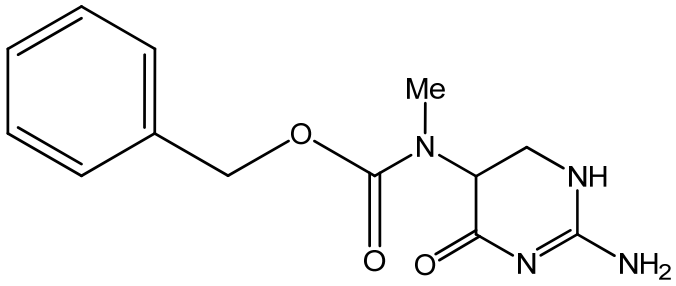
Spectrum 2.18: Compound **11** (ES-063) ^{1}H NMR, CD_3OD, 400 MHz



Spectrum 2.19: Compound **11** (ES-063) ^{13}C NMR, CD_3OD, 400 MHz



Compound **12** (ES-102A)



Chemical Formula: $C_{13}H_{16}N_4O_3$

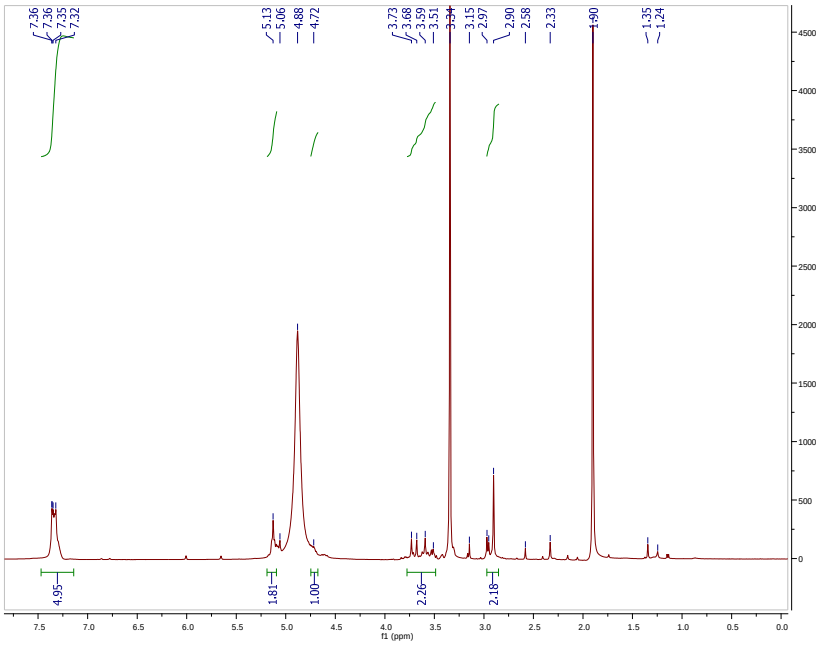
Exact Mass: 276.12. Found $[M+H]^+$ 277.1301 Delta ppm=2.2 (HR-ESI-TOFMS).

NMR Assignments

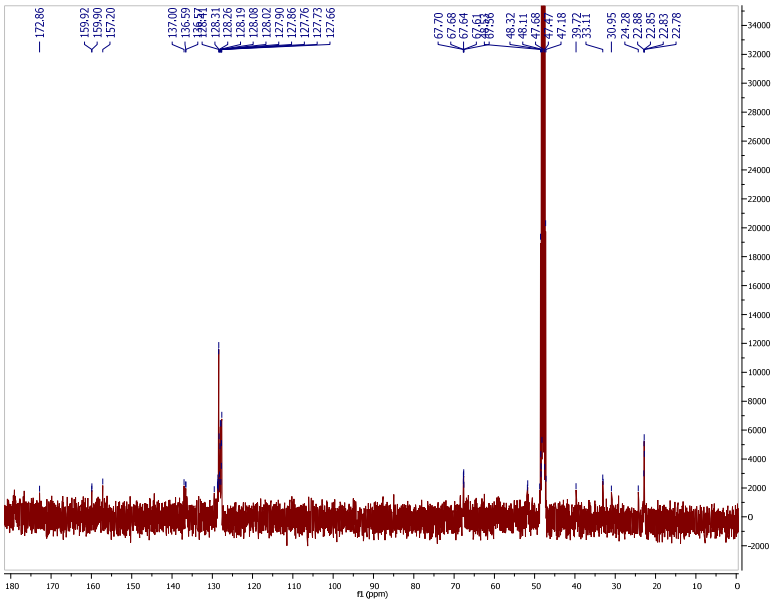
^{1}H NMR: δ 7.36-1.32 (m, 5H), 5.13 (m, 2H), 4.72 (m, 1H) ,3.73-3.51 (m, 2H), 2.90 (m, 3H).

^{13}C NMR: δ 172.86, 159.92, 157.20, 137.00, 128.31, 128.19, 128.02, 127.76, 127.66, 67.70 48.32, 33.11, 30.95.

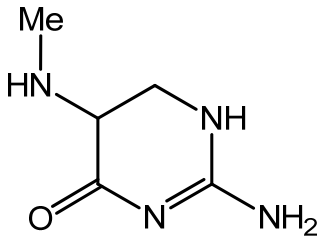
Spectrum 2.20: Compound **12** (ES-111A) ^{1}H NMR, CD_3OD, 400 MHz



Spectrum 2.21: Compound **12** (ES-247) ^{13}C NMR, DMSO, 500 MHz



Compound **13** (ES-139B)



Chemical Formula: $C_5H_{10}N_4O$

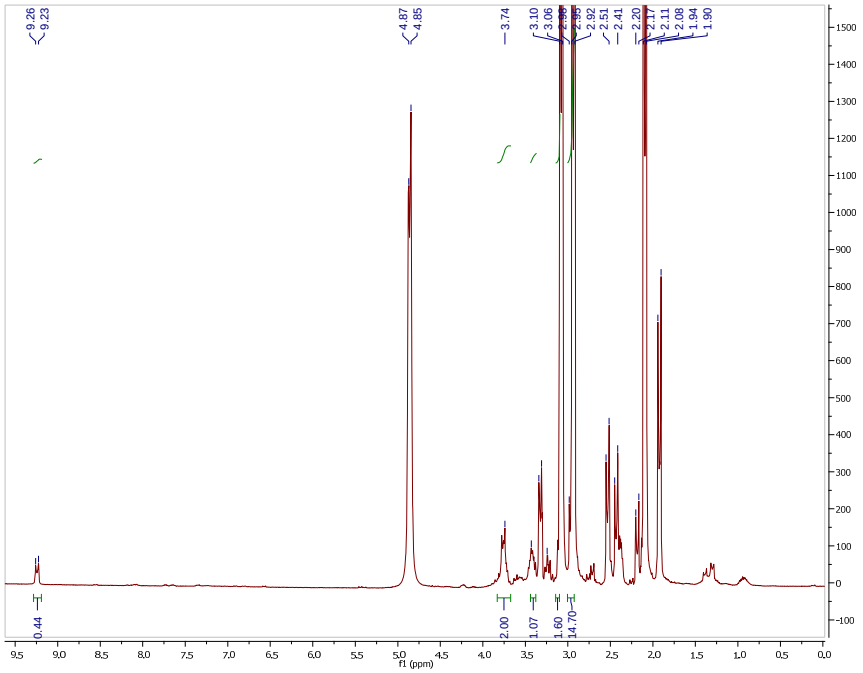
Exact Mass: 142.09. Found $[M+MeOH+H]^+$ 175.18 (ESI Positive Ion Mode).

NMR Assignments

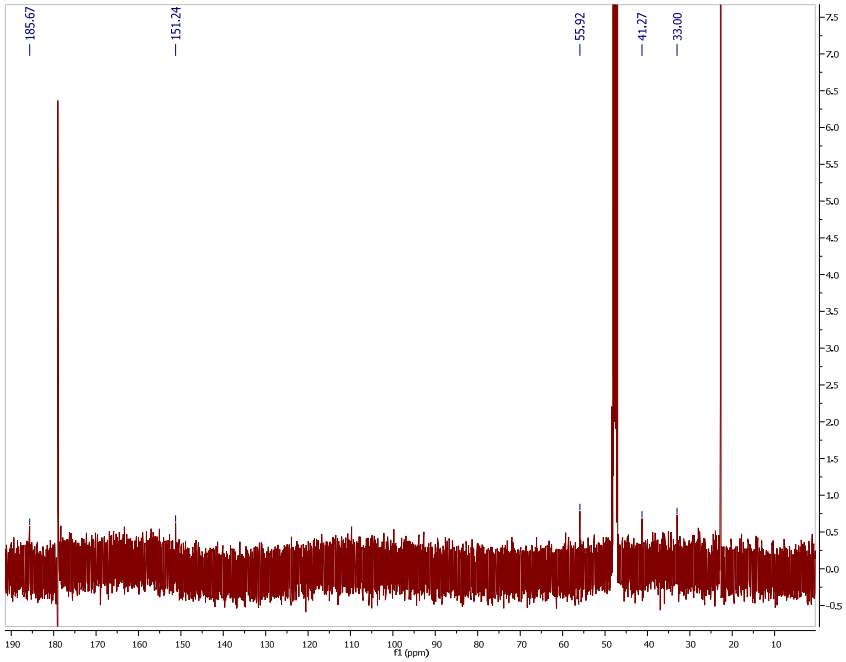
^{1}H NMR: δ 9.26-9.23 (J=14.01Hz, bd, 2H), 3.77-3.74 (m, 2H), 3.43-3.42 (m, 1H), 2.55-2.51 (J-14.5, d, 3H).

^{13}C NMR: δ 185.67, 151.24, 55.92, 41.27, 33.00.

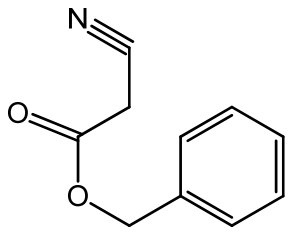
Spectrum 2.22: Compound **13** (ES-217) ^{1}H NMR, CD_3OD, 400 MHz



Spectrum 2.23: Compound **13** (ES-247) ^{13}C NMR, CD_3OD, 500 MHz



Compound **14** (ES-103A)



Chemical Formula: $C_{10}H_9NO_2$

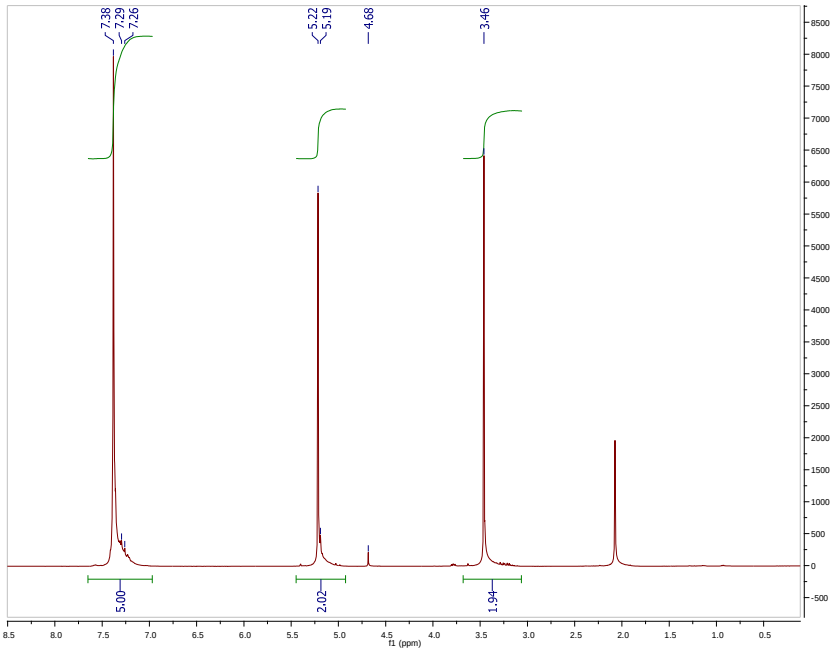
Exact Mass: 175.06. Found $[M+Na]^+$ 197.86 (ESI Positive Ion Mode).

NMR Assignments

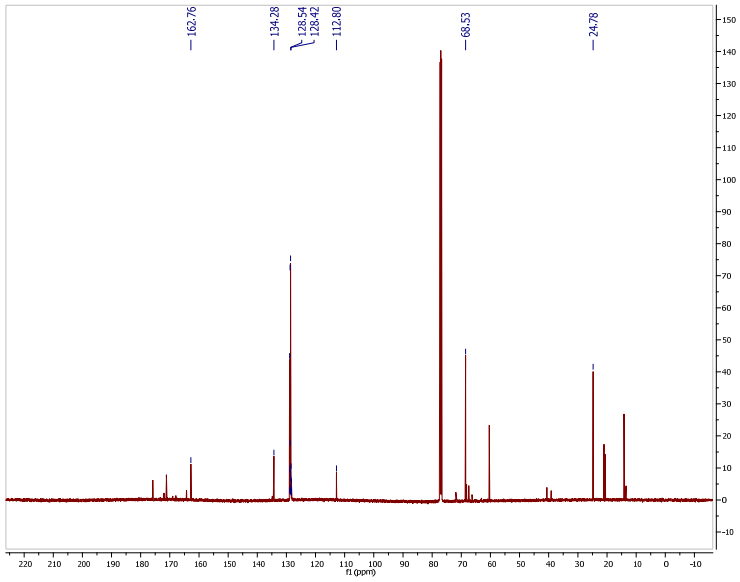
^{1}H NMR: δ 7.38-7.26 (m, 5H), 5.22(s, 2H), 3.46 (s, 2H).

^{13}C NMR: δ 162.76, 134.28, 128.75, 128.42, 112.80, 68.53, 24.78.

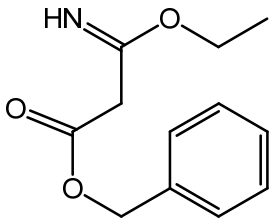
Spectrum 2.24: Compound **14** (ES-MA-57.2) ^{1}H NMR, $CDCl_3$, 400 MHz



Spectrum 2.25: Compound **14** (ES-MA-30C) ^{13}C NMR, $CDCl_3$, 500 MHz



Compound **15** (ES-MA-37)



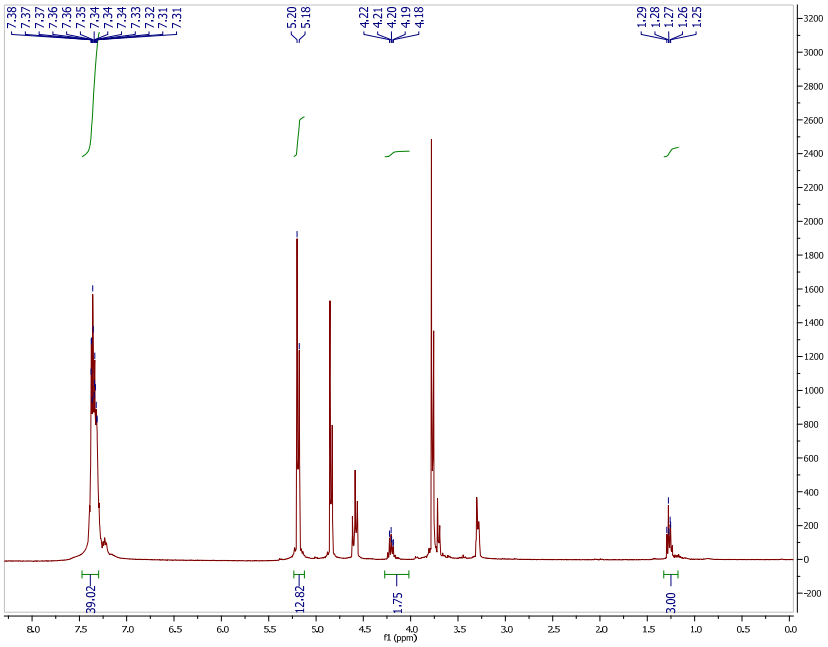
Chemical Formula: $C_{12}H_{15}NO_3$

Exact Mass: 221.11. Found $[M+H]^+$ 222.26 (ESI Positive Ion Mode).

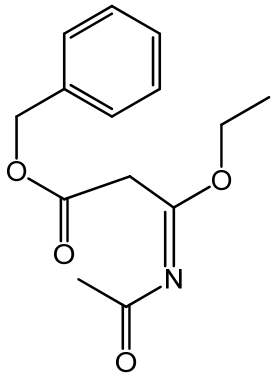
NMR Assignments

^{1}H NMR: δ 7.40-7.29 (m, 5H), 5.20-5.17 (J=9.25Hz, d, 2H), 4. (m 2H), 1.28-1.26 (m, 3H).

Spectrum 2.26: Compound **15** (ES-MA-17) ^{1}H NMR, CD_3OD, 400 MHz



Compound **16** (ES-164A)



Chemical Formula: $C_{14}H_{17}NO_4$

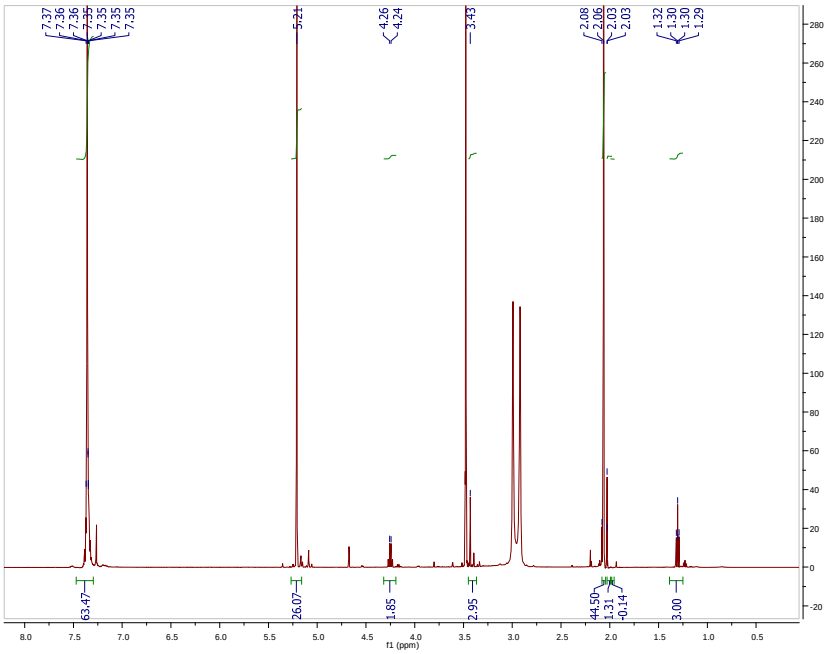
Exact Mass: 263.12. Found $[M+H]^+$ 264.07 (ESI Positive Ion Mode).

NMR Assignments

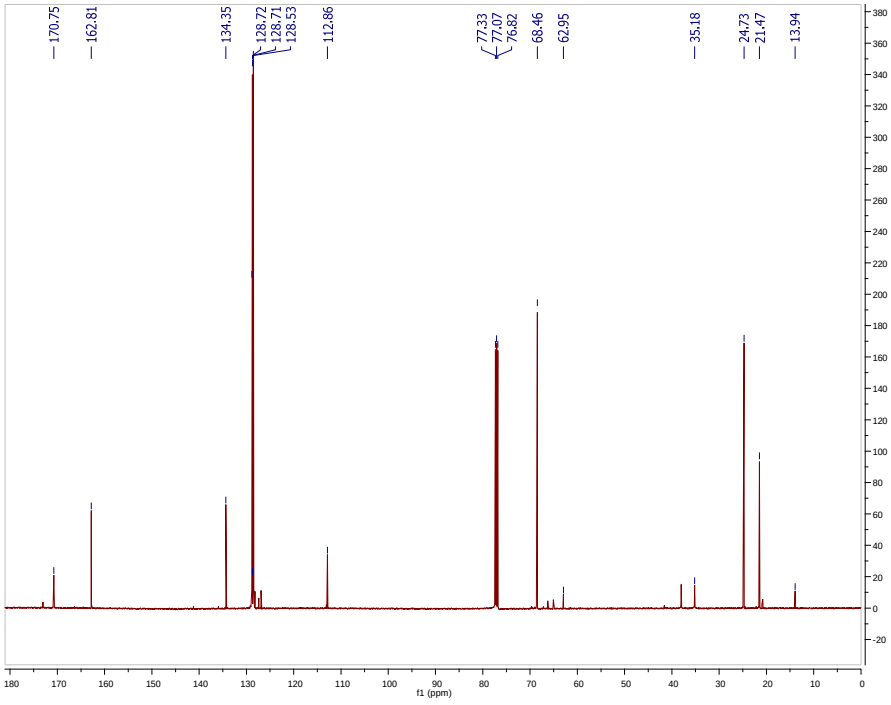
^{1}H NMR: δ 7.38-7.26 (m, 5H), 5.23(s, 2H), 4.28-4.26 (m, 2H), 3.47-3.44 (m, 2H), 2.10 (s, 3H), 1.34-1.31 (m, 3H).

^{13}C NMR: δ 170.76, 162.83, 134.36, 128.86-127.41, 68.47, 62.96, 35.18, 24.74, 13.94.

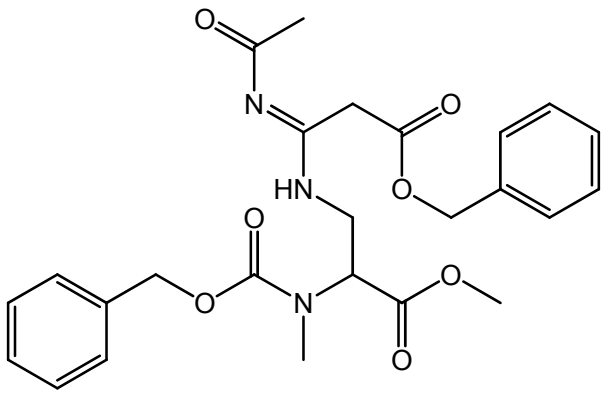
Spectrum 2.27: Compound **16** (ES-MA-221) ^{1}H NMR, $CDCl_3$, 400 MHz



Spectrum 2.28: Compound **16** (ES-MA-21C) ^{13}C NMR, $CDCl_3$, 500 MHz



Compound **17** (ES-228)



Chemical Formula: $C_{25}H_{29}N_3O_7$

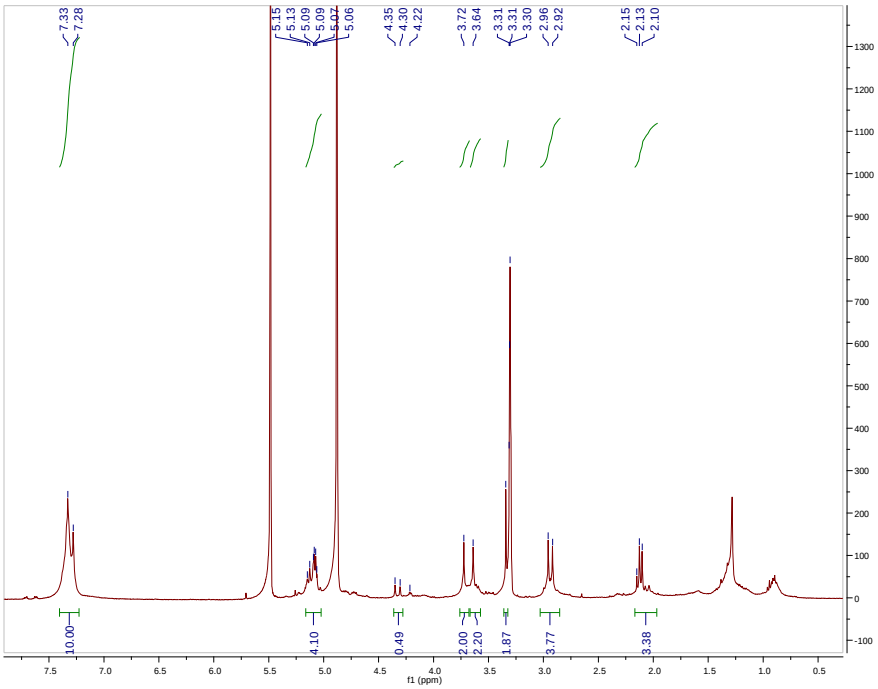
Exact Mass: 483.20. Found $[M+Na]^+$ 506.19 , Delta ppm =0.2 (HR-ESI-TOFMS).

NMR Assignments

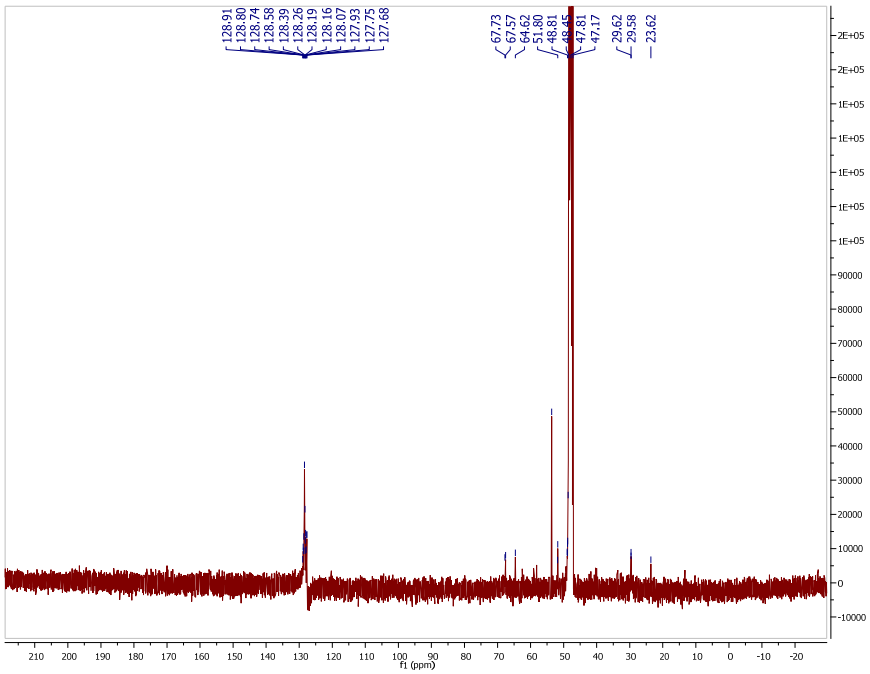
^{1}H NMR: δ 7.34-7.28 (m, 10H), 5.15-5.06 (m, 4H), 4.35-4.22 (m, 1H), 3.72 (s, 2H), 3.64 (m, 2H), 3.31 (s, 1H), 2.96 (J=15.74, d, 3H), 2.15-2.10 (m, 3H).

^{13}C NMR: δ 128.41, 128.27, 127.76, 127.70, 67.73, 64.63, 51.80, 40.02, 29.58, 23.62. (carbonyl carbons not observed due to a combination of low concentration and not enough delay times for the heteroatom nature of the compound).

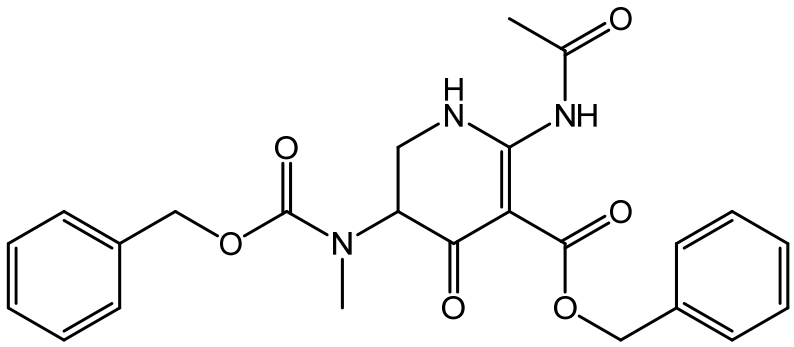
Spectrum 2.29: Compound **17** (ES-228) ^{1}H NMR, CD_3OD, 400 MHz



Spectrum 2.30: Compound **17** (ES-228C) ^{13}C NMR, CD_3OD, 400 MHz



Compound **18** (ES-257)



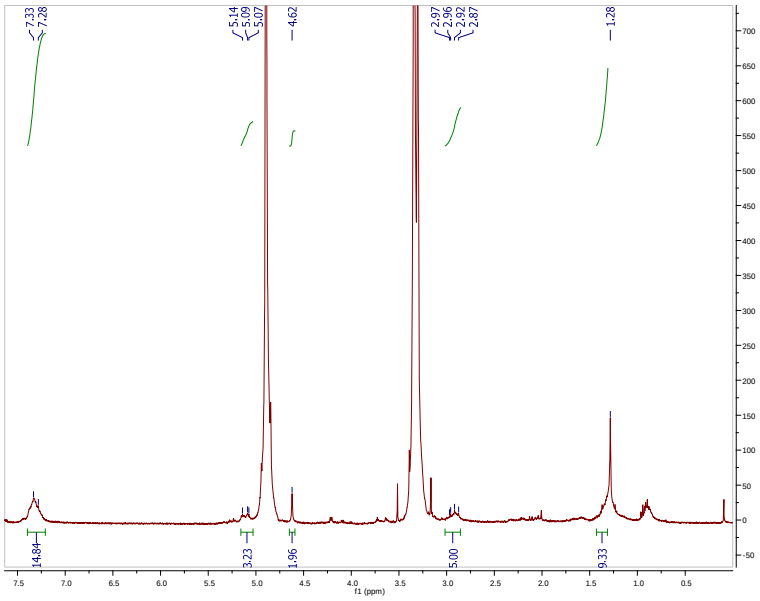
Chemical Formula: $C_{24}H_{25}N_3O_6$

Exact Mass: 451.17. Found $[M+Na]^+$ 474.16 , Delta ppm =0.6 (HR-ESI-TOFMS).

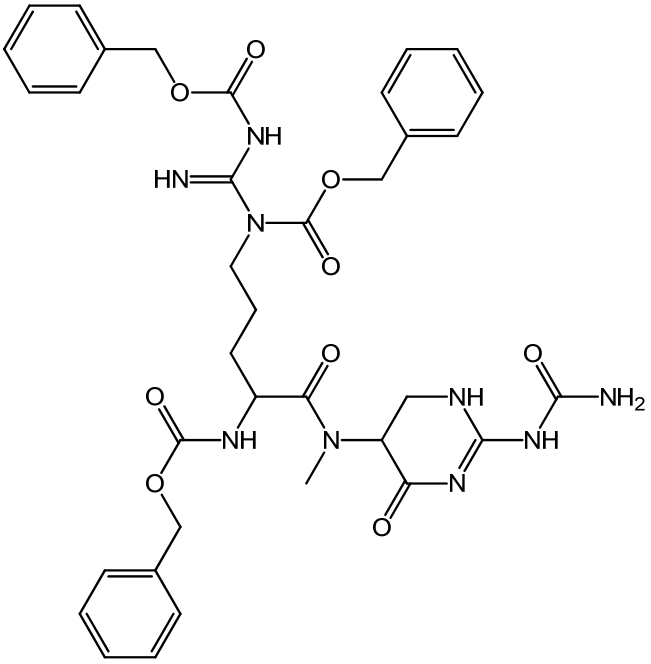
NMR Assignments

^{1}H NMR: δ 7.33-7.28 (m, 10H), 5.14-5.07 (m, 4H), 4.62 (s, 1H), 2.97-2.87 (m, 5H), 1.28 (s, 3H).

Spectrum 2.31: Compound **18** (ES-260) ^{1}H NMR, CD_3OD, 400 MHz



Compound **19** (ES-140A)



Chemical Formula: $C_{36}H_{41}N_9O_9$

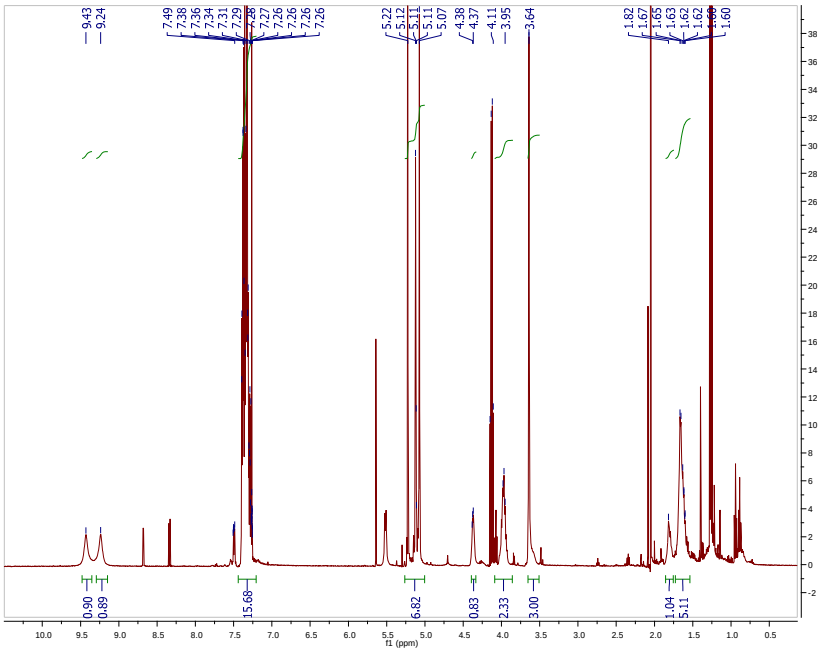
Exact Mass: 743.30. Found $[M+H]^+$ 744.29 (ESI Positive Ion Mode).

NMR Assignments

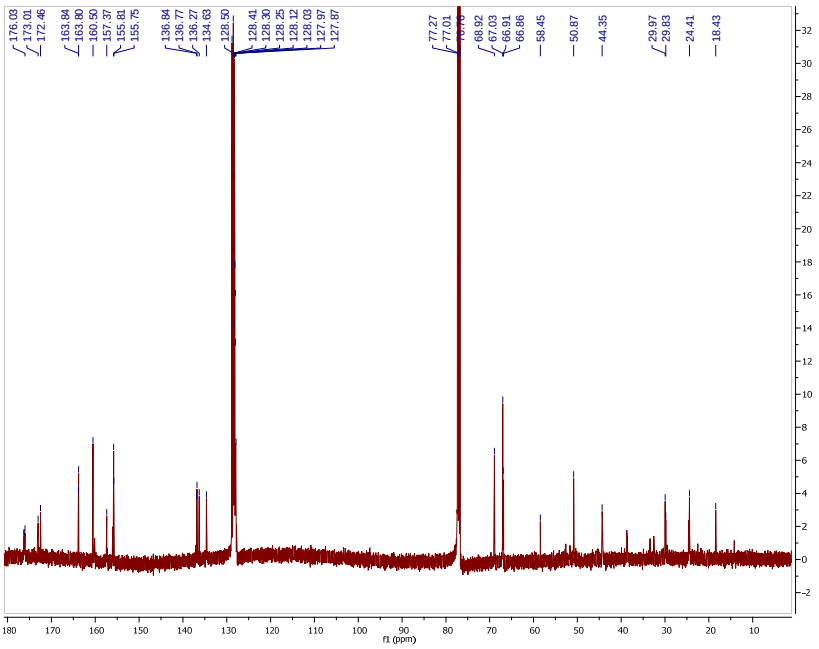
^{1}H NMR: δ 9.43 (bs, 1H), 9.24 (bs, 1H), 7.49-7.26 (m, 15H), 5.22-5.07 (m, 6H), 4.39-4.34 (m, 1H), 4.00-3.92 (m, .2H), 3.64 (s, 3H), 1.82 (m, 1H), 1.70-1.55 (m, 5H).

^{13}C NMR: δ 176.27, 176.05, 173.03, 163.86, 163.82, 160.16, 157.39, 155.98, 155.83, 155.22, 136.99, 136.86, 136.78, 134.64, 128.80-127.88, 76.05, 66.92, 66.86, 58.45, 44.36, 44.28, 38.75, 38.58, 33.42, 32.59, 29.97, 29.84, 24.61, 24.42. (mixture of rotamers).

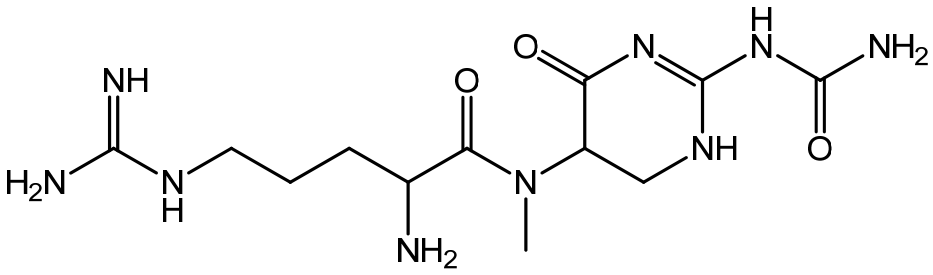
Spectrum 2.32: Compound **19** (ES-140A) ^{1}H NMR, $CDCl_3$, 400 MHz



Spectrum 2.33: Compound **19** (ES-140A) ^{13}C NMR, $CDCl_3$, 500 MHz



Compound **20** (ES-144B)



Chemical Formula: $C_{12}H_{23}N_9O_3$

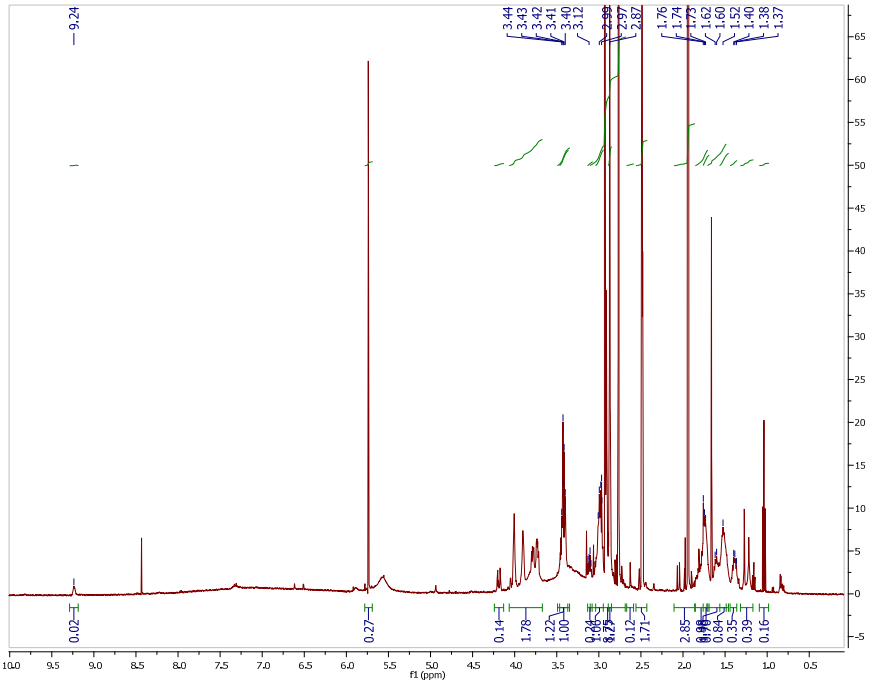
Exact Mass: 341.19. Found $[M+H]^+$ 342.17 (ESI Positive Ion Mode).

NMR Assignments

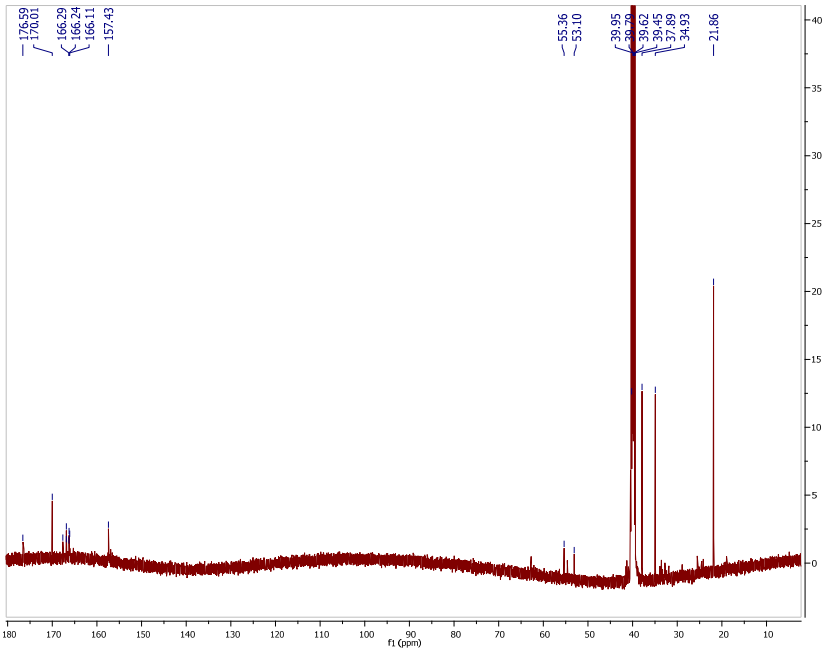
^{1}H NMR: δ 3.45-3.40 (m, 2H), 3.15-3.06 (m, 1H), 3.01-2.97 (m, 2H), 2.87 (s, 3H), 1.76-1.73 (m, 2H), 1.53-1.51 (m, 2H).

^{13}C NMR: δ 170.03, 167.67, 166.87, 166.26, 166.13, 157.51, 157.44, 156.96, 62.76, 54.64, 53.11, 34.94, 32.76, 31.89, 25.55, 24.55, 24.18. (mixture of rotamers).

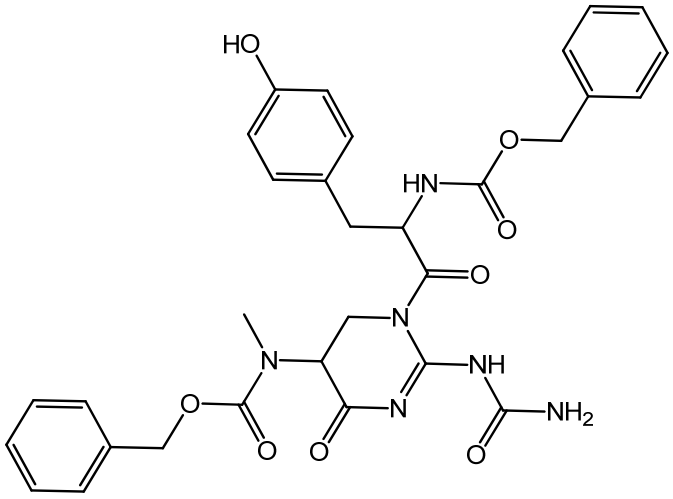
Spectrum 2.34: Compound **20** (ES-144B) ^{1}H NMR, DMSO, 500 MHz



Spectrum 2.35: Compound **20** (ES-144B) ^{13}C NMR, DMSO, 500 MHz



Compound **21** (ES-148A)



Chemical Formula: $C_{31}H_{32}N_6O_8$

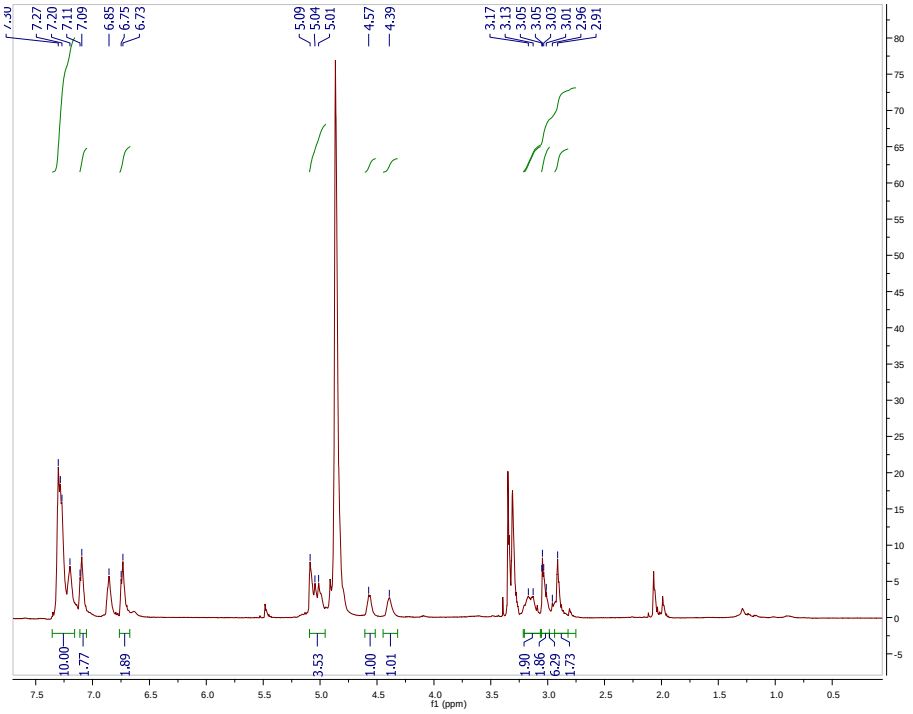
Exact Mass: 616.23 Found $[M+H_2O+H]^+$ 635.29 (ESI Positive Ion Mode).

NMR Assignments

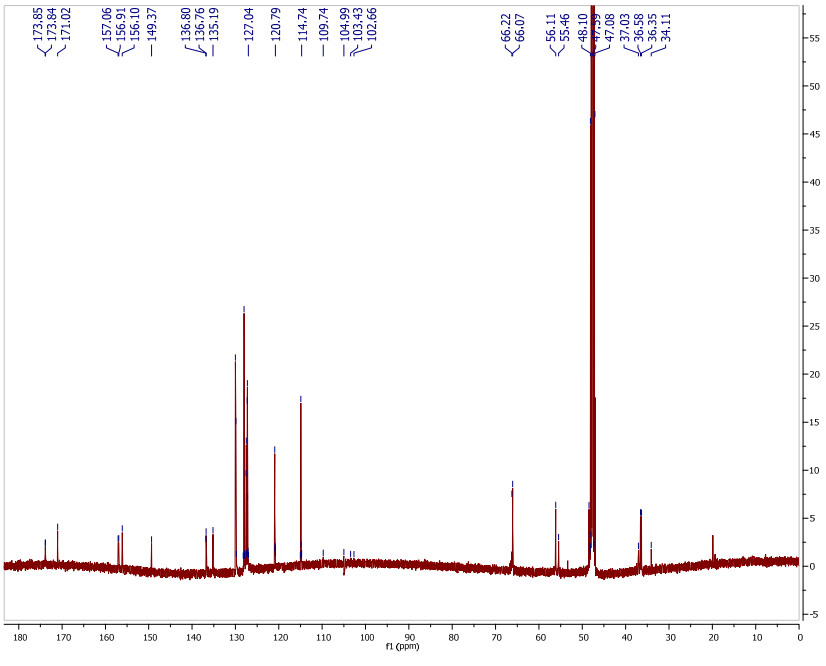
^{1}H NMR: δ 7.26-7.16 (m, 10H), 7.07-7.05 (J=7.78 Hz, d, 2H), 6.75-6.73 (J=7.88, d, 2H), 5.09-5.01 (m, 4H), 4.57 (s, 1H), 4.39 (s, 1H), 3.17-3.13 (m, 2H), 3.05-3.01 (m, 3H), 2.92-2.90 (m, 2H).

^{13}C NMR: δ 173.87, 171.04, 157.08, 156.93, 156.12, 148.39, 136.78, 135.20, 130.04, 129.91, 128.04, 127.26, 114.94, 66.23, 56.12, 55.47, 36.59, 36.36, 34.11.

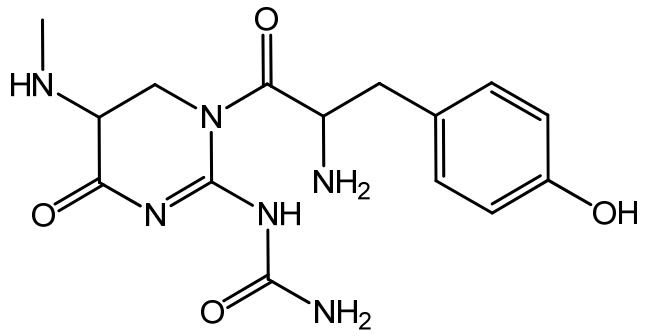
Spectrum 2.36: Compound **21** (ES-148A) ^{1}H NMR, CD_3OD, 400 MHz



Spectrum 2.37: Compound **21** (ES-148A) ^{13}C NMR, CD_3OD, 500 MHz



Compound **22** (ES-151B)



Chemical Formula: $C_{15}H_{20}N_6O_4$

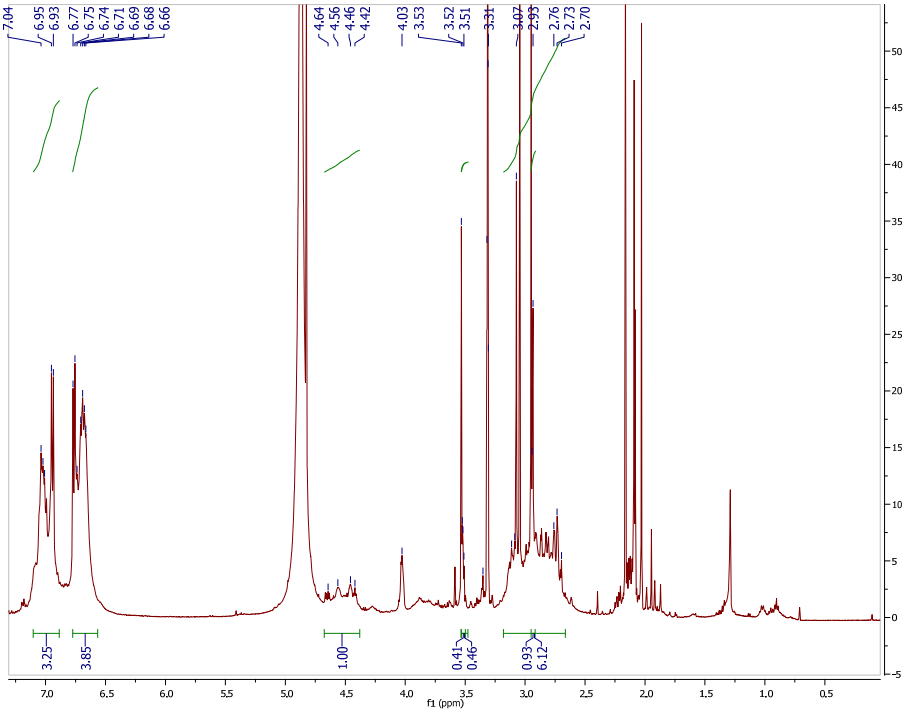
Exact Mass: 348.15 Found $[M+ H]^+$ 349.16 (ESI Positive Ion Mode).

NMR Assignments

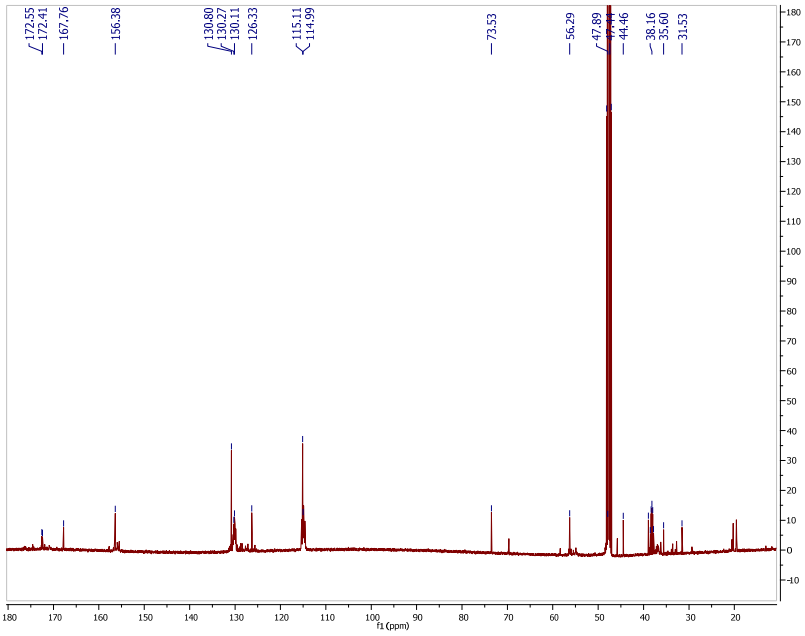
^{1}H NMR: δ 7.04-6.93 (m, 2H), 6.77-6.66 (m, 2H), 4.64-4.42 (m, 1H), 3.53-3.51 (m, 1H), 3.07-2.71 (m, 7H).

^{13}C NMR: δ 172.43, 167.78, 156.54, 156.40, 130.80, 130.27, 130.11, 126.33, 115.37, 69.68, 56.30, 38.16, 35.60, 31.53.

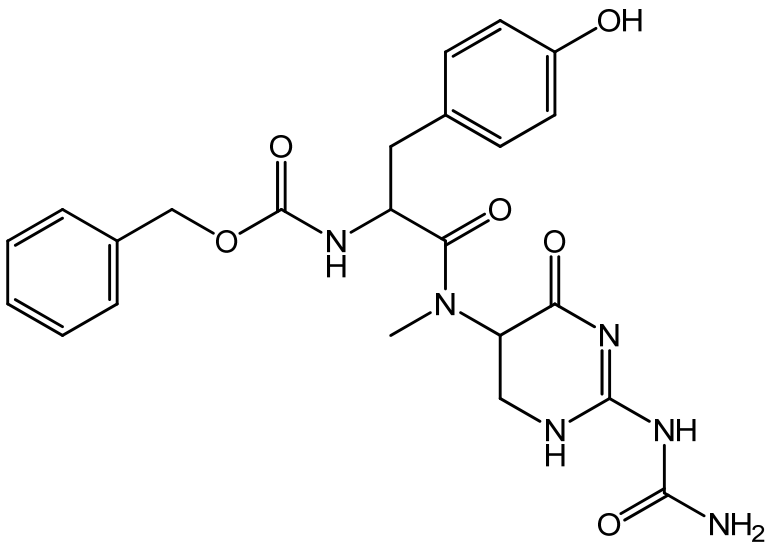
Spectrum 2.38: Compound **22** (ES-151B) ^{1}H NMR, CD_3OD, 500 MHz



Spectrum 2.39: Compound **22** (ES-151B) ^{13}C NMR, CD_3OD, 500 MHz



Compound **23** (ES-152B)



Chemical Formula: $C_{23}H_{26}N_6O_6$

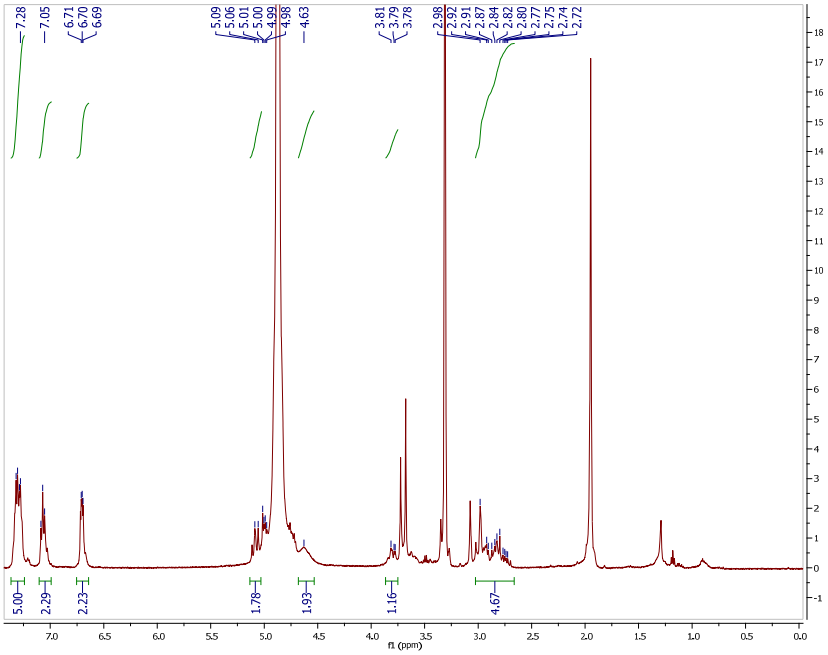
Exact Mass: 482.19 Found $[M+MeOH+H]^+$ 515.18 (ESI Positive Ion Mode).

NMR Assignments

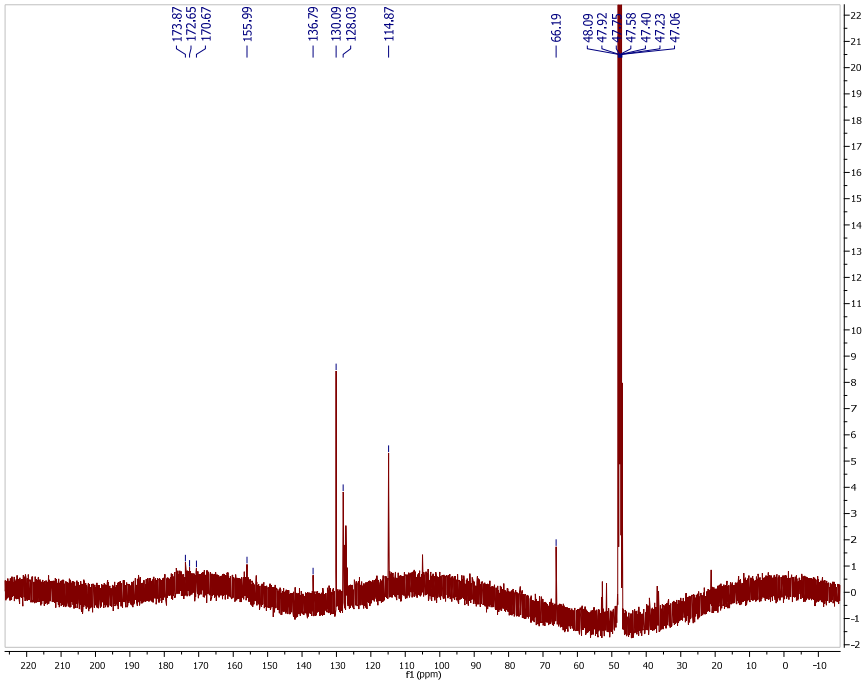
^{1}H NMR: δ 7.32-7.28 (m, 5H), 7.09-7.03 (m, 2H), 6.72-6.69 (m, 2H), 5.11-5.06 (m, 2H), 4.63 (bs, 2H), 3.81-3.77 (m, 1H), 3.08-2.80 (m, 5H).

^{13}C NMR: δ 173.87, 172.65, 170.67, 155.99, 136.79, 130.09, 128.03, 127.53, 127.19, 114.87, 66.19, 53.02, 52.74, 39.10, 36.83, 36.46.

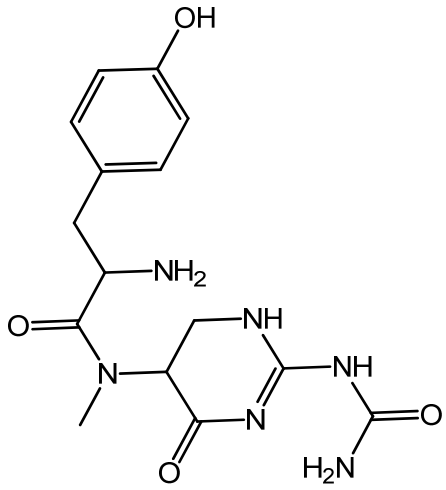
Spectrum 2.40: Compound **23** (ES-148A-F5) ^{1}H NMR, CD_3OD, 500 MHz



Spectrum 2.41: Compound **23** (ES-148A-F5) ^{13}C NMR, CD_3OD, 500 MHz



Compound **24** (ES-153A)



Chemical Formula: $C_{15}H_{20}N_6O_4$

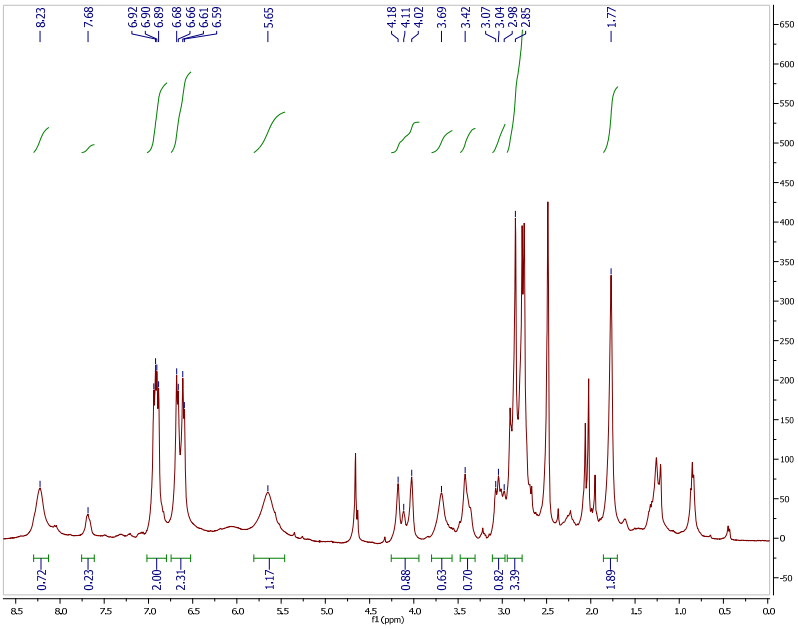
Exact Mass: 348.15 Found $[M+ H]^+$ 349.21 (ESI Positive Ion Mode).

NMR Assignments

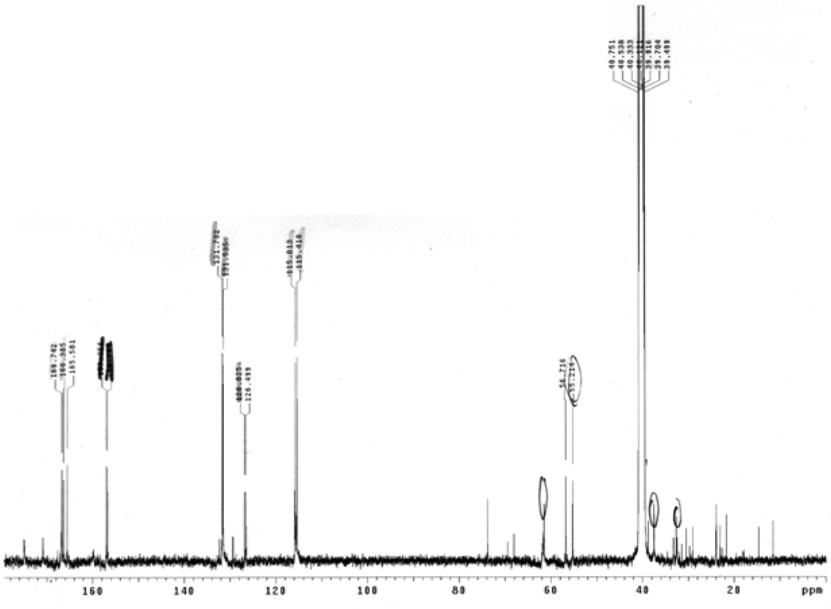
^{1}H NMR: δ 8.23 (bs, 1H), 7.68 (bs, 1H), 6.94-6.89 (m, 2H), 6.68-6.59 (m, 2H), 4.18-4.02 (m, 1H), 3.69 (bs, 1H), 3.42 (m, 1H), 3.10-2.98 (m, 1H), 2.94-2.70 (m, 4H), 1.77 (bs, 1H).

^{13}C NMR: δ 166.74, 166.39, 157.06, 156.80, 131.70, 131.54, 126.83, 115.81, 115.42, 62.20, 55.21, 37.80, 33.21.

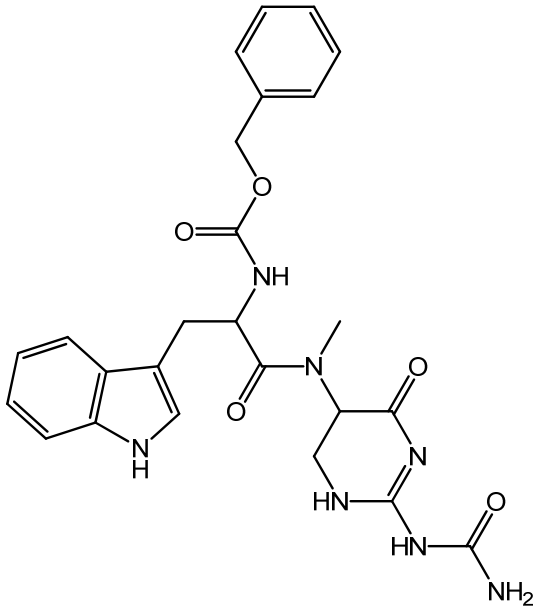
Spectrum 2.42: Compound **24** (ES-153A) ^{1}H NMR, DMSO, 400 MHz



Spectrum 2.43: Compound **24** (ES-153A) ^{13}C NMR, DMSO, 400 MHz



Compound **25** (ES-150B)



Chemical Formula: $C_{25}H_{27}N_7O_5$

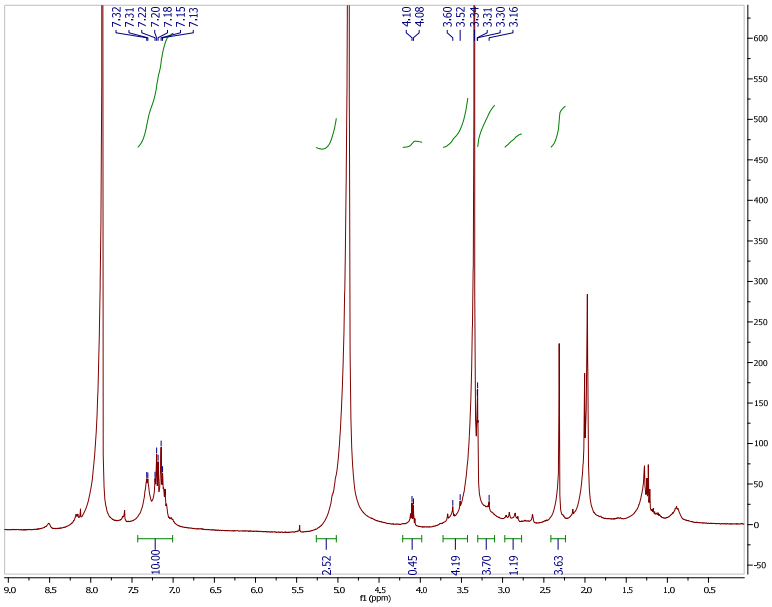
Exact Mass: 505.21 Found $[M+H]^+$ 506.20 (ESI Positive Ion Mode).

NMR Assignments

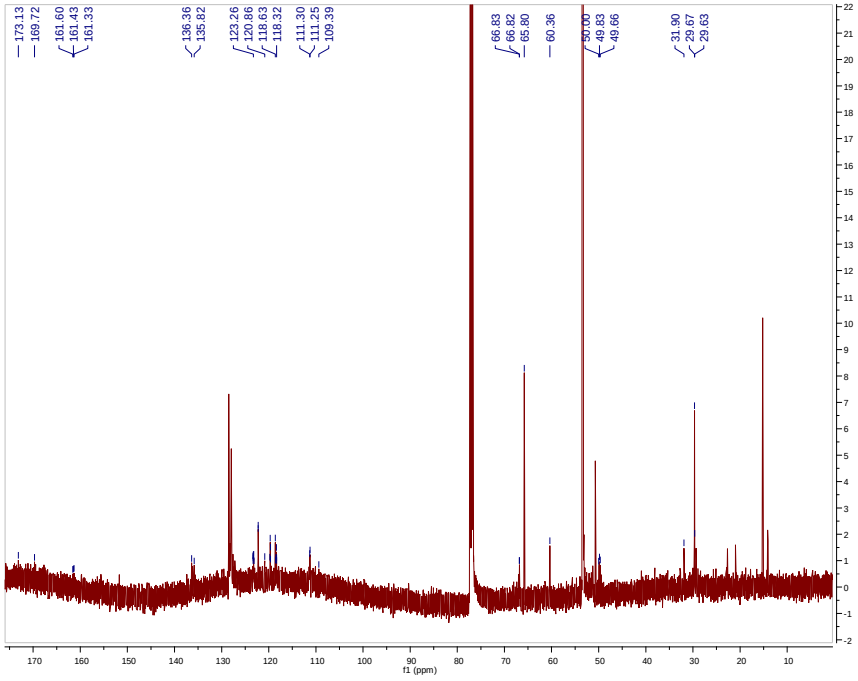
^{1}H NMR: δ 7.32-7.13 (m, 10H), 4.87 (m, 2H), 4.10-4.08 (m, 1H), 3.75-3.40 (m, 4H), 3.30-3.10 (m, 4H).

^{13}C NMR: δ 173.13, 169.72, 161.60, 161.43, 161.33, 136.36, 128.66, 128.041, 127.564, 127.41, 126.83, 123.26, 120.86, 118.63, 118.32, 111.25, 109.40, 66.83, 65.80, 60.36, 49.83, 31.90, 29.67.

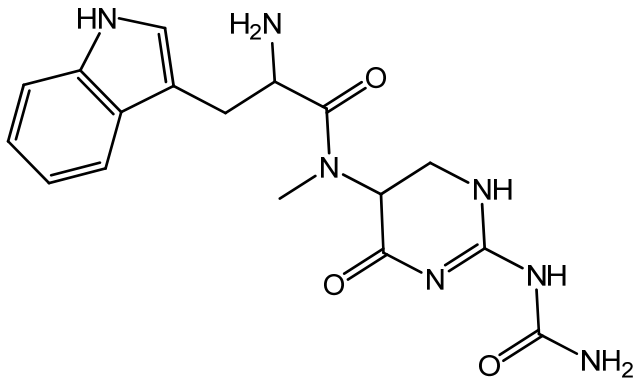
Spectrum 2.44: Compound **25** (ES-150B) ^{1}H NMR, CD_3OD, 400 MHz



Spectrum 2.45: Compound **25** (ES-136B/137A) ^{13}C NMR, CD_3OD, 500 MHz



Compound **26** (ES-154B)



Chemical Formula: $C_{17}H_{21}N_7O_3$

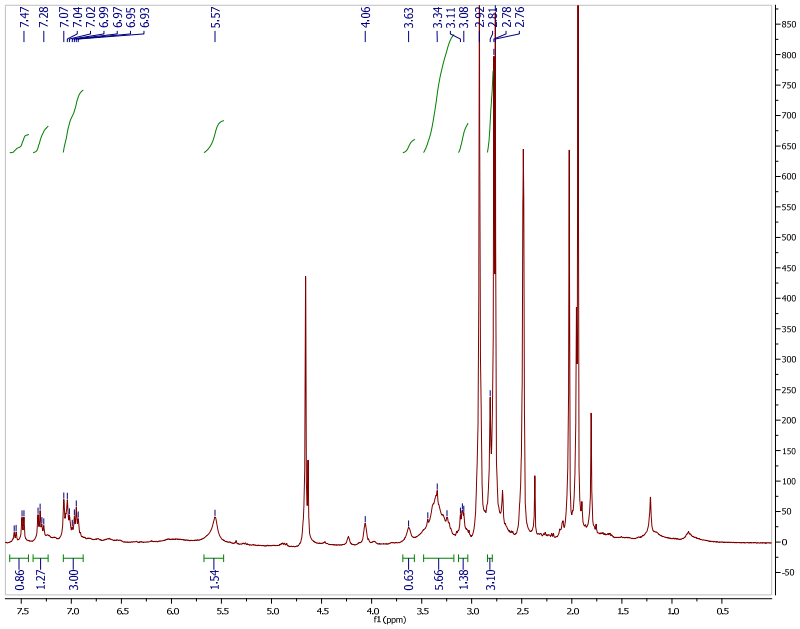
Exact Mass: 371.17 Found $[M+H]^+$ 372.25 (ESI Positive Ion Mode).

NMR Assignments

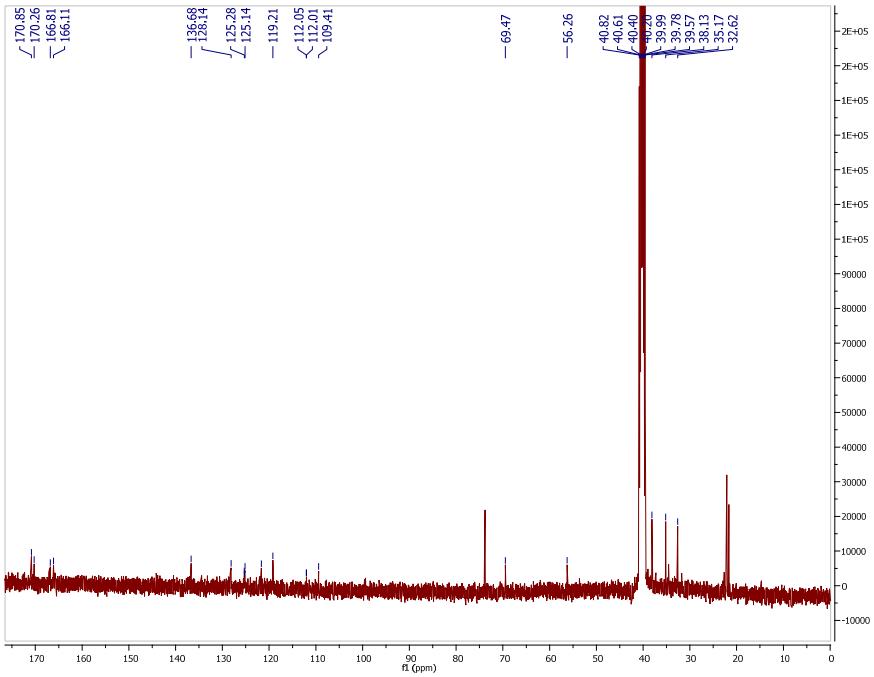
^{1}H NMR: δ 7.57-7.47 (m, 1H), 7.33-7.28 (m, 1H), 7.07-6.93 (m, 3H), 5.57 (bs, 2H), 3.63 (s, 1H), 3.44-3.25 (m, 4H), 3.11-3.08 (m, 1H), 2.81 (s, 3H).

^{13}C NMR: δ 170.85, 170.26, 166.81, 166.11, 136.68, 128.14, 125.28, 125.14, 119.21, 112.05, 112.01, 109.41, 69.47, 56.26, 38.13, 35.17, 32.62.

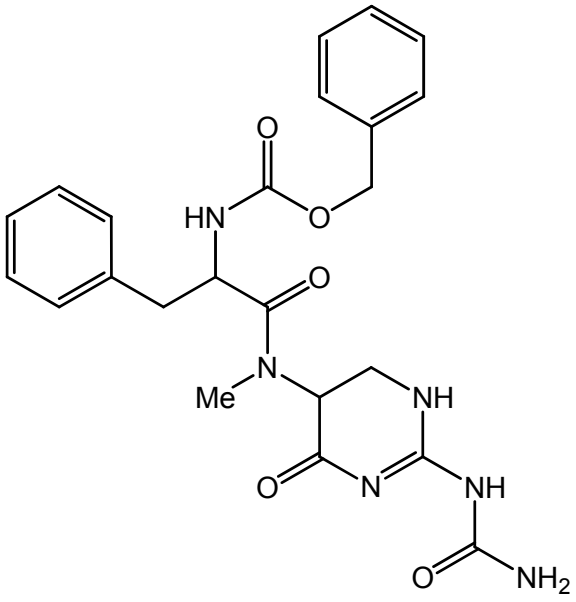
Spectrum 2.46: Compound **26** (ES-154B) ^{1}H NMR, DMSO, 400 MHz



Spectrum 2.47: Compound **26** (ES-154B) ^{13}C NMR, DMSO, 400 MHz



Compound **27** (ES-181B)



Chemical Formula: $C_{23}H_{26}N_6O_5$

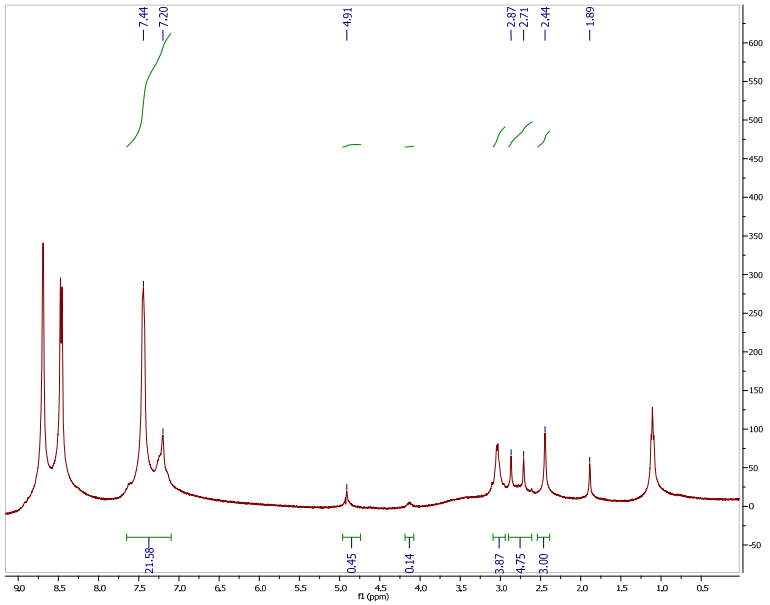
Exact Mass: 466.20 Found $[M+H]^+$ 467.27 (ESI Positive Ion Mode).

NMR Assignments

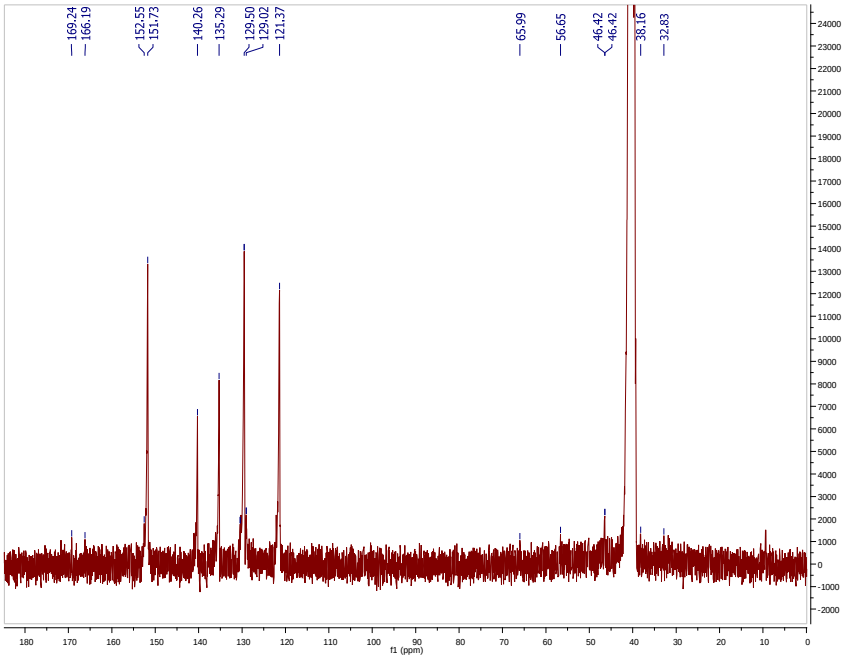
^{1}H NMR: δ 7.44-7.20 (m, 10H), 4.91 (m, 2H), 4.15 (m, 2H), 3.05 (m, 2H), 3.03 (m, 1H), 2.87 (m, 1H), 2.45 (s, 3H).

^{13}C NMR: δ 169.24, 166.19, 152.55, 151.73, 140.26, 135.29, 129.50, 129.02, 121.37, 65.99, 56.65, 46.42, 38.16, 32.83.

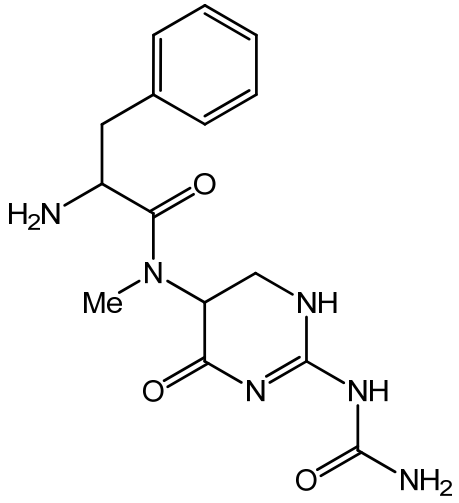
Spectrum 2.48: Compound **27** (ES-181B) ^{1}H NMR, DMSO, 400 MHz



Spectrum 2.49: Compound **27** (ES-181B) ^{13}C NMR, DMSO, 300 MHz



Compound **28** (ES-184A)



Chemical Formula: $C_{15}H_{20}N_6O_3$

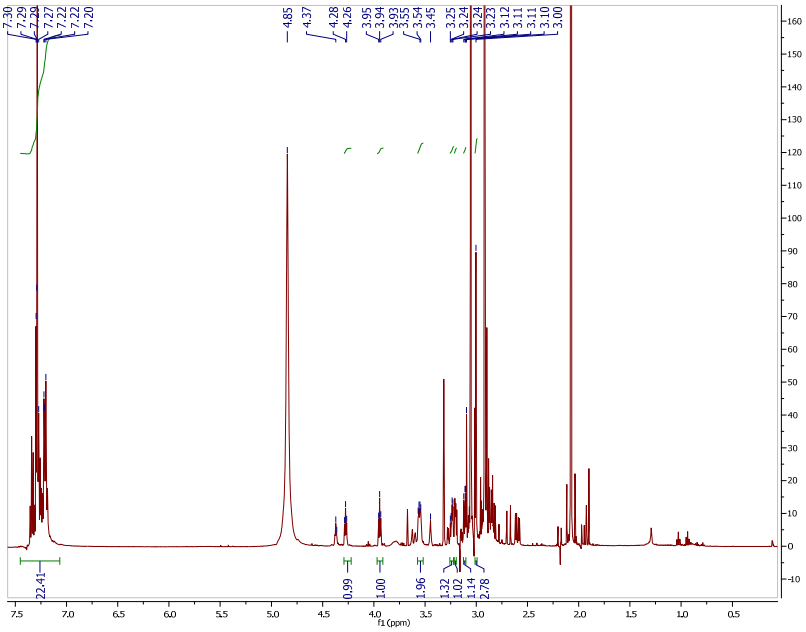
Exact Mass: 332.16 Found $[M+H]^+$ 333.17 (ESI Positive Ion Mode).

NMR Assignments

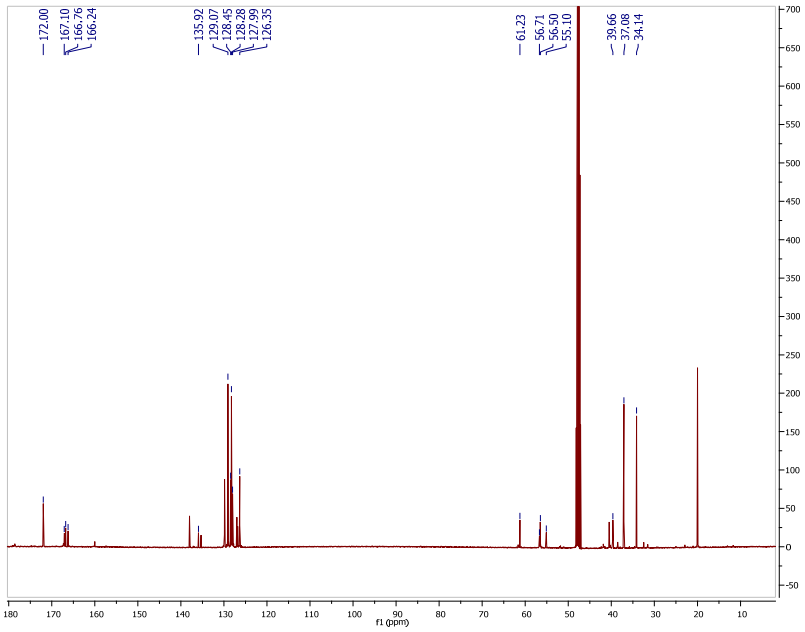
^{1}H NMR: δ 7.30-7.20 (m, 5H), 4.29-4.26 (J=6.17Hz, t, 1H), 3.95-3.93 (J=5.61, t, 1H), 3.57-3.45 (J_1=4.17Hz, J_2=6.32Hz, dd, 2H), 3.25-3.23 (m, 1H), 3.12-3.11 (m, 1H), 3.00 (s, 3H).

^{13}C NMR: δ 172.02, 167.11, 166.78, 155.26, 135.94, 129.09, 128.46, 128.29, 128.00, 127.04, 61.24, 56.71, 55.11, 39.67, 37.08, 34.14.

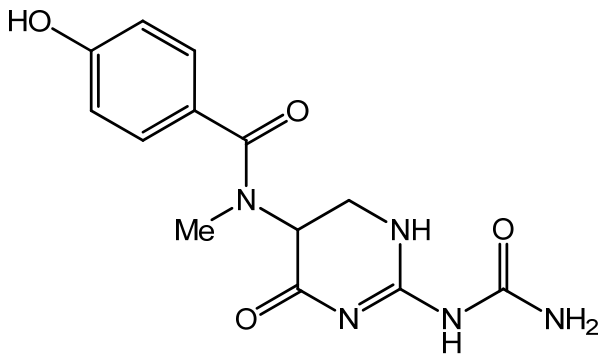
Spectrum 2.50: Compound **28** (ES-184A) ^{1}H NMR, CD_3OD, 500 MHz



Spectrum 2.51: Compound **28** (ES-184A) ^{13}C NMR, CD_3OD, 500 MHz



Compound **29** (ES-199A)



Chemical Formula: $C_{13}H_{15}N_5O_4$

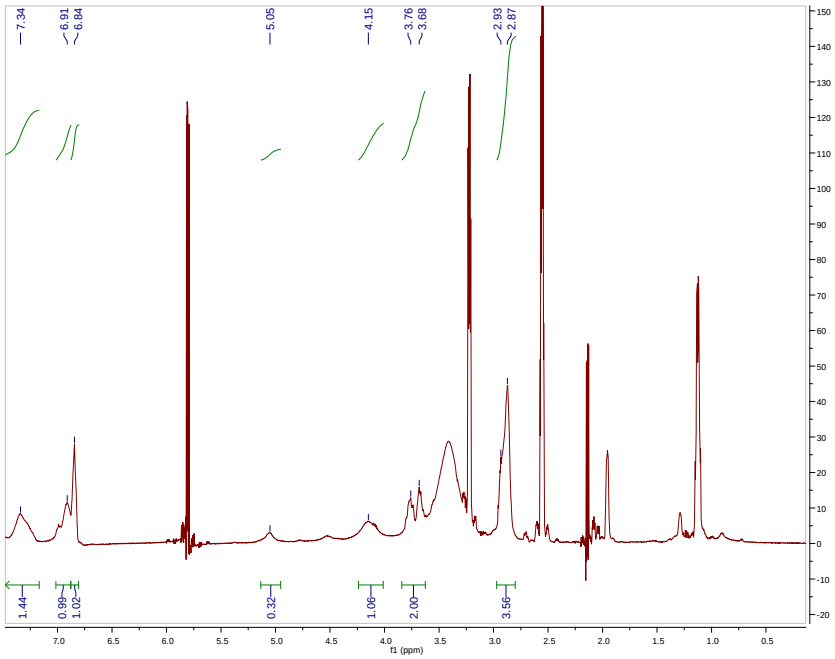
Exact Mass: 305.11 Found $[M+H]^+$ 306.1195, Delta ppm = -0.7 (HR-ESI-FT-MS (Orbit-Trap-MS)).

NMR Assignments

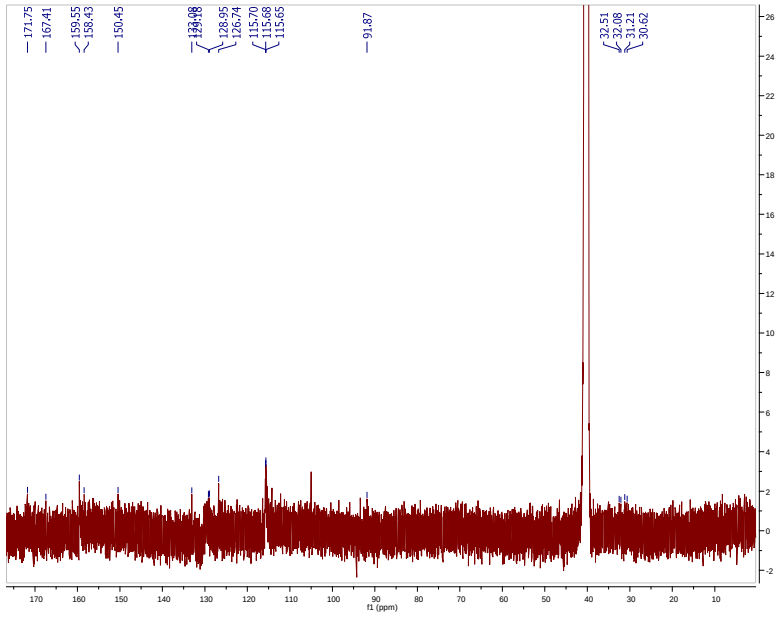
^{1}H NMR: δ 7.34 (bs, 2H), 6.91 (m, 1H), 6.84 (s, 1H), 5.05 (bs, 1H), 4.15 (s, 1H), 3.76-3.68 (m, 2H), 2.87 (s, 3H).

^{13}C NMR: δ 171.75, 167.41, 159.55, 158.43, 150.45, 133.08, 129.18, 129.05, 128.95, 126.74, 115.70, 115.68, 91.87, 32.51, 31.21.

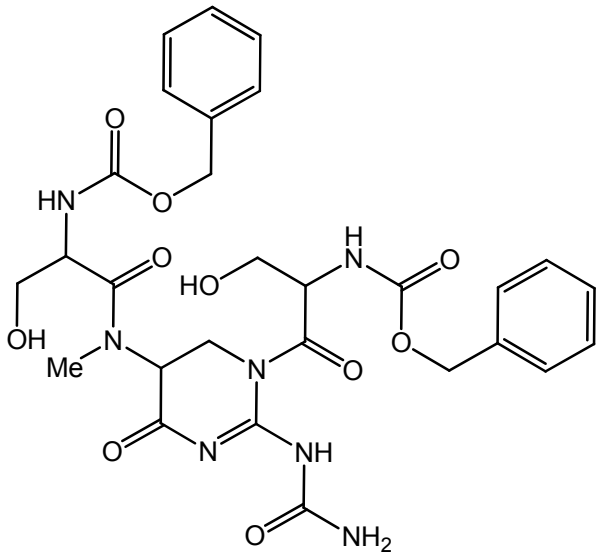
Spectrum 2.52: Compound **29** (ES-199A) ^{1}H NMR, DMSO, 500 MHz



Spectrum 2.53: Compound **29** (ES-199A) ^{13}C NMR, DMSO, 500 MHz



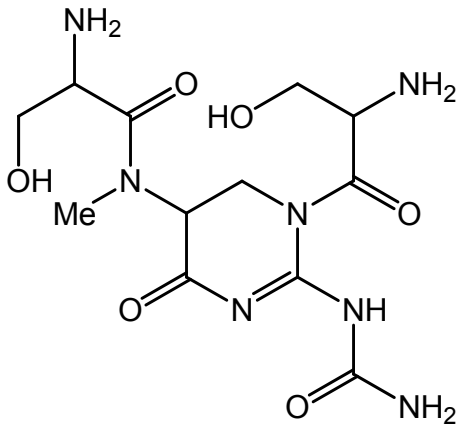
Compound **30** (ES-198B)



Chemical Formula: $C_{28}H_{33}N_7O_{10}$

Exact Mass: 627.23 Found $[M+H]^+$ 628.20 (ESI Positive Ion Mode).

Compound **31** (ES-215)



Chemical Formula: $C_{12}H_{21}N_7O_6$

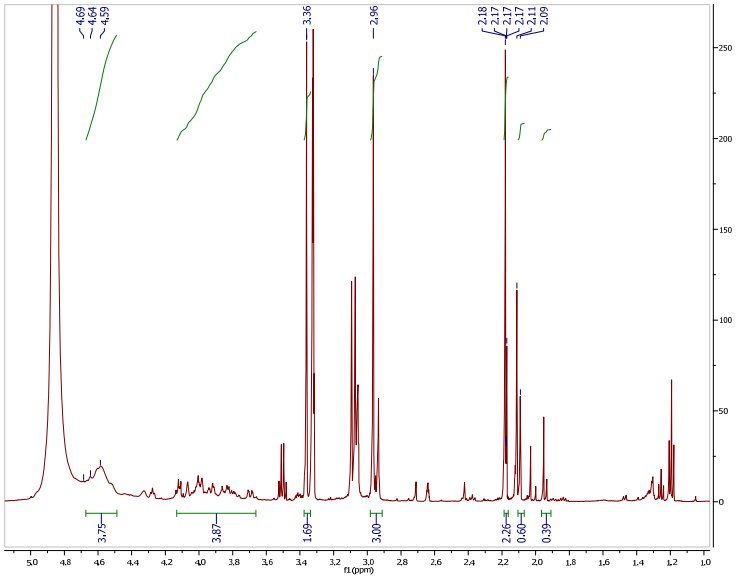
Exact Mass: 359.16 Found $[M+H]^+$ 359.98 (ESI Positive Ion Mode).

NMR Assignments

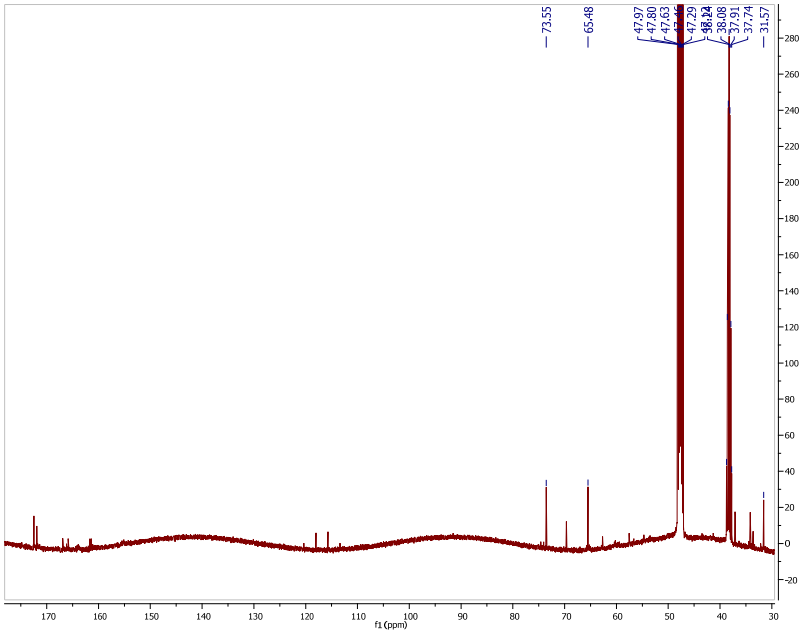
^{1}H NMR: δ 4.69-4.59 (m, 3H), 4.12-3.69 (m, 4H), 3.36 (s, 2H), 2.96 (s, 3H).

^{13}C NMR: δ 172.50, 171.95, 166.83, 166.20, 161.45, 73.56, 69.67, 65.49, 34.17, 31.57.

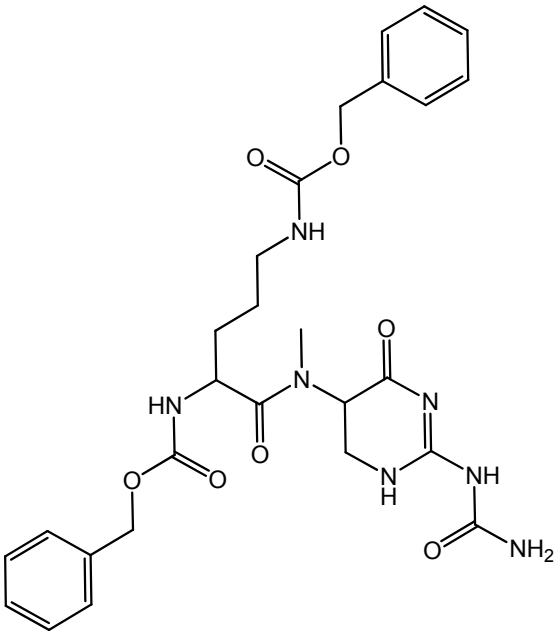
Spectrum 2.54: Compound **31** (ES-215) ^{1}H NMR, CD_3OD, 500 MHz



Spectrum 2.55: Compound **31** (ES-215) ^{13}C NMR, CD_3OD, 500 MHz



Compound **32** (ES-256)



Chemical Formula: $C_{27}H_{33}N_7O_7$

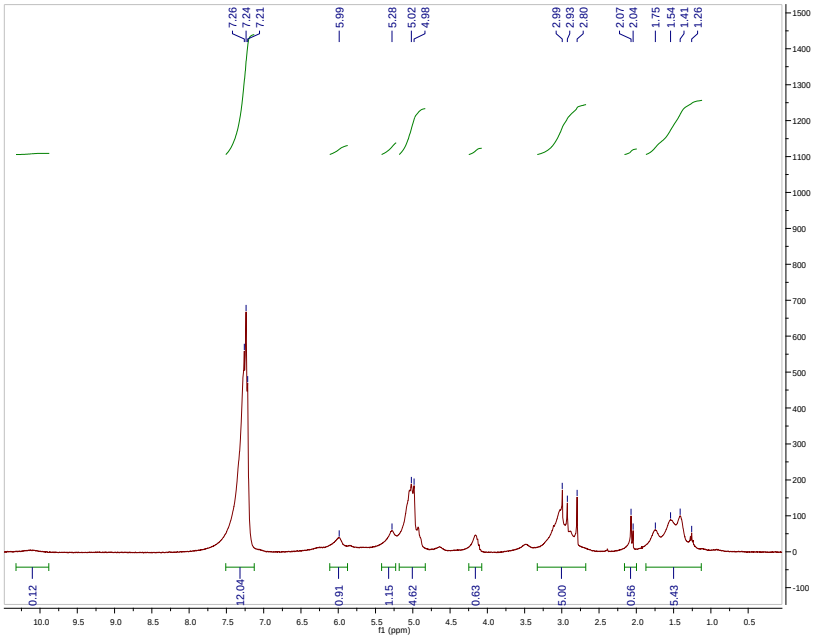
Exact Mass: 567.24 Found $[M+H]^+$ 568.25, Delta ppm =0.2 (HR-ESI-TOFMS).

NMR Assignments

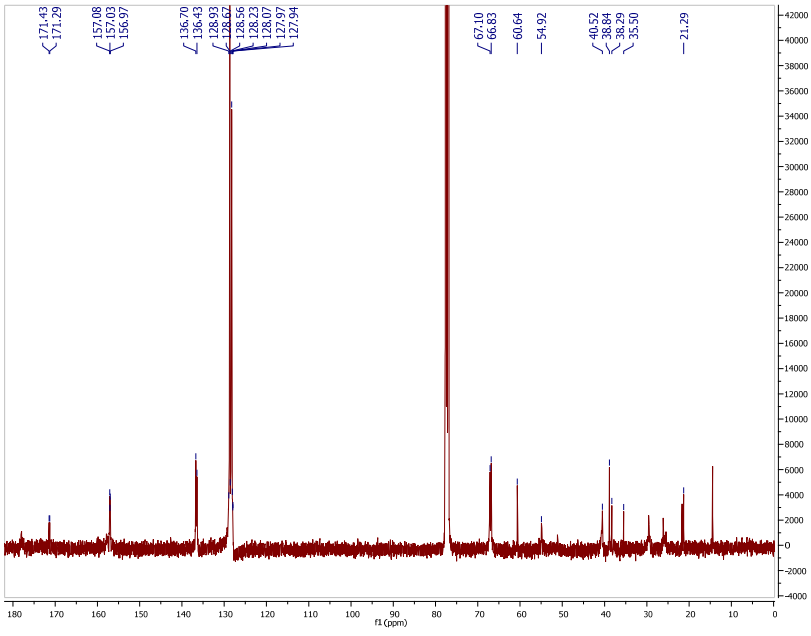
^{1}H NMR: δ 10.2 (bs, 1H), 7.26-7.214 (m, 10H), 5.99 (bs, 1H), 5.28 (bs, 1H), 5.08-4.97 (m, 4H), 4.16 (bs, 1H), 2.99-2.80 (m, 5H), 2.07-2.04 (J=10.92Hz, d, 1H), 1.80-1.25 (m, 6H).

^{13}C NMR: δ 171.43, 171.29, 157.08, 157.03, 156.97, 136.70, 136.43, 128.93, 128.67, 128.56, 128.23, 128.07, 127.97, 127.94, 67.10, 66.83, 60.64, 54.92, 40.52, 38.84, 38.29, 35.50, 21.29.

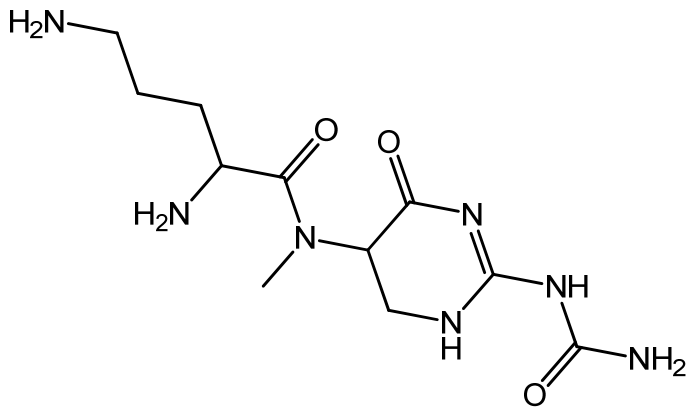
Spectrum 2.56: Compound **32** (ES-256) ^{1}H NMR, $CDCl_3$, 400 MHz



Spectrum 2.57: Compound **32** (ES-256) ^{13}C NMR, $CDCl_3$, 400 MHz



Compound **33** (ES-256)



Chemical Formula: $C_{11}H_{21}N_7O_3$

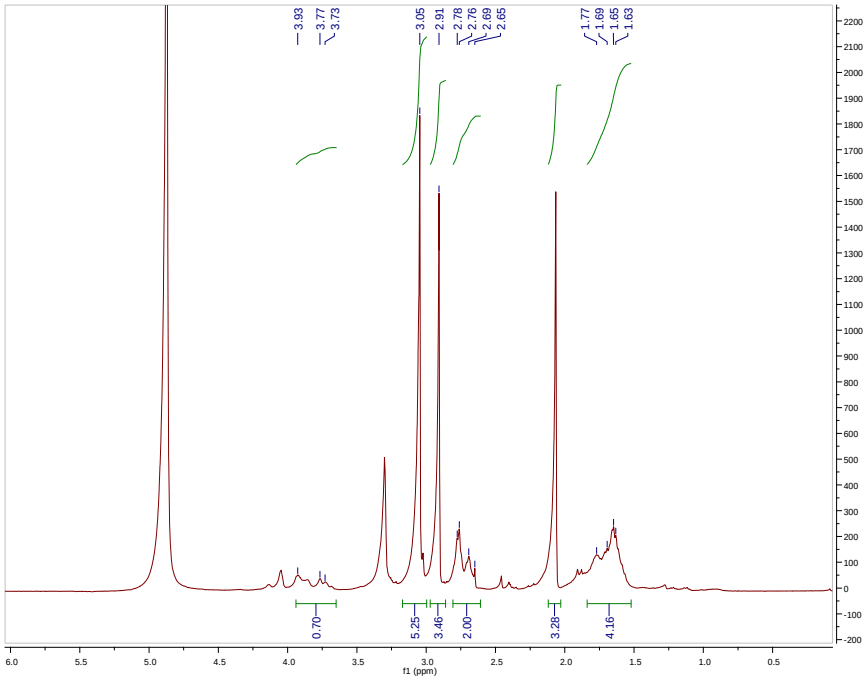
Exact Mass: 299.17 Found $[M+H]^+$ 300.1781, Delta ppm =0.7 (HR-ESI-TOFMS).

NMR Assignments

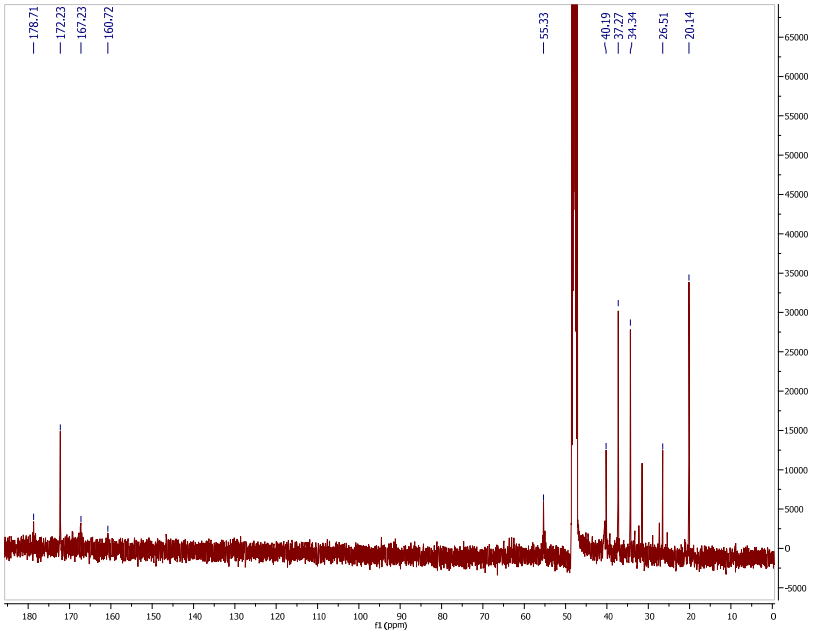
^{1}H NMR: δ 3.93 (m, 1H), 3.08-3.02 (m, 2H), 2.91 (s, 3H), 2.80-2.65 (m, 2H), 1.79-1.55 (m, 4H).

^{13}C NMR: δ 178.71, 172.23, 167.23, 160.72, 55.33, 40.19, 37.27, 34.34, 26.51, 20.14.

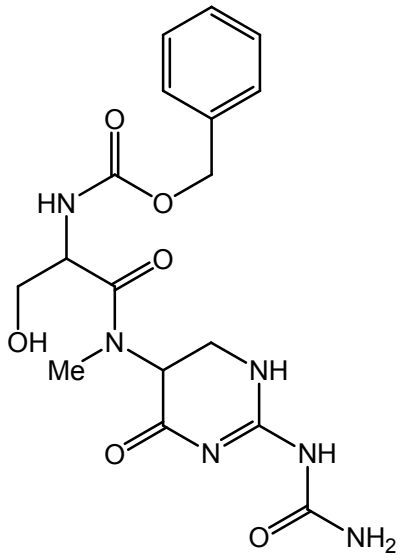
Spectrum 2.58: Compound **33** (ES-259) ^{1}H NMR, CD_3OD, 400 MHz



Spectrum 2.59: Compound **33** (ES-259) ^{13}C NMR, CD_3OD, 400 MHz



Compound **34** (ES-246)



Chemical Formula: $C_{17}H_{22}N_6O_6$

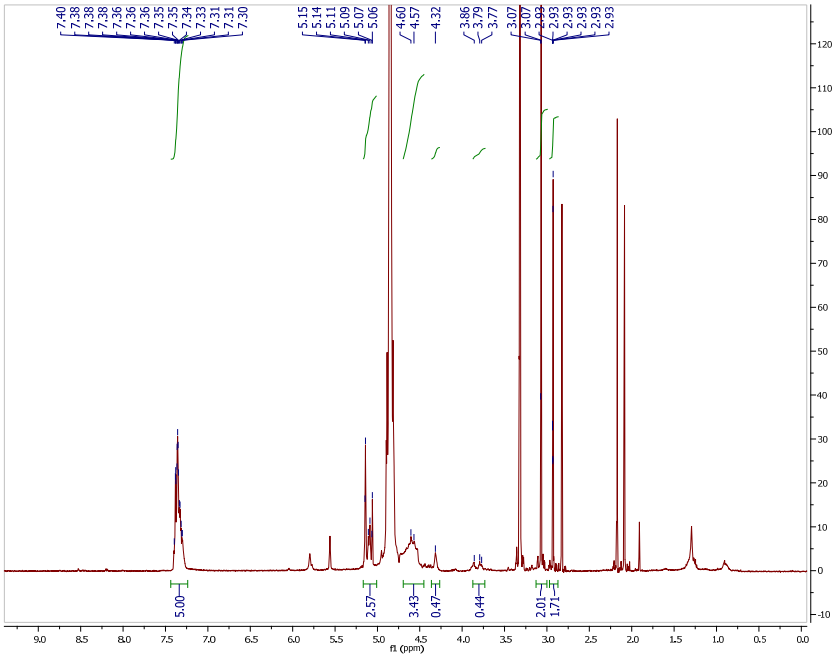
Exact Mass: 406.16 Found $[M+H]^+$ 407.1675, Delta ppm =0.2 (HR-ESI-TOFMS).

NMR Assignments

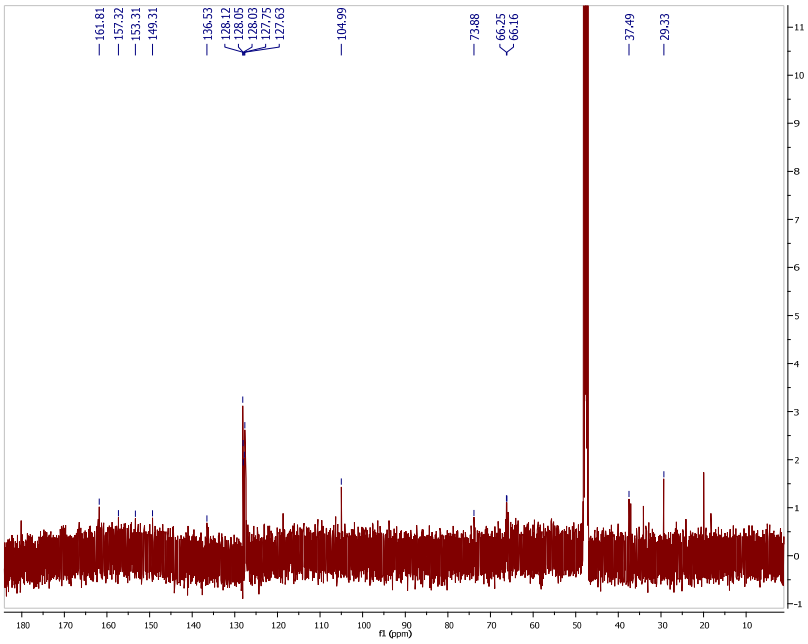
^{1}H NMR: δ 7.40-7.30 (m, 5H), 5.15-5.06 (m, 2H), 4.65-4.52 (m, 3H), 4.48-4.29 (m, 1H), 3.88-3.75 (m, 1H), 3.12-3.02 (m, 2H), 2.93 (s, 3H).

^{13}C NMR: δ 161.81, 157.32, 153.31, 149.31, 136.53, 128.12, 128.05, 128.03, 127.75, 127.63, 104.99, 73.88, 66.25, 66.16, 37.49, 29.33.

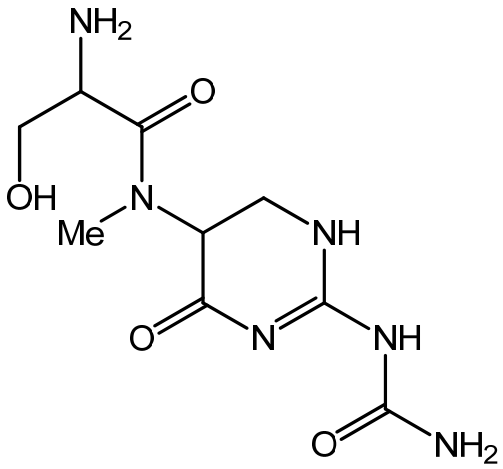
Spectrum 2.60: Compound **34** (ES-181A2) ^{1}H NMR, CD_3OD, 500 MHz



Spectrum 2.61: Compound **34** (ES-181A2) ^{13}C NMR, CD_3OD, 400 MHz



Compound **35** (ES-185B)



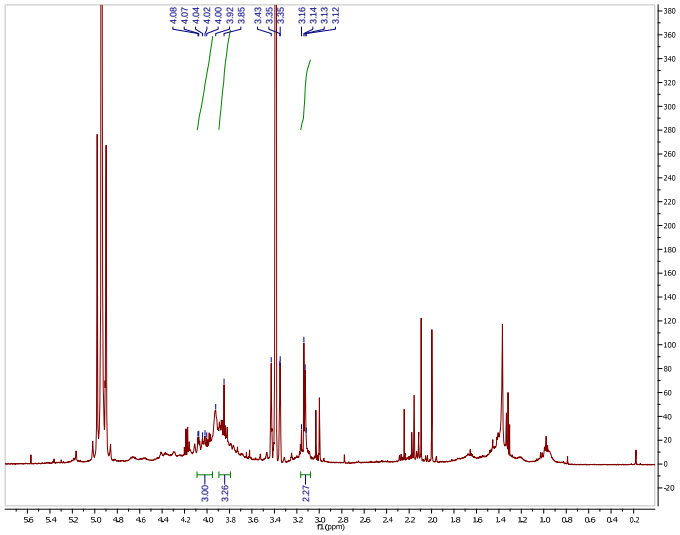
Chemical Formula: $C_9H_{16}N_6O_4$

Exact Mass: 272.12 Found $[M+H]^+$ 273.37 (ESI Positive Ion Mode).

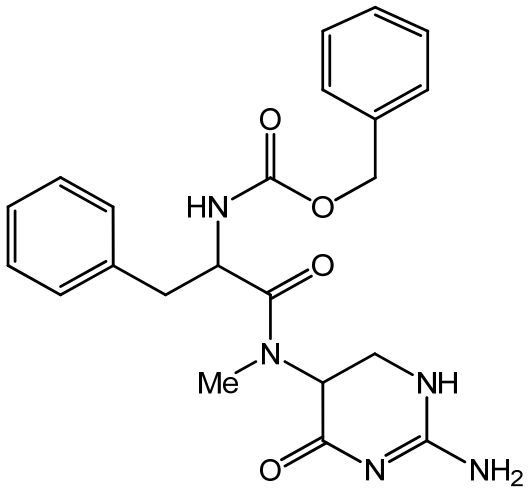
NMR Assignments

^{1}H NMR: δ 4.10-3.95 (m, 3H), 3.90-3.75 (m, 3H), 3.16-3.12 (m, 3H).

Spectrum 2.62: Compound **35** (ES-185B) ^{1}H NMR, CD_3OD, 500 MHz



Compound **36** (ES-211)



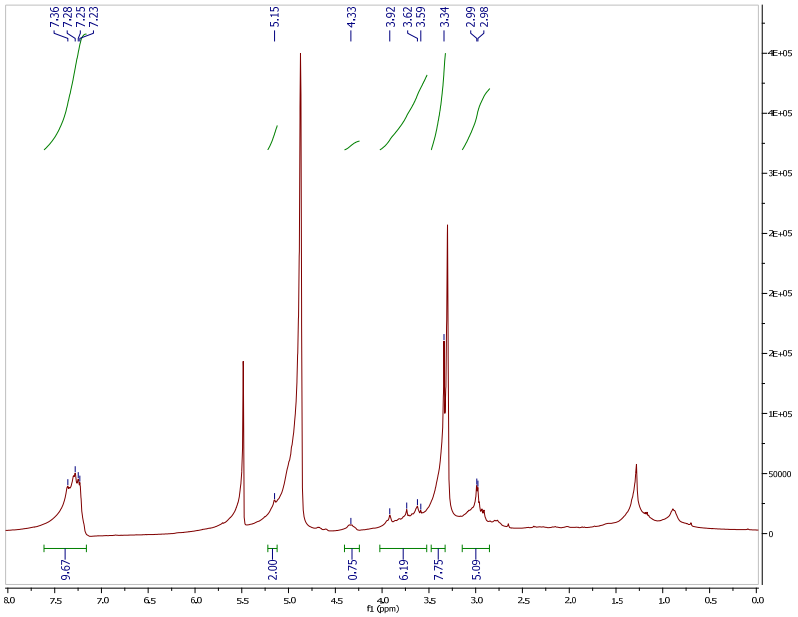
Chemical Formula: $C_{22}H_{25}N_5O_4$

Exact Mass: 423.19 Found $[M+Na]^+$ 446.1790, Delta ppm =2.0 (HR-ESI-TOFMS).

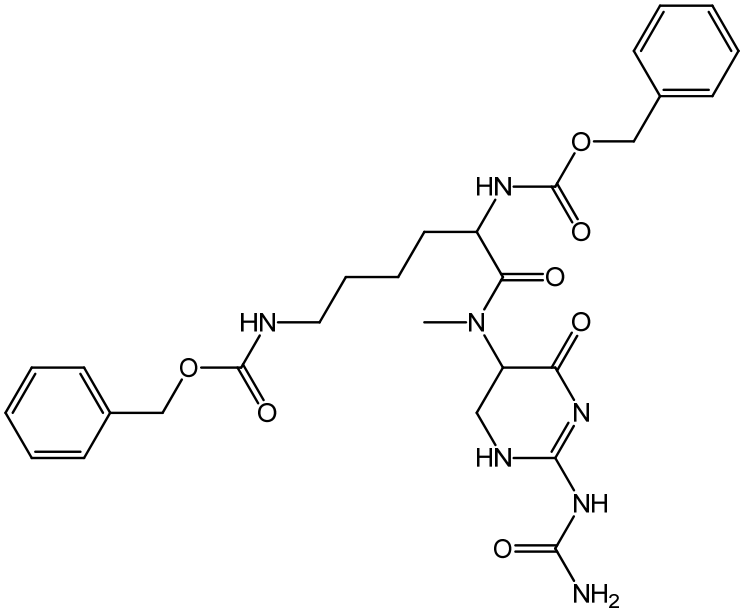
NMR Assignments

^{1}H NMR: δ 7.36-7.23, 5.15 (s, 2H), 4.33 (m, 1H), 3.92-3.59 (m, 5H), 2.99-2.98 (m, 3H).

Spectrum 2.63: Compound **36** (ES-211) ^{1}H NMR, CD_3OD, 400 MHz



Compound **37** (ES-147A)



Chemical Formula: $C_{28}H_{35}N_7O_7$

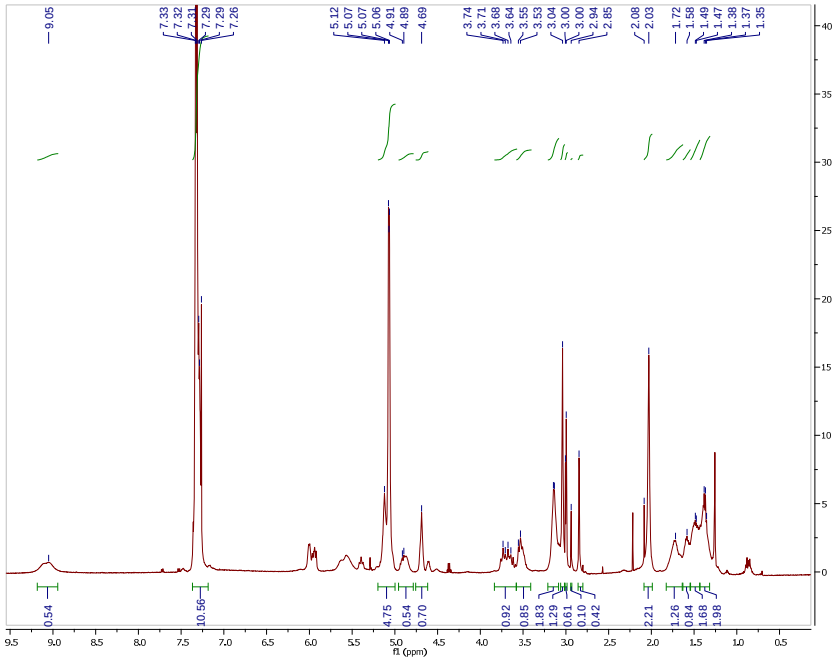
Exact Mass: 581.26 Found $[M+H]^+$ 582.23 (ESI Positive Ion Mode).

NMR Assignments

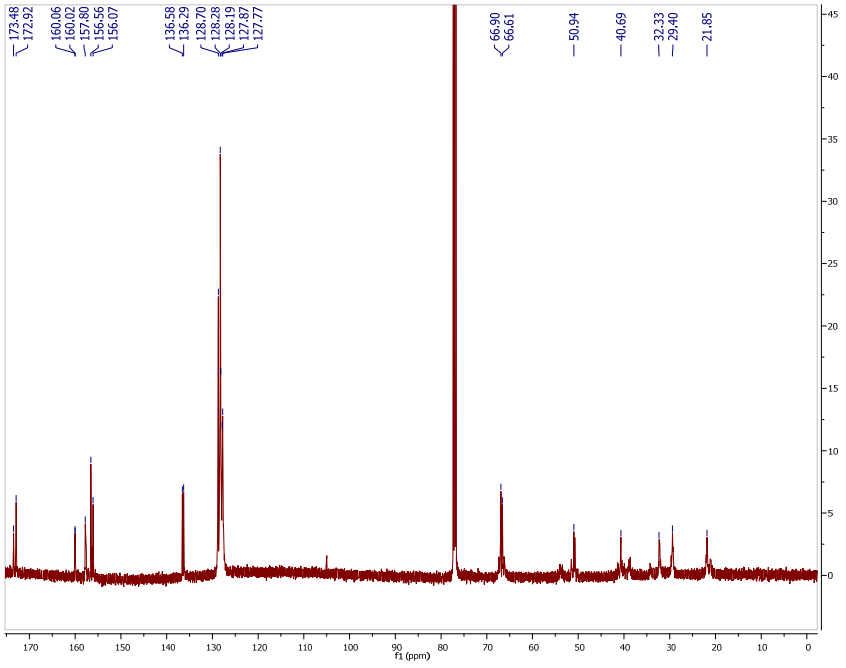
^{1}H NMR: δ 9.05 (bs, ,1H), 7.33-7.26 (m, 10H), 5.12-5.06 (m, 4H), 4.91-4.89 (m, 1H), 3.74-3.64 (m, 1H), 3.55-3.53 (m, 1H), 3.14-3.13 (m, 2H), 3.0 (J=5.44Hz, d, 1H), 2.03 (s, 3H), 1.80-1.65 (m, 1H), 1.62-1.56 (m, 1H), 1.54-1.44 (m, 2H), 1.43-1.32 (m, 2H).

^{13}C NMR: δ.173.48, 172.92, 160.06, 160.02, 157.80, 156.56, 156.07, 136.58, 136.29, 128.70, 128.28, 128.19, 127.87, 127.77, 66.90, 66.61, 50.94, 40.69, 32.33, 29.40, 21.85.

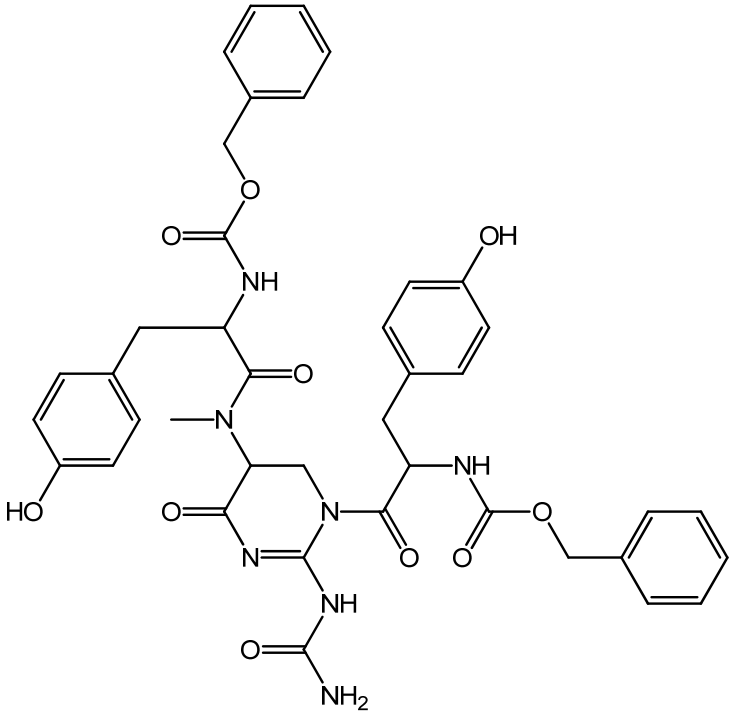
Spectrum 2.64: Compound **37** (ES-147A) ^{1}H NMR, $CDCl_3$, 500 MHz



Spectrum 2.65: Compound **37** (ES-147A) ^{13}C NMR, $CDCl_3$, 500 MHz



Compound **38** (ES-148A-F3)



Chemical Formula: $C_{40}H_{41}N_7O_{10}$

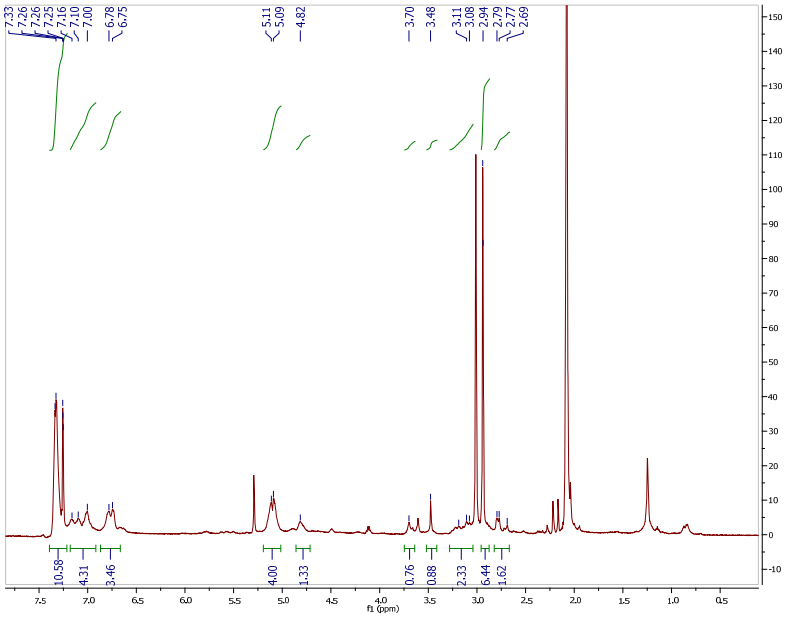
Exact Mass: 779.29 Found $[M+H]^+$ 780.21 (ESI Positive Ion Mode).

NMR Assignments

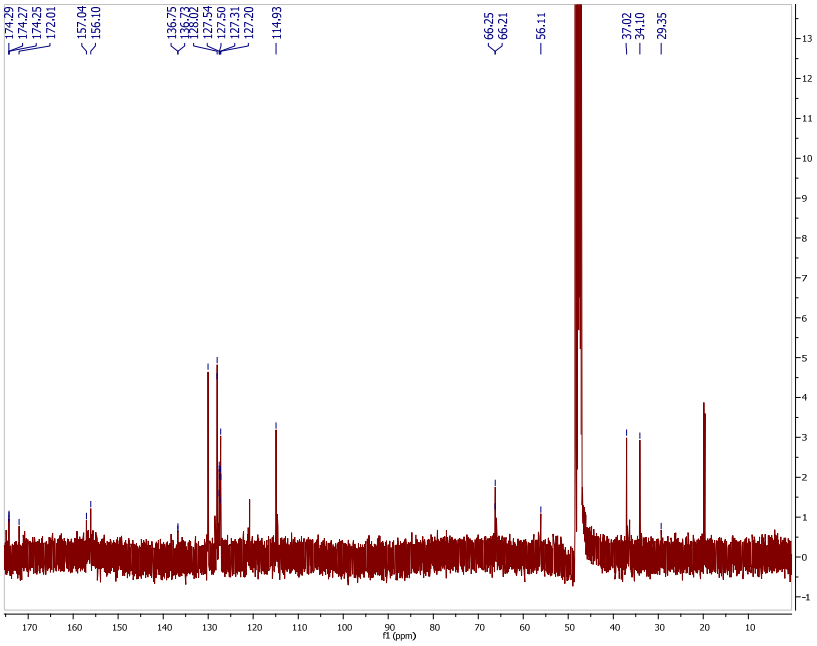
^{1}H NMR: δ 7.33-7.16 (m, 10H), 7.10-7.00 (m, 4H), 6.78-6.75 (m, 4H), 5.11-5.09 (m, 4H), 4.82 (s, 1H), 3.70 (m, 1H), 3.48 (m, 1H), 3.25-3.05 (m, 2H), 2.94 (s, 3H), 2.79-2.69 (m, 2H).

^{13}C NMR: δ 174.29, 174.27, 174.25, 172.01, 157.04, 156.10, 136.75, 136.73, 128.02, 127.54, 127.50, 127.31, 127.20, 114.93, 66.25, 66.21, 56.11, 37.02, 34.10, 29.35.

Spectrum 2.66: Compound **38** (ES-148A-F3) ^{1}H NMR, CD_3OD, 500 MHz



Spectrum 2.67: Compound **38** (ES-148A-F3) ^{13}C NMR, CD_3OD, 500 MHz



Conclusions

The synthesis of three cores from a common methyl ester precursor was performed. Couplings of various carboxylic acids to the DHP and DHP Urea cores were successful and yielded 10 final compounds. The final compounds as well as the individual cores, both Cbz-protected and unprotected, were tested in the FRET assay for binding in HCV IRES domain IIa, and in the IVT assay for translational disruption caused by small molecule binding within the TS and the IRES. Results of the FRET assay show no interference with the bend of domain IIa of the HCV IRES. The IVT assay identified compounds **5** and **13** as potentially acting on the TS hairpin, and compounds **24**, **26**, **28**, **29**, **31**, and **36** as potential IRES inhibitors. Further tests are necessary for many of these compounds to validate the activity and determine EC_{50} values. Future work will include exploring other analogs of the natural DHP Urea core of TAN 1057 A/B, specifically those with an exocyclic non-urea free amine or an aromatic peptide attached at the N-methyl position of the core.

Acknowledgements

Chapter 2 is currently being prepared for submission for publication. The dissertation author was the primary author. Co-authors include Andrea Potocny, Maria Alvarado, Jesus Moreno, and Thomas Hermann.

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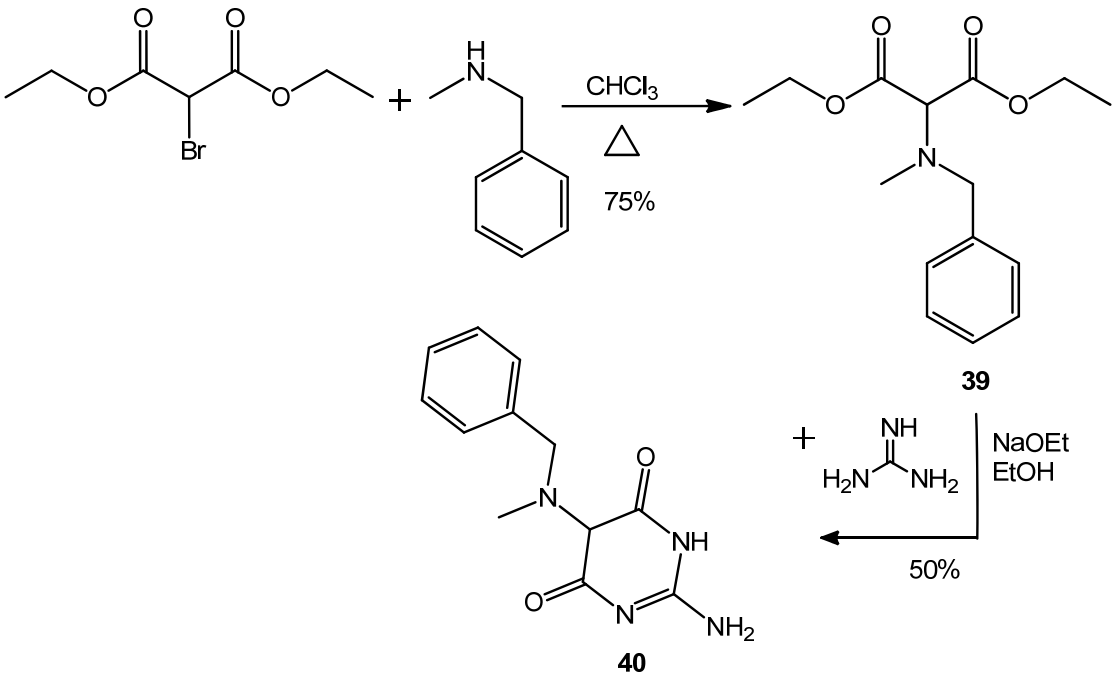
CHAPTER 3: Derivatives of the DHP core of TAN-1057 A/B: Synthesis and Binding of 2-methylamino-5-amino-4,6-dihydroxypyrimidine Derivatives

Design and Synthesis

In this work, a set of compounds were synthesized based on carbonyl derivatization at position 6 of the TAN 1057 A/B heterocyclic core. The purpose of this synthesis was to further investigate the potential of DHP derivatives as RNA-friendly molecules and expand our exploration of the heterocycle with respect to RNA binding ability. In particular, the design was aimed at synthesizing more stable, soluble analogs of TAN 1057 compared to those discussed in chapter 2.

Derivatization at position 6 of the TAN 1057 A/B heterocyclic core was proposed based on similar schemes found in literature starting from commercially available diethyl bromomalonate (Scheme 3.1)[1-3]. Substitution of the bromide with N-methylbenzylamine is carried out forming the benzyl malonate **39**, followed by a double condensation of the diethyl N-methylbenzylaminomalonate with guanidine to yield the dione heterocycle 2-methylbenzylamino-5-amino-4,6-dihydroxypyrimidine **40**. The possibility for further derivatization through the introduction of various N-Methyl and guanidinyl substituents suggested potential for rapid development of a library of compounds useful for SAR studies (Figure 3.1). In order to establish a synthetic route, three compounds were synthesized containing the dione core. These molecules were then tested for binding with the HCV IRES and

Thymidylate Synthase (TS) RNA using assays previously developed in our lab, which have been described in detail in Chapter 1.



Scheme 3.1: Design of dione derivatives of DHP

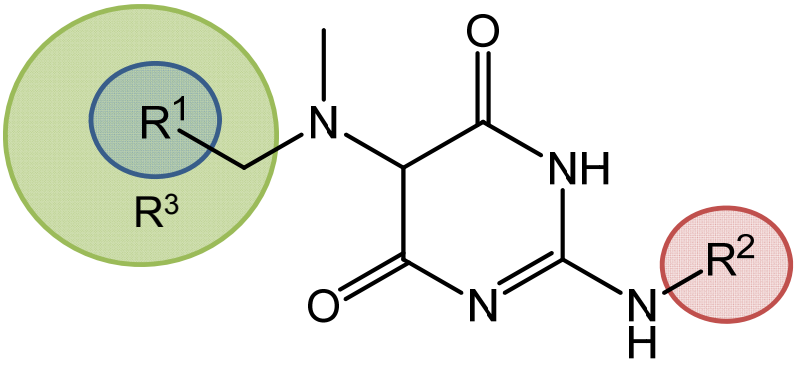
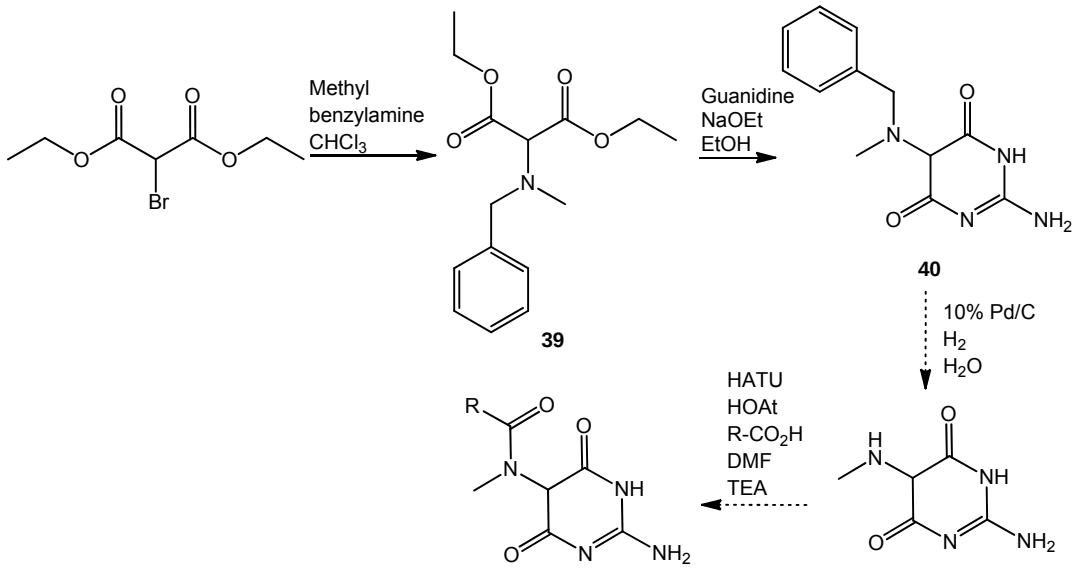


Figure 3.1: Areas of potential variation of the dione derivative of DHP.

Attempts at Further Derivatization

In an attempt to expand the possibilities for potential derivatives, another set of synthetic routes were attempted (Schemes 3.2, 3.3, 3.4). In these schemes, the benzyl group of scheme 3.1 could be removed pre- or post-cyclization to yield a free amine, which could then undergo peptide coupling with an array of carboxylic acids to further expand the library of compounds. However, all proposed synthetic routes were unsuccessful.

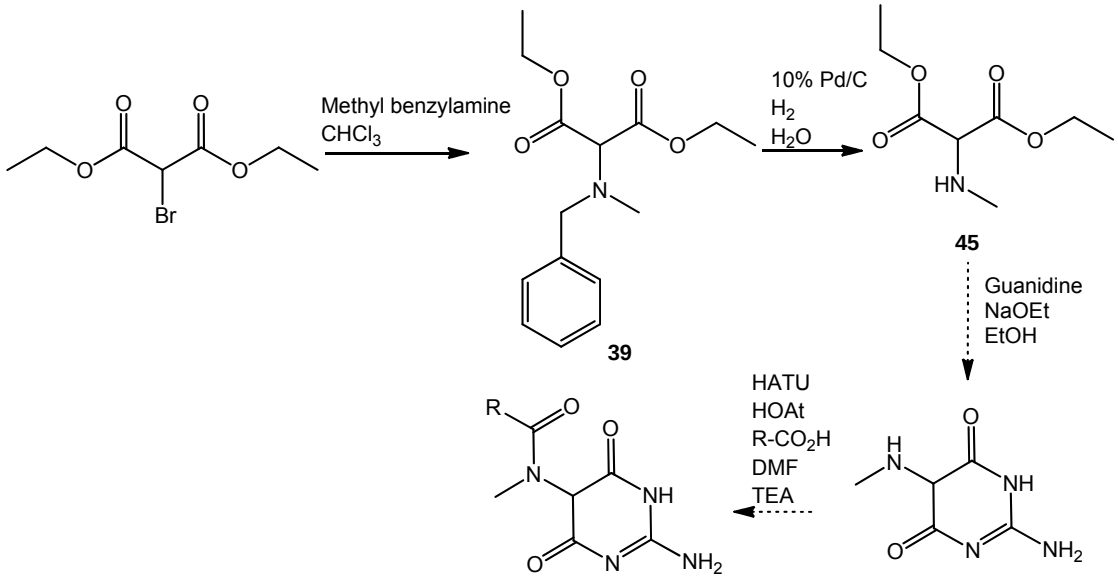
In scheme 3.2, removal of the benzyl group post-cyclization resulted in a loss of material. Tracking the reaction via TLC, the reaction appears to have been successful. However, the poor solubility of this dione heterocycle inhibits it from being filtered through celite for removal of the palladium catalyst, and the product is lost.



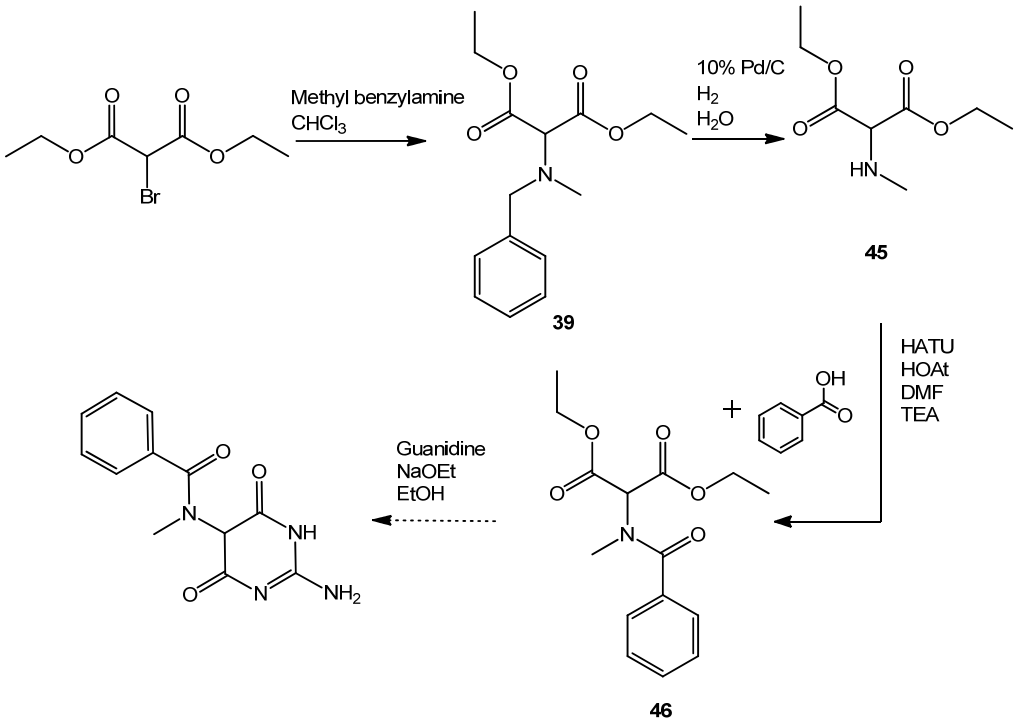
Scheme 3.2: Proposed post-cyclization route for additional dione heterocycle derivatives.

In scheme 3.3, removal of the benzyl group pre-cyclization resulted in diethyl-N-methylaminomalonate **45**. Unfortunately, condensation of **45** with guanidine proved unsuccessful. I propose this is due to a lack of reactivity caused by the removal of the bulky benzyl group, revealing the free N-methyl amine, and in turn affecting the electronics of the molecule. In base, the formation of an electrophilic imine can lead to the formation of a malonic ester instead of the desired dione product. As a means of solving this problem, a scheme involving the peptide coupling of the free amine to carboxylic acids before cyclization was attempted (Scheme 3.4). Benzoic acid was chosen as the carboxylic acid to provide a bulky substituent similar to the benzyl group successfully used in scheme 3.1. Peptide coupling of the malonate was

carried out using standard peptide coupling conditions to yield the benzoic acid malonate **46**. The condensation of **46** with guanidine was then performed. Even with multiple attempts and variations in the procedure including an increased concentration of base and the addition of heat, the cyclization failed. I propose the reason for this failure is the carbonyl group of the benzoic acid, with the potential to interfere through hydrogen bonding interactions or by interfering with the electronics of the malonate.



Scheme 3.3: Proposed pre-cyclization route for additional dione heterocycle derivatives.

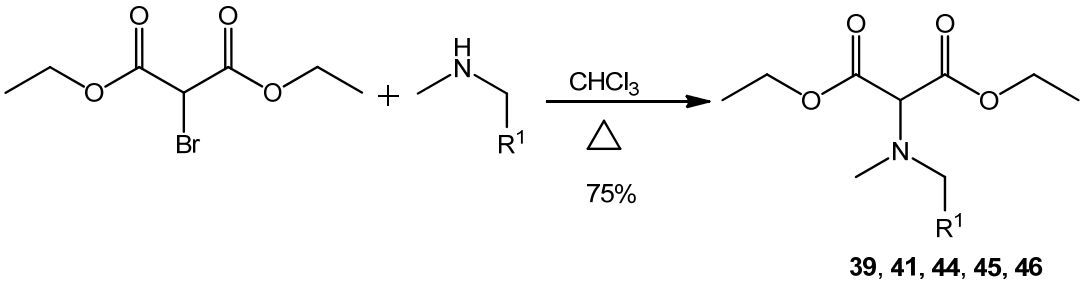


Scheme 3.4: Proposed pre-cyclization peptide coupling route to additional derivatives of the dione heterocycle.

Based on the results of schemes 3.2, 3.3, and 3.4, it was decided that this route of derivatization would be abandoned, and the focus of derivatization would be carried out via scheme 3.1.

Materials and Methods

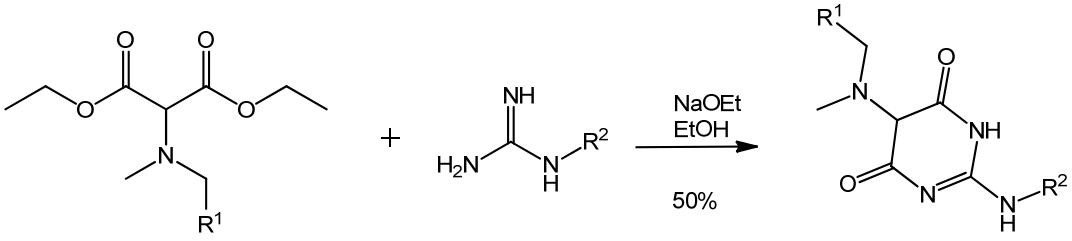
Compound Synthesis



39, **41**, **44**, **45**, **46**

Scheme 3.5: Substitution of diethyl bromo malonate to form diethyl N-methyl malonates

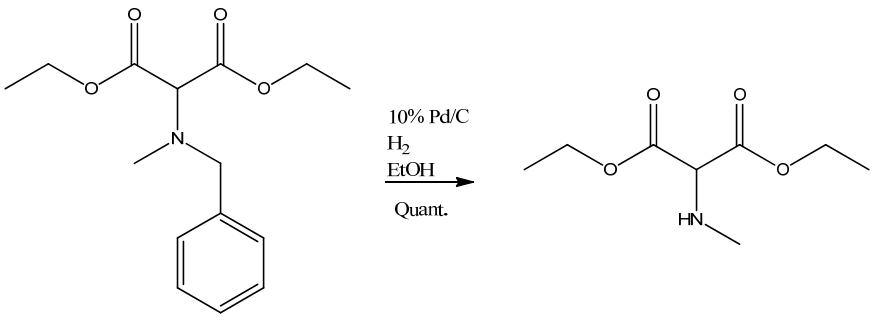
Chloroform was washed through a column of basic alumina (10g/14mL) to remove traces of water, acid, and ethanol. After washing, chloroform (14M) was added to a mixture of diethyl bromomalonate (1 eq.), and substituted N-methyl amine (2 eq.). The reaction was heated to reflux and followed by TLC. Upon completion, the reaction mixture is removed from heat and concentrated. The residue is then washed with ether. The ether filtrate is concentrated in vacuo to yield **39, 41, 44, 45, or 46** as an oil. In certain N-methyl amine substituents, the product needs to be washed with dichloromethane followed by concentration of the filtrate. The product is then purified from the unreacted N-methyl amine via column purification.



39, **41**, **44**, **45**, **46** **40**, **42**, **43**

Scheme 3.6: Dione core condensation

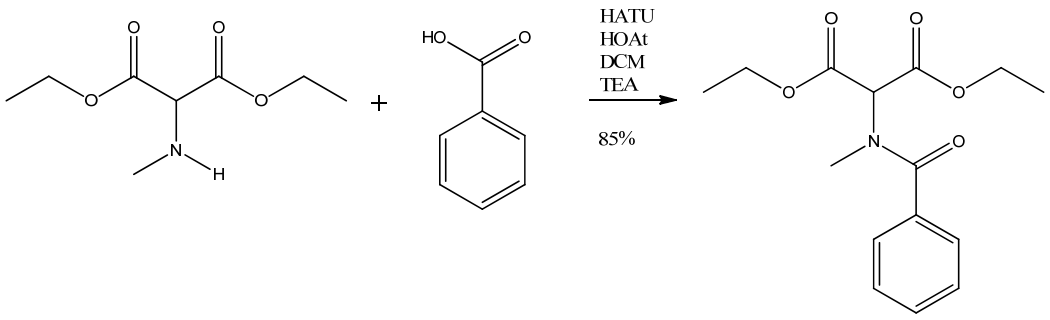
To a round bottom flask was added substituted diethyl N-methyl amino malonate (1 eq.), substituted guanidine (1.7 eq.), sodium ethoxide (4M as a 21% solution in ethanol), and ethanol (5.6M). The reaction was stirred at room temperature under argon overnight. The reaction was then concentrated in vacuum and the residue was taken up in water and washed with ethyl acetate. Upon extraction, a solid precipitate forms out of the aqueous layer. The solid product is filtered off to yield **40, 42, 43** as a solid.



39 **45**

Scheme 3.7: Benzyl deprotection

Compound **39** was dissolved in anhydrous ethanol (0.03M) and purged with argon. To this was added 10% Pd/C (0.5 eq.), and the reaction was purged again with argon. Next, the reaction was purged with hydrogen gas for 15 minutes, and was then left to stir at room temperature under a hydrogen balloon. The reaction was left to stir at room temperature under hydrogen overnight, and was monitored by TLC. Upon complete reaction of all starting material **39**, the reaction was purged with argon, filtered through celite to remove the catalyst, and was concentrated in vacuo to yield product **45** as an oil.



45 **46**

Scheme 3.8: Peptide coupling of diethyl N-methyl amino malonate

Compound **45** (1 eq.) was combined with benzoic acid (2.9 eq.), 1-Hydroxy-7-azabenzotriazole (HOAt) (2.9 eq.), 2-(1H-7-Azabenzotriazol-1-yl)--1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU) (2.9 eq.), and triethylamine (7 eq.) in dichloromethane (DCM) (0.07M). The reaction mixture was stirred under argon at room temperature, and was followed by TLC. Once compound **45** is no longer detected by TLC, the reaction was diluted with DCM. The sample was then washed with 1M

hydrochloric acid, saturated sodium bicarbonate, and saturated sodium chloride. The DCM layer was then dried under magnesium sulfate, filtered, and concentrated in vacuo to yield **46** as an oil.

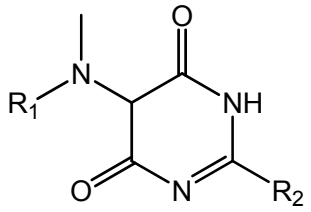
HCV and IVT Protocols

Protocols for measuring compound binding with TS and the HCV IRES were carried out as described in chapter 2 of this thesis.

Activity Testing

Overall, three model compounds synthesized to establish a synthetic route were tested in the FRET and IVT assays as described in chapter 2 of this thesis (Table 3.1).

Table 3.1: Structure Activity Relationship for all tested compounds in the FRET and IVT assay. n.a. stands for no activity. IVT results are shown at 100 uM compound.



Cmpd	R_1	R_2	FRET % decrease at 416.6 uM	IVT Relative Firefly Luc.	IVT Relative Renila Luc.	IVT Ren. Luc. /FF Luc.
40	benzyl	NH_2	n.a.	1.509	0.9792	0.6491
42	2-methoxybenzyl	NH_2	n.a.	1.0584	0.8991	0.8494
43	2-methoxybenzyl	Acetyl	n.a.	1.268	0.9363	0.7382

Results Testing Binding at the HCV IRES IIa Target

Data for compound **42** is shown below as a representative set of FRET data for the set of dione heterocycles (Figure 3.2). As observed in figure 3.2, a lack of change in eFRET provides evidence of the inability to change the angle of domain IIa of the HCV IRES. This inactivity was seen with all of the dione heterocycle compounds synthesized.

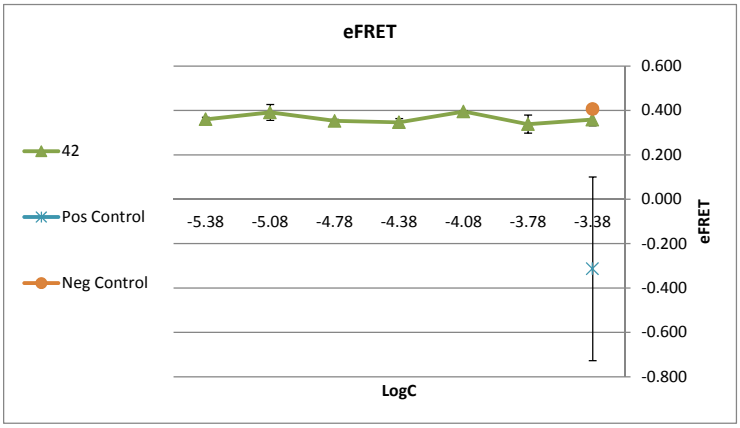


Figure 3.2: eFRET of representative compound **42**.

In case change was observed in eFRET, the CT, DE, and EE were plotted against concentration (Figures 3.3-3.5). This allows one to determine if a change in eFRET is in fact due to the compound interacting directly with the IIa angle of the HCV IRES resulting in a change in the distance between the FRET pair, or if the compound may be interfering with one of the dyes. If compound interference was observed, a change in signal would coincide with an increase or decrease in concentration. The lack of activity observed in eFRET was further verified by the lack of interference seen between the compound and the dyes.

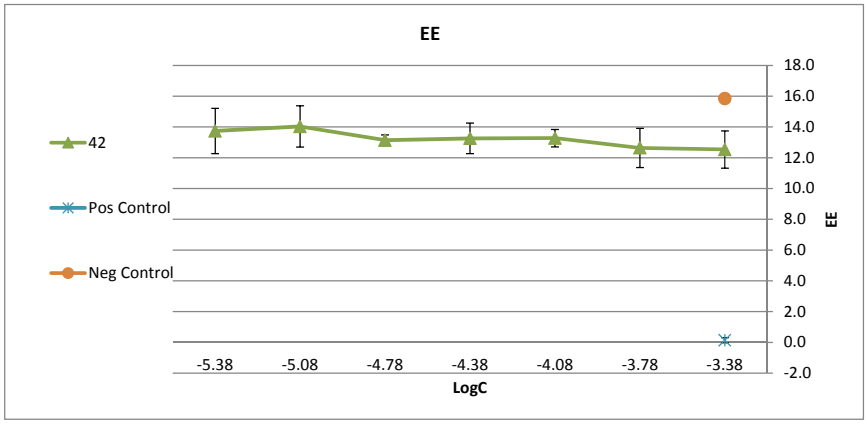


Figure 3.3: EE measurement for representative compound **42**.

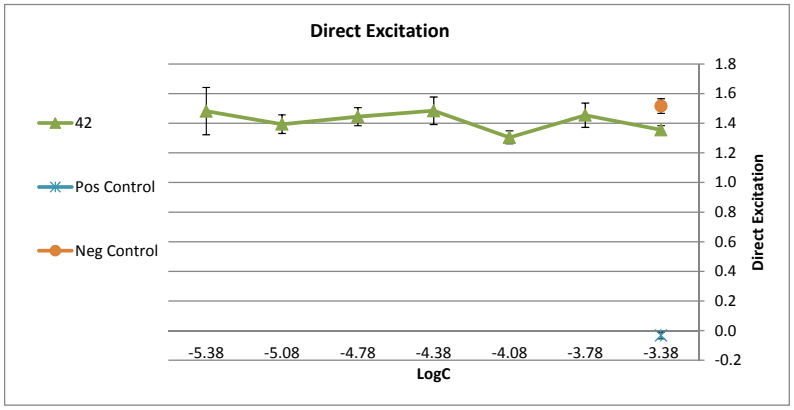


Figure 3.4: Direct excitation of compound **42** as a representative compound.

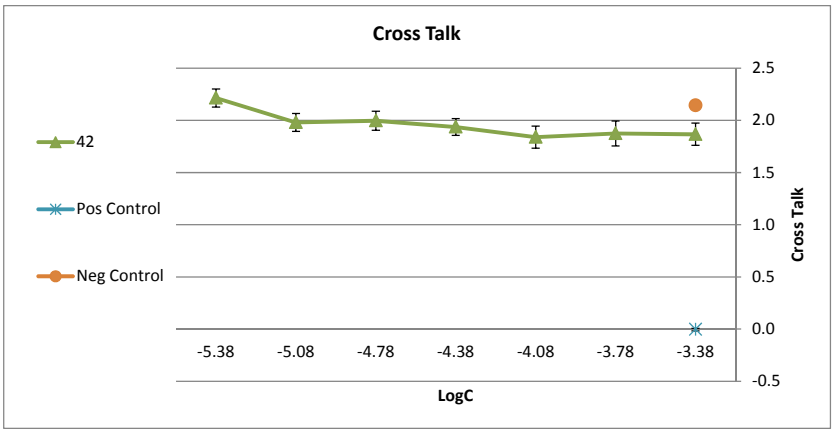


Figure 3.5: Cross talk of compound **42**.

Since none of the dione compounds show activity, no structure activity relationships (SAR) can be realized. Plans for further synthesis to expand this small library of compounds in an attempt to discover more about binding potential of TAN 1057 derivatives is discussed in the future projects section of this chapter.

Results of Translation Inhibition from the IVT Assay

For compounds tested in the IVT assay, the results are shown as normalized IRES/CAP ratios (Figures 3.6-3.7). As described in detail in chapter 2, in cases of active compounds, it is necessary to further evaluate where the effect on translation occurs by looking at the individual normalized firefly and renilla signals (Figures 3.8-3.9).

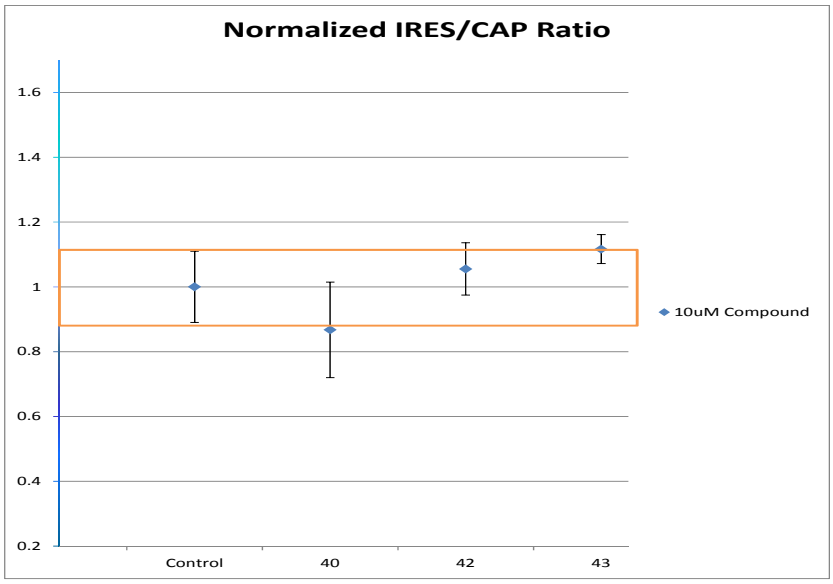


Figure 3.6: Normalized IRES/CAP Ratio for compounds **40-43** at 10uM compound, where the orange box signifies 3σ standard deviation of the control.

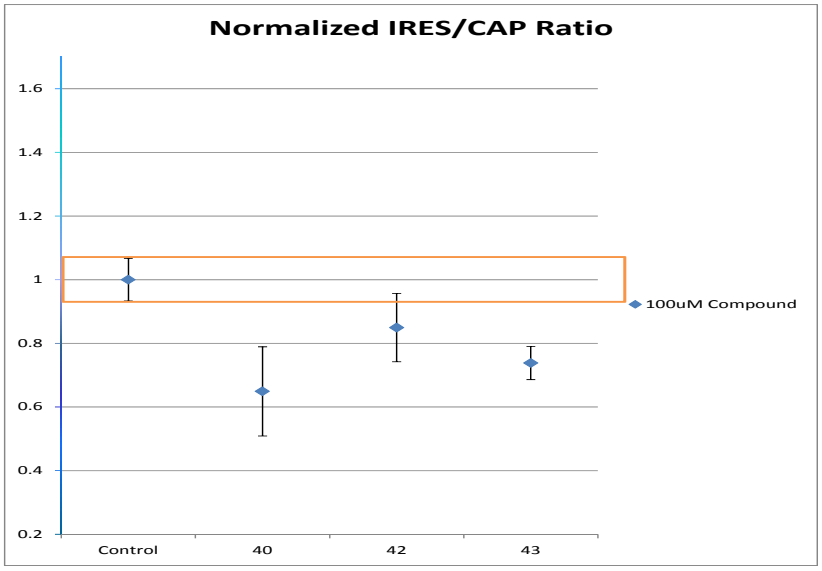


Figure 3.7: Normalized IRES/CAP Ratio for compounds **40-43** at 100uM compound, where the orange box signifies 3σ standard deviation of the control.

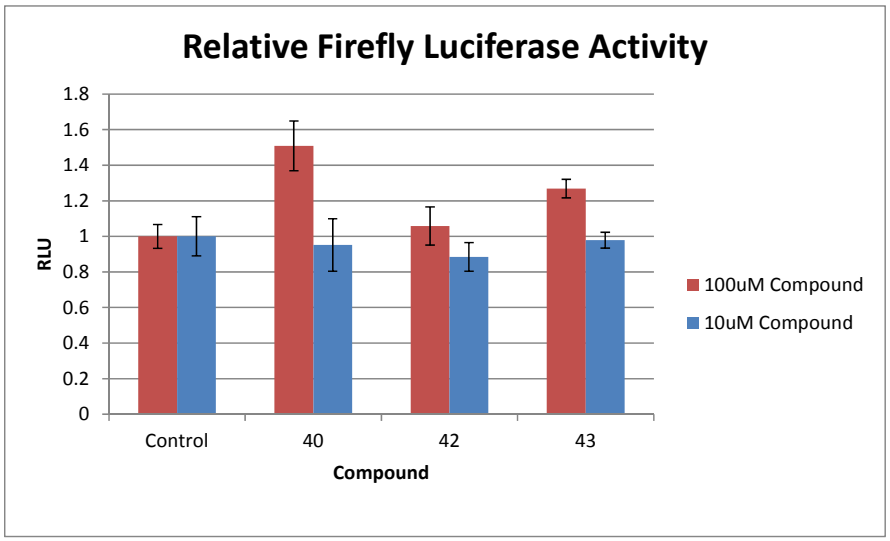


Figure 3.8: Relative Firefly Luciferase Activity of Compounds **40**-**43**.

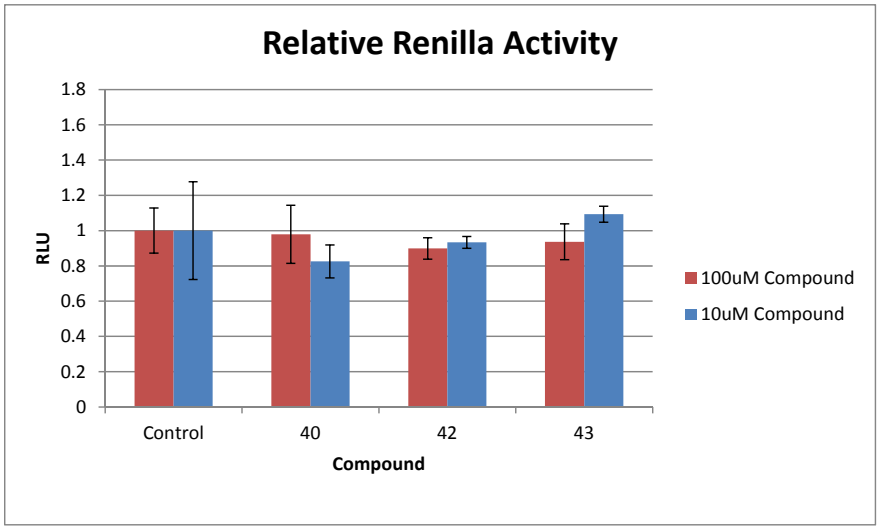


Figure 3.9: Relative Renilla Luciferase Activity of Compounds **40**-**43**.

The results of translation inhibition from the IVT assay show two interesting compounds. Compound **43**, 2-methyl-(2-methoxy-benzylamino)-5-

acetyl-4,6-dihydroxypyrimidine at 100 uM is above 3σ of the control. Upon further investigation, we see the decrease compared to the control is caused by an increase in firefly signal caused by an effect on the TS. This indicates a potential interaction with TS in which the hairpin is destabilized resulting in an increase in translation. However, when looking at renilla signal, we see that the compound may also have an effect on the IRES. In order to rule this out, retesting is necessary. Compound **40**, 2-methylbenzylamino-5-amino-4,6-dihydroxypyrimidine, is by far the most interesting of the compounds in my small collection of compounds, from both chapter 2 and 3. This compound shows a large increase in firefly activity, indicating the compounds interactions with TS causing an increase in translation. Although our focus was to find inhibitors of translation, it is interesting that this small molecule must be destabilizing the hairpin in order to make the start codon more accessible, therefore resulting in an increase in translation.

Future Directions

While the RNA-binding activity of the three model dione compounds is discouraging in the HCV FRET assay it shows promise in regards to TS translation, and therefore I feel further steps should be taken in this project. Modeling studies of the natural DHP core illustrates a potential to bind with RNA, and through expansion of a derivative library, such an RNA-binding molecule may be revealed. Future derivatives should be designed to include moieties often found in known RNA-binding small molecules such as ethyl

dimethyl amine side chains, positively charged amines, and additional phenyl rings. Based on the IVT results, retesting of compound **43**, and further testing and possibly co-crystalization of the TS hairpin with compound **40** may provide us with further important structural information.

In addition to looking at this library of compounds as RNA binders against the HCV IRES and TS, this derivative set of compounds should be analyzed as potential antibacterials. Much has been published examining derivatives of TAN 1057 A/B as antibiotics. However in all of my searches I have not found any analogs containing this change at position 6 of the heterocycle. For this reason, I would like to examine the potential of these derivatives as potential antibiotics, and compare the activity to both the natural TAN 1057 A/B and previously reported analogs.

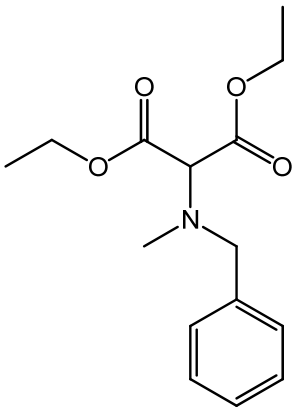
Conclusions

This chapter discussed compounds synthesized as potential RNA-binding molecules. Three compounds, all containing a change at position 6 of the original DHP core from a methyl to a carboxyl group, were synthesized in an attempt to establish a synthetic route providing the opportunity for rapid development of a small library of TAN 1057 A/B derivatives. Although none of the three model compounds show binding activity against the HCV IRES, two showed potential for affecting translation in TS. Further testing of these potentially active compounds as well as the synthesis and testing of additional

derivatives, particularly those which expand the exploration of guanidine substituents, may provide further information towards yielding biologically relevant RNA-binding molecules.

Spectral Data

Compound **39** (ES-JM-73)



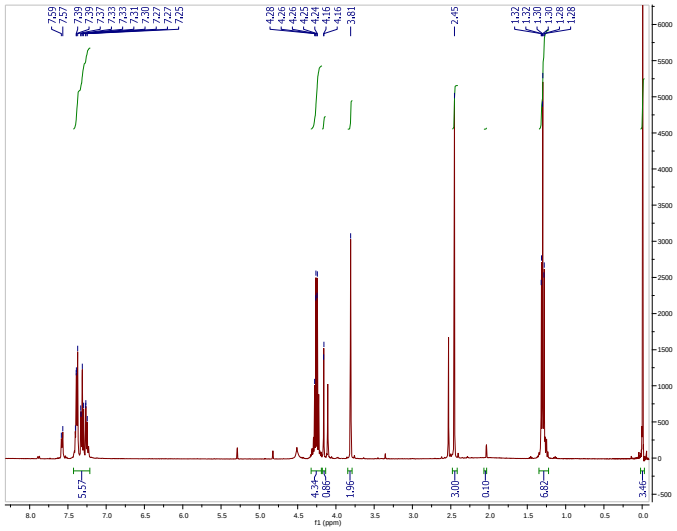
Chemical Formula: $C_{15}H_{21}NO_4$

Exact Mass: 279.15. Found $[M+H]^+$ 280.11 (ESI Positive Ion Mode).

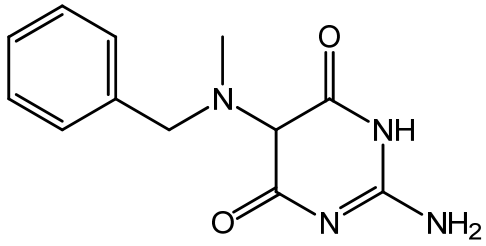
NMR Assignments

^{1}H NMR: δ 7.39-7.25 (m, 5H), 4.28-4.24 (m, 4H), 4.16 (s, 1H), 3.81 (s, 2H), 2.45 (s, 3H), 1.32-1.28 (m, 6H).

Spectrum 3.1: Compound **39** (ES-JM-75) ^{1}H NMR, $CDCl_3$, 400 MHz



Compound **40** (ES-291)



Chemical Formula: $C_{12}H_{14}N_4O_2$.

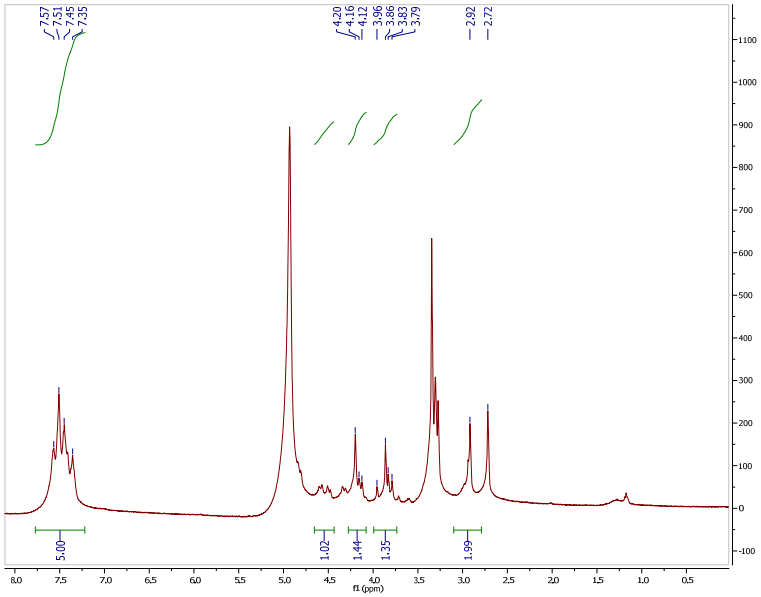
Exact Mass: 246.11. Found $[M+H]^+$ 247.1190, Delta ppm = -0.19 (HR-ESI-TOFMS).

NMR Assignment

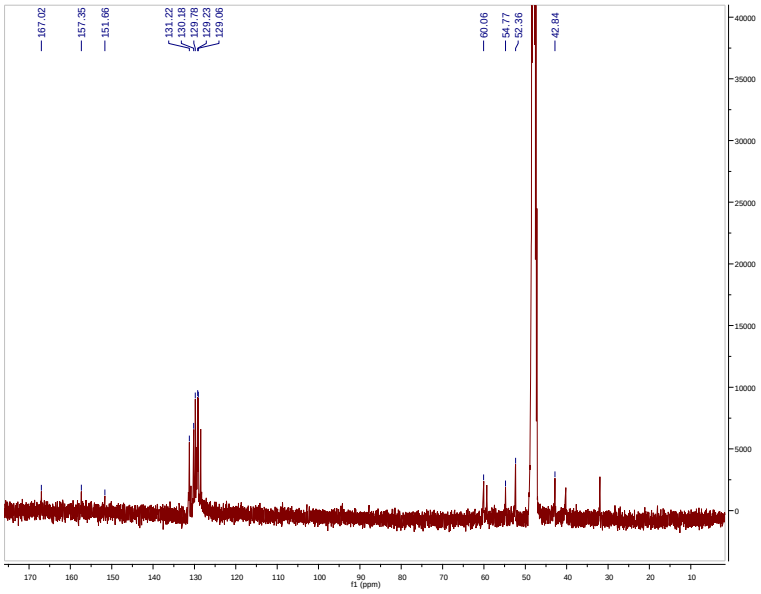
^{1}H NMR: δ 7.57-7.35 (m, 5H), 4.65-4.57 (J=11.73Hz, d, 1H), 4.20-4.12 (m, 1H), 3.96-3.79 (m, 1H), 2.92 (s, 3H).

^{13}C NMR: δ 167.02, 157.35, 151.66, 131.22, 130.18, 129.78, 129.23, 129.06, 60.06, 54.77, 52.36, 42.84.

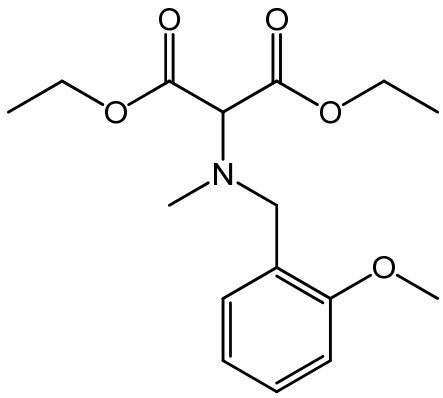
Spectrum 3.2: Compound **40** (ES-JM-91) ^{1}H NMR, CD_3OD, 400 MHz



Spectrum 3.3: Compound **40** (ES-JM-91) ^{13}C NMR, CD_3OD, 400 MHz



Compound **41** (ES-283)



Chemical Formula: $C_{16}H_{23}NO_5$

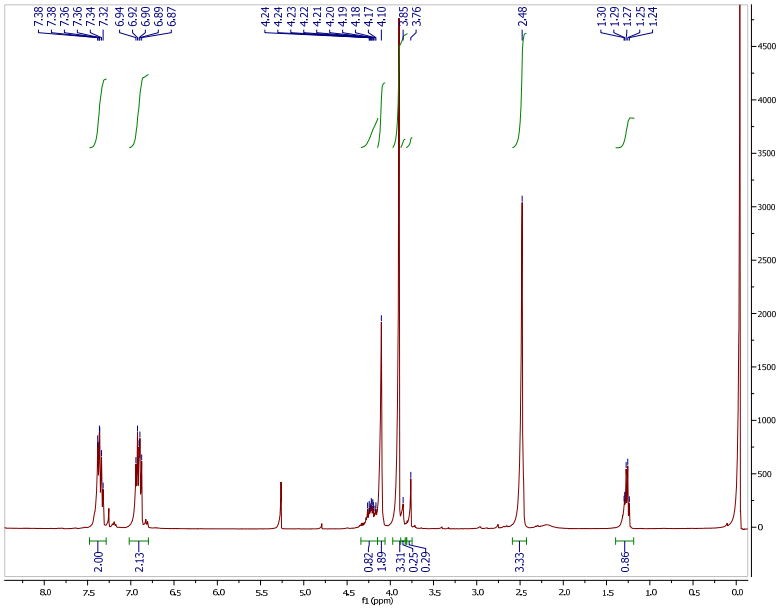
Exact Mass: 309.16. Found $[M+H]^+$ 310.18 (ESI Positive Ion Mode).

NMR Assignments

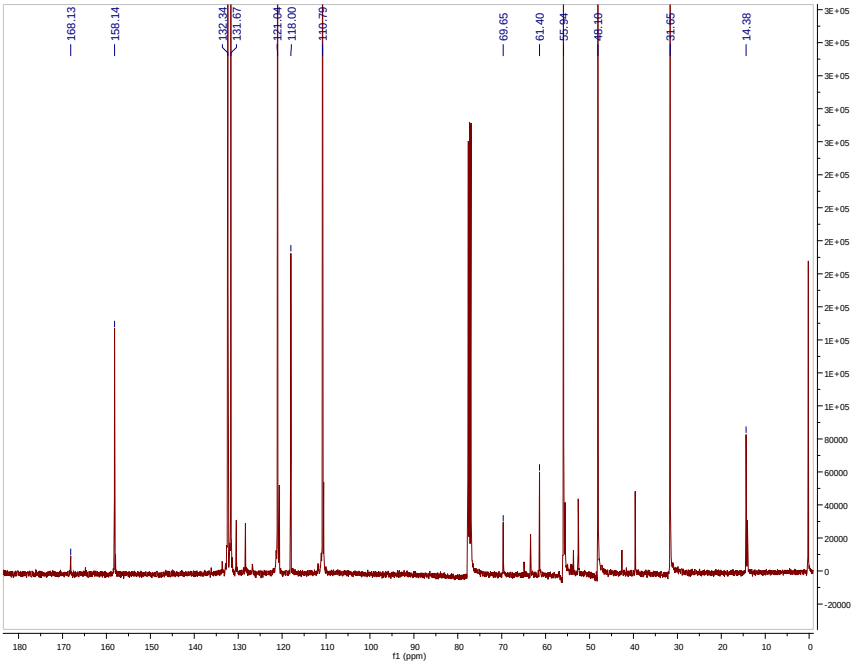
^{1}H NMR: δ 7.38-7.32 (m, 2H), 6.94-6.87 (m, 2H), 4.26-4.17 (m, 4H), 4.10 (s, 2H), 3.90 (s, 3H), 3.76 (s, 1H), 2.48 (s, 3H), 1.23-1.25 (m, 6H).

^{13}C NMR: δ 168.13, 158.14, 132.34, 131.67, 121.04, 118.00, 110.79, 69.65, 61.40, 55.94, 48.10, 31.65, 14.38.

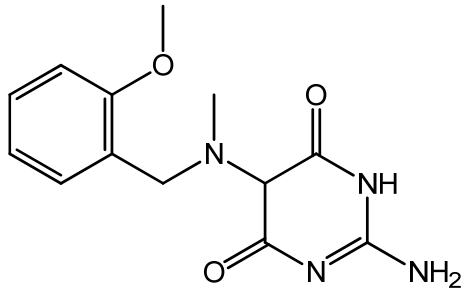
Spectrum 3.4: Compound **41** (ES-283) ^{1}H NMR, CD_3OD, 400 MHz



Spectrum 3.5: Compound **41** (ES-283) ^{13}C NMR, CD_3OD, 400 MHz



Compound **42** (ES-290-S)



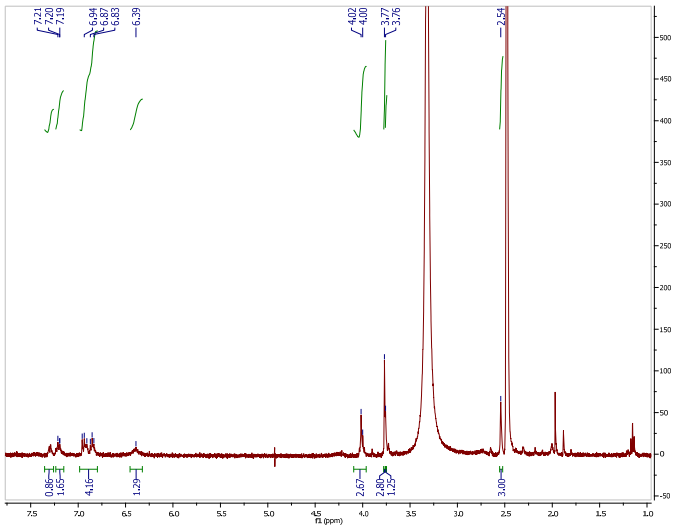
Chemical Formula: $C_{13}H_{16}N_4O_3$

Exact Mass: 276.12. Found $[M+H]^+$ 277.1295, Delta ppm = -0.4 (HR-ESI-TOFMS)

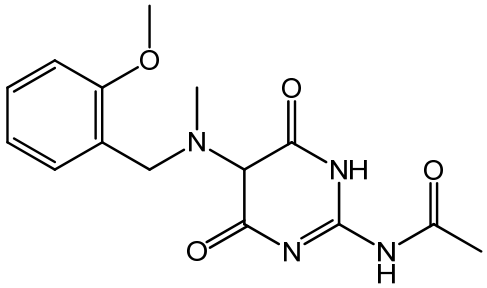
NMR Assignments

^{1}H NMR: δ 7.21-7.19 (m, 1H), 6.94-6.83 (m, 3H), 4.02-4.00 (m, 2H), 3.77 (s, 3H), 3.76 (s, 1H) 2.54 (s, 3H).

Spectrum 3.6: Compound **42** (ES-290-S) ^{1}H NMR, DMSO, 400 MHz



Compound **43** (ES-290-SW)



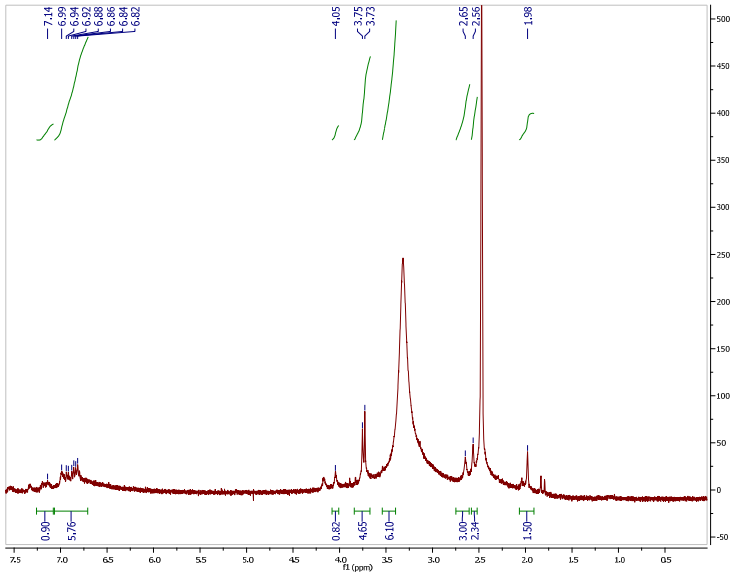
Chemical Formula: $C_{15}H_{18}N_4O_4$

Exact Mass: 318.13. Found $[M+H]^+$ 319.1401, Delta ppm = 0.0 (HR-ESI-TOFMS).

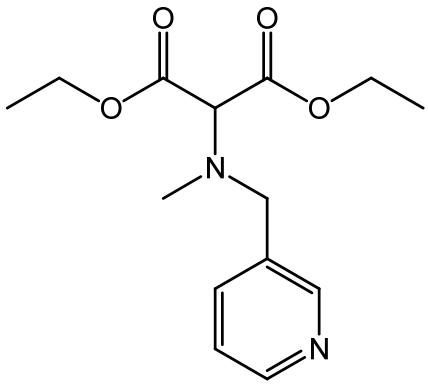
NMR Assignments

^{1}H NMR: δ 7.25-7.10 (m, 1H), 6.99-6.82 (m, 3H), 4.05 (s, 1H), 3.75-3.73 (J= 10.55Hz, d, 2H), under solvent (water) peak (s, 3H), 2.65 (s, 3H), 1.98 (s, 3H).

Spectrum 3.7: Compound **43** (ES-081B) ^{1}H NMR, DMSO, 400 MHz



Compound **44** (ES-284)



Chemical Formula: $C_{14}H_{20}N_2O_4$

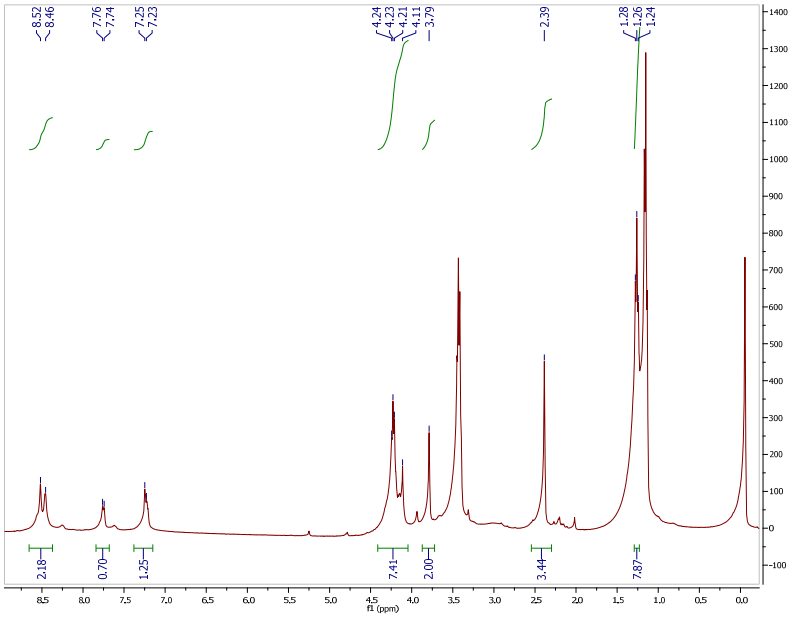
Exact Mass: 280.14 Found $[M+H]^+$ 281.12 (ESI Positive Ion Mode).

NMR Assignments

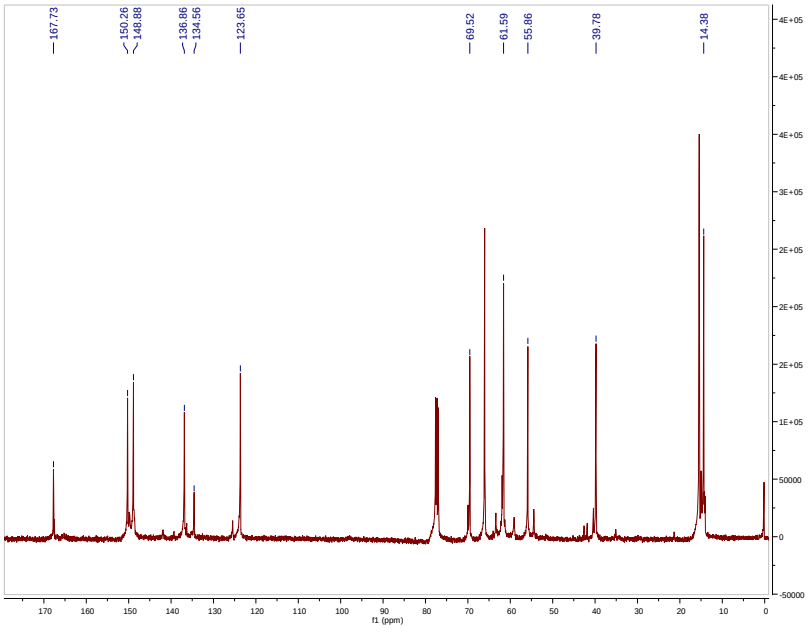
^{1}H NMR: δ 8.52-8.46 (m, 2H), 7.75 (J=6.15Hz, d, 1H), 7.25-7.23 (m, 1H), 4.24-4.21 (m, 4H), 4.11 (s, 1H), 3.79 (s, 2H), 2.39 (s, 3H), 1.28-1.24 (m, 6H).

^{13}C NMR: δ 167.73, 150.26, 148.88, 136.86, 134.56, 123.65, 69.52, 61.59, 55.86, 39.78, 14.38.

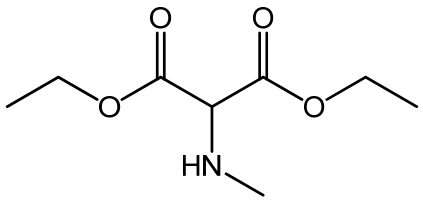
Spectrum 3.8: Compound **44** (ES-284) ^{1}H NMR, $CDCl_3$, 400 MHz



Spectrum 3.9: Compound **44** (ES-284) ^{13}C NMR, $CDCl_3$, 400 MHz



Compound **45** (ES-MA-99)



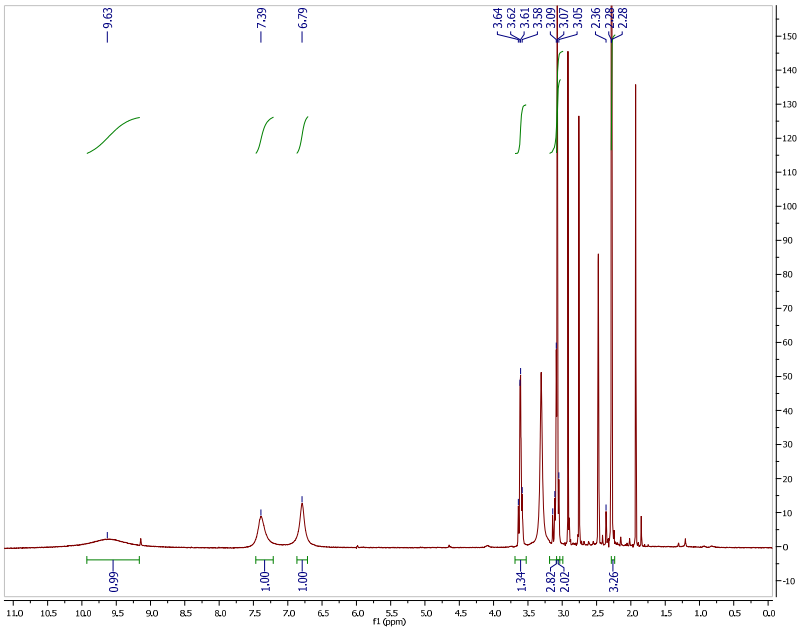
Chemical Formula: $C_8H_{15}NO_4$

Exact Mass: 189.10. Found $[M+H]^+$ 190.01 (ESI Positive Ion Mode).

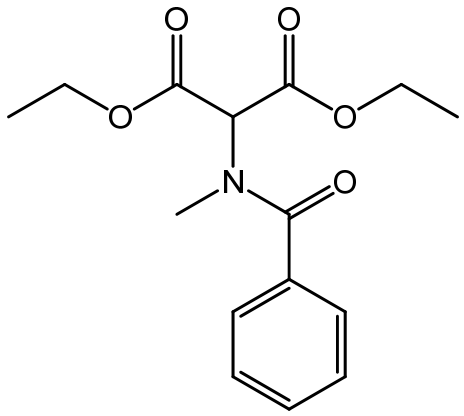
NMR Assignments

^{1}H NMR: δ 4.35-4.25 (m, 4H), 2.99 (s, 3H), 2.68 (s, 1H), 1.34-1.29 (m, 6H).

Spectrum 3.10: Compound **45** (ES-MA-99) ^{1}H NMR, $CDCl_3$, 400 MHz



Compound **46** (ES-277)



Chemical Formula: $C_{15}H_{19}NO_5$

Exact Mass: 293.13. Found $[M+H]^+$ 294.11 (ESI Positive Ion Mode).

References

(1) Legraverend, M.; Boumchita, H.; Bisagni, E. *Synthesis-Stuttgart* **1990**, 587.

(2) Baruah, P. K.; Gonnade, R.; Phalgune, U. D.; Sanjayan, G. J. *The Journal of Organic Chemistry* **2005**, *70*, 6461.

(3) Kampe, K. D. *Annalen Der Chemie-Justus Liebig* **1974**, 593.

CHAPTER 4: PharmaChemistry in the Classroom: A Drug Discovery Experiment for the High School Chemistry or Biotechnology Classroom

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Abstract:

In this laboratory experiment, students learn about the processes by which drugs are discovered and designed, and are then provided an opportunity to simulate those processes. The students first perform a synthesis of oil of wintergreen from aspirin, and analyze the physical properties of both the starting material (aspirin) and product (oil of wintergreen) to validate their reaction was successful. Unique to this lab experiment, students further scrutinize the synthesis by performing a biological assay investigating the antibacterial properties of their samples. Through the use of these analyses, students are able to play the roles of chemists, biologists, and team members involved in the drug discovery pipeline. The results reveal to the students that small changes in molecular structure may result in significant changes in medicinal activity. Through data analysis, discussion, and problem sets, students evaluate various steps of the experiment as they simulate the drug discovery process.

Introduction

Incorporating a theme of drug discovery within a high school science curriculum provides opportunities for relevant experimentation and the potential to enhance student learning. It has been shown that including topics in the classroom which encourage students to make connections between their lives and their science course content increases student interest.[1] In addition, topics interesting to students result in increased motivation and academic performance.[1,2] With this in mind, we sought to exploit the relevance and student interest of the medical field.[3] As a result, we developed an experiment incorporating various aspects of the drug discovery process in high school chemistry or biotechnology classrooms.

The discovery of new medicines is a complex process. First, scientists must recognize a medical problem and identify a target within the body, which if hit, can lead to a treatment or cure. Chemists then synthesize and characterize molecules designed to potentially hit the biological target. Small changes or additions to these molecules may cause a large change in their therapeutic effectiveness. For this reason, an average of ten thousand new molecules are developed before an ideal drug candidate is found.[4] Each newly made molecule must then be tested in biological assays, animal testing, and human testing to ensure that the molecule is safe for use, and that the desired effect is produced.

In the activity presented here, students execute various roles of scientists within the drug discovery pipeline: identifying a medical problem, performing a chemical synthesis to make a new molecule, characterization of the new molecule, and running a biological assay comparing their starting material to their product (Figure 1). More specifically, this set of experiments introduces disease-causing bacteria as a medical problem, and involves the search for a "new" antibacterial agent. Students are guided through a chemical synthesis where they transform aspirin into methyl salicylate, more commonly known as oil of wintergreen. Unlike previously published articles on the synthesis of oil of wintergreen from aspirin,[5,6] this experiment further investigates the starting material and product. Based on observations of physical properties before and after the synthesis, students see evidence that their reaction was successful and that their molecule has changed (solid to oil, bitter smell to minty smell, white color to colorless). The students then grow bacteria in the presence of the two different molecules, and observe the effect each molecule has on bacterial survival by measuring the zone of inhibition.[7] Following the activity, students convene to discuss their results and talk about the importance of those results in light of the drug discovery process.

When developing this experiment, the authors specifically chose the synthesis of oil of wintergreen from aspirin because of its relatively straightforward nature including its adaptability to the time, equipment, and chemical constraints of the high school classroom. Additionally, the drastic physical property changes observed provides confidence in the success of the

reaction. When implementing in the classroom, students are only given the chemical name of the product, methylsalicylate. Therefore, upon observation of smell, students recognize the familiar wintergreen scent further contributing to the relevance of the experiment to the students' lives. While oil of wintergreen is popularly known as a flavoring agent in gum, mints, and soft drinks, this experiment allows the students to "discover" the antibacterial properties of oil of wintergreen, and evaluation of its medicinal use in mouthwashes and topical analgesics is discussed.

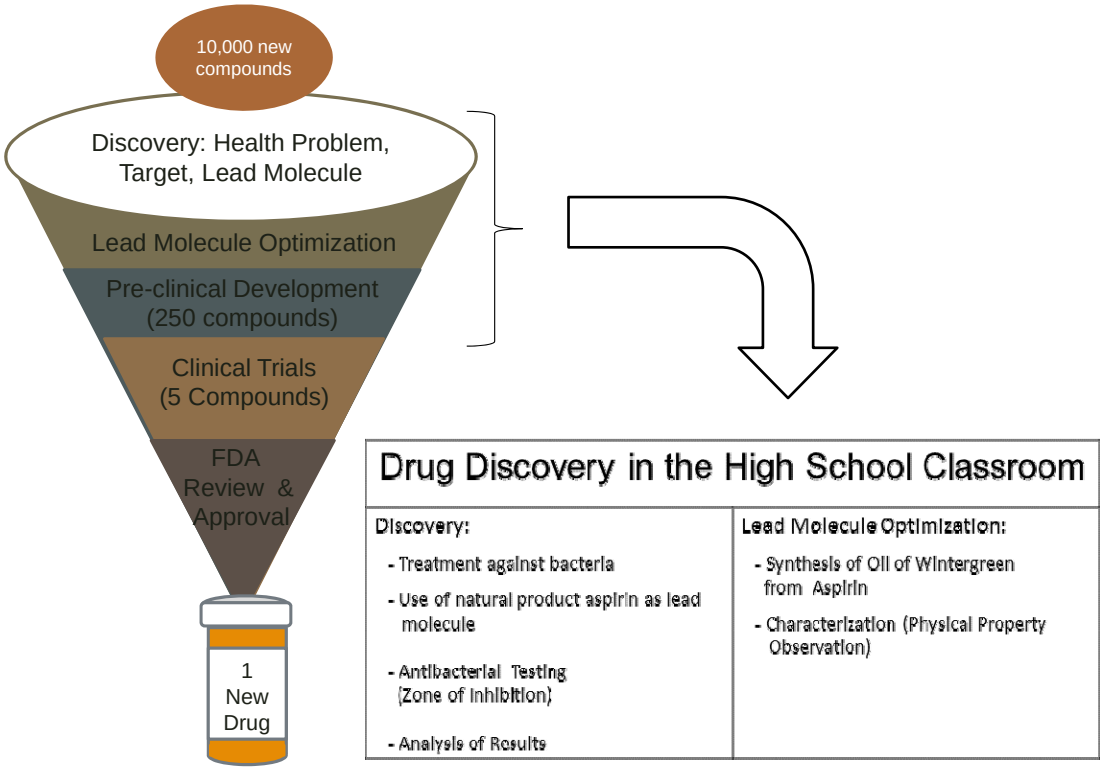


Figure 4.1: The drug discovery process can be outlined in 5 main steps: Discovery, Lead Molecule Optimization, Pre-Clinical Trials, Clinical Trials, and FDA Review and Approval. In the lab experiment described herein, two of these steps have been adapted for the high school classroom.

Experimental Section

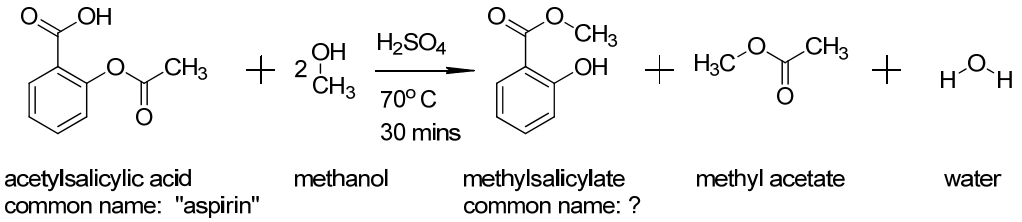
Materials

Methanol, sulfuric acid, salicylic acid, and ethanol were purchased from Sigma-Aldrich. Filter paper, large (90 cm diameter) petri dishes, agar, and well plates purchased from Flynn Scientific. Aspirin (Regular Strength 325mg tablets, non coated, non buffered), cotton swabs, and bleach were purchased from a local grocery store. Gloves were purchased from the local hardware store. Bacteria stab used for making pre-plated lawns was provided by ScienceBridge.[8] Paper discs were made from standard filter paper using a ½" hole cutter purchased from an online retailer. Sterile water purified using a mega-pure water purification system from Flynn Scientific. The experiments require a fume hood, water bath, and incubator.

Synthesis of Oil of Wintergreen from Aspirin Tablets

Students are provided with two tablets of 325mg regular strength aspirin. After grinding up the tablets using a mortar and pestle, students analyze the physical properties of the powder to be compared with the product in order to verify a successful reaction. Next, students dissolve the active ingredient, acetylsalicylic acid, in methanol and filter off the inactive ingredients. In the fume hood, students add two drops of the catalyst, concentrated sulfuric acid, to the test tube containing their sample, and place it in a water bath set to 70 °C. After 30 minutes, the transesterification-Fisher

esterification reaction will be complete and the sample can be removed from the water bath (Scheme 1). In order to fit into the time constraints, equipment availability, and approved chemical list of the school district, the lab was adapted accordingly from previously published procedures.[9]



Scheme 4.1: Reaction scheme of synthesis of methylsalicylate from acetylsalicylic acid.

Antibacterial Testing

Students are provided with agar plates, pre-plated colonies to create their own bacterial lawn, and solutions of aspirin and salicylic acid for preparation of paper discs for antibacterial testing. Necessary items are prepared ahead of time accordingly.[10] Students prepare their samples by placing 2-3 drops of prepared solutions onto appropriate paper discs.[11] While allowing the discs to dry, students create a lawn of bacteria on large agar plates using a cotton swab and spreading a colony of pre-grown bacteria onto their sample plate. After allowing five minutes for paper discs to dry, students place paper discs onto appropriately labeled quadrants and place in an incubator set to 37° C overnight. On day two, students take the agar plates

out of the incubator and use a ruler to measure the zone of inhibition of samples from paper discs.

Hazards

Sulfuric acid is corrosive and must be handled with care. Methanol is extremely flammable and toxic by inhalation, ingestion, or absorption through the skin. Therefore, gloves should be used. Acetylsalicylic acid and methylsalicylate are irritants. Bacteria strands used should be mild, and students should be instructed to wash hands thoroughly after handling. Heating of methanol results in evaporation and should be carried out in a fume hood. All procedures should be carried out wearing safety glasses.

Results and Discussion

Comparing Physical Properties between Starting Material and Product

The reaction of aspirin (acetylsalicylic acid) to form oil of wintergreen (methylsalicylate) is a convenient synthesis utilizing relatively common chemical reagents, heat, and a sulfuric acid catalyst. By analyzing the physical properties before and after the reaction, students are able to observe notable physical changes between starting material and product (Table 1). From class discussions and pre- and post-lab questions, students study the synthesis and are able to observe that small changes in a molecule can lead

to substantial differences in physical nature. As students will observe, acetylsalicylic acid is a white solid exhibiting a bitter odor; however methylsalicylate is a colorless oil with a minty odor, indicating a successful synthesis. The synthetic procedure allows students to play the role of a medicinal chemist in the search for new potential drugs.

Table 4.1: Sample data table of physical properties before and after chemical synthesis. The example of smell as a physical property is provided for them, and they are responsible to fill in the others.

Physical Property to Observe	Observations of Starting Material (acetylsalicylic acid/aspirin) **before Synthesis**	Observations of Product (methyl salicylate) **after Synthesis**
Example: Smell	Bitter	Minty
Color	White	Colorless
State	Solid	Liquid

Testing Oil of Wintergreen for Antibacterial Activity

The biological assay is performed by plating the starting material (aspirin), the product (oil of wintergreen), a positive control (salicylic acid, a known antibacterial substance), and a negative control (evaporated ethanol exhibiting no antibacterial effect) to show a comparison between antibacterial and non-antibacterial substances.[8] After incubation, the students are able to observe whether a substance is antibacterial or not through the absence or presence of a zone of inhibition, or a clear area where bacterial growth is inhibited around the impregnated paper discs.[4] Aspirin is not antibacterial,

and will therefore exhibit bacterial growth up to the aspirin-soaked paper disc. Oil of wintergreen however, is antibacterial, and will exhibit a clear ring around the paper disc soaked with the compound. The diameter of the zone of inhibition can be compared to known antibiotics and will be proportional to the strength of antibacterial activity. In comparison with the positive and negative controls, students observe that oil of wintergreen kills bacteria, while aspirin does not (Figure 2). Students also compare data between groups to come to the conclusion they have created a new molecule with different medicinal properties. In completing their observations and participating in useful discussions, students come full circle in mirroring the processes used in drug discovery. Through further discussion, students are directed to analyze the active ingredients from the labels of hygienic products such as Listerine® and Bengay® and are able to observe and discuss standard uses for methylsalicylate (Figure 3).[12]

Figure 4.2: A sample student plate illustrates the antibacterial effects of evaporated ethanol (upper left quadrant), salicylic acid (upper right quadrant),acetylsalicylic acid starting material (lower right), and methylsalicylate product (lower left)

Active Ingredients	Purpose
Eucalyptol 0.092%	Antiplaque/antigingivitis
Menthol 0.042%	Antiplaque/antigingivitis
Methyl salicylate 0.060%	Antiplaque/antigingivitis
Thymol 0.064%	Antiplaque/antigingivitis

Figure 4.3: Students discuss the use of methylsalicylate (oil of wintergreen) in common products used. One example used is looking at the active ingredients of Listerine.13

Conclusions

The integration of medicinal chemistry in the high school classroom can be accomplished by taking students through various steps of the drug discovery process. In this way, students are exposed to a more real-world approach to science and to the integration of chemistry and biology so commonly found in many aspects of our lives. Through use of a topic relevant to students' lives, we are able to promote interest and consequently improve student performance and achievement.[1,14]

We have found students to be very receptive to this laboratory. Following the experiments, students were asked to fill out a survey in order to better gauge student interest and understanding. Overall, sixty-seven percent of the students found the activity to be exciting or 'cool'.[15] Students provided

an average ranking of 3.65 out of 5.0 for the ability of the activity to keep them engaged, providing further evidence of student interest. Inquiring about general understanding, students were asked what they thought they were supposed to learn from this science activity, and eighty-eight percent of students gave correct, relevant responses. The results of the survey help to reflect the influence of interest in overall student understanding. Unfortunately, more data is required in order to say with confidence that a positive correlation is found between student interest and understanding in this particular activity. However it has been shown that topics maintaining high levels of student interest, as observed here, consequently result in an increase in student achievement and future recall.[16]

Associated Content

A curriculum packet complete with teacher implementation guide, powerpoint, and student handouts is available in the appendix.

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Our research was funded by the Socrates program at UCSD, a GK-12 grant (NSF 0742551). Special thanks to PI, Maarten Chrispeels, and the Socrates 2010-2011 cohort and staff, especially Shelley Glenn Lee and Johnnie Lyman for their support and discussion throughout the development of the activity.

Chapter 4 is currently being prepared for submission for publication, and the dissertation author was the primary author. The co-author is Jewyl Clarke.

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(9) Instead of refluxing, the reaction was performed in an open test tube heated at 70ºC in the fume hood allowing for the reaction and evaporation of excess methanol. By performing the reaction just above the boiling point, the methanol maintains a gentle boil instead of violently bumping, avoiding a potentially hazardous situation. We have found that after 30 minutes under these conditions, the methanol is evaporated and the reaction is completed (MS, H NMR analysis). Following heating, students analyze the physical properties of the oil of wintergreen product and compare against the physical properties of their starting material, acetylsalicylic acid. In regards to purification, previous procedures perform an extraction involving dichloromethane. Unfortunately, the use of dichloromethane within the classrooms of our school district is prohibited. Our attempts to purify the oil of wintergreen while remaining respectful of classroom time and safety restrictions, were unsuccessful. However, a comparison of appropriately diluted pure oil of wintergreen with the impure product yielded comparable results. While we recognize that there may be other factors affecting the antibacterial nature of our impure product, this serves to provide a good general example within our means, and also leads to productive scientific discussion of results and error.

(10) Detailed instructions on preparing bacteria colonies and chemical solutions can be found in the appendix within the curriculum packet.

(11) The product is diluted after the reaction is complete, before antibacterial testing is performed

(12) To promote further student discussion and for more information, see powerpoint slides provided in the supplemental information.

(13) Information and image obtained from http://www.listerine.com/products/product-cool-mint

(14) Schwartz-Boom, R. D.; Halpin, M. J.; Reiter, J. P. *Journal of Chemical Education* **2011**, *88*, 744.

(15) Of 123 students surveyed, 82 answered 'Yes' to the question " Do you feel the activity contained a "WOW" factor?".

(16) Sandoval, J. *Annual Review of Psychology* **1995**, *46*, 355.

Appendix 1: Supporting Information for Chapter 4. Curriculum Packet for Pharma-Chemistry in the Classroom: Synthesis and Analysis of an Antibacterial from Aspirin

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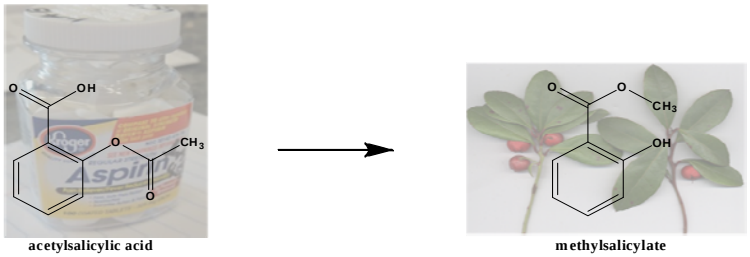
ABSTRACT



acetylsalicylic acid
common name: aspirin

methylsalicylate
common name:
oil of wintergreen

In this laboratory activity, students learn about the processes by which drugs are discovered and designed. Students are first asked to think about where medicines come from, and are prompted to think about how medicines improve lives. They next learn that the drug discovery process is lengthy, complex, and costly. The students are then provided an opportunity to mimic the discovery process through performing a synthesis and subsequent analysis. The students perform a synthesis of wintergreen oil from aspirin followed by a biological assay involving plating bacteria. This assay is used to determine the new molecule's effectiveness as an antibacterial. Through analysis, students are able to validate that they successfully synthesized

wintergreen oil from aspirin, and are able to compare physical qualities as well as antibacterial properties of the two.

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Adapted from article: Hartel, A.M, and Hanna Jr., J.M *J. Chem. Ed.*86, 475-476.

NOTE: This activity was developed as a product of the Socrates Fellows program at UC San Diego, a project of ScienceBridge, supported by funds from the National Science Foundation GK12 STEM Fellows in Education, awarded to Maarten Chrispeels, Division of Biological Sciences.

GRADE LEVEL: 10-12

DURATION: 3 periods (50 minute periods)

LEARNING OBJECTIVES:

- Students will be able to use lab practices such as measuring, pouring, heating, pipeting.
- Students will be able to perform a synthesis reaction to change one molecule into something different.
- Students will be able to run biological and physical property tests to examine the antibiotic properties of the molecules used in the synthesis as well as the product.
- Students will show through discovery that a new molecule can be synthesized with very different interactions from the starting material.

STANDARDS ADDRESSED:

<u>California Science Standards</u>

CHEMISTRY

Standard 2a: Students know that atoms combine to form molecules to form covalent bonds.

Standard 3a: Students know how to describe chemical reactions by writing balanced equations.

Standard 3d. Students know how to determine the molar mass of a molecule from its chemical formula and a table of atomic masses, and calculate its percentage composition and how to convert the mass of a molecular substance to moles, number of particles or volume of gas at standard temperature and pressure.

Standard 3e. Students know how to calculate the masses of reactants and products in a chemical reaction from the mass of one of the reactants or products and the relative atomic masses.

Standard 3f: Students know how to calculate the percentage yield in a chemical reaction.

Standard 8b: Students know how reaction rates depend on such factors as concentration, temperature, and pressure.

Standard 8c: Students know the role a catalyst plays in increasing the reaction rate.

Standard 10b: Students know the bonding characteristics of carbon that result in the formation of a large variety of structures ranging from simple hydrocarbons to complex polymers and biological molecules.

INVESTIGATION and EXPERIMENTATION

Standard 1a: Students select and use appropriate tools to perform tests, collect data, analyze relationships, and display data

Standard 1b: Identify and communicate sources of unavoidable experimental error.

Standard 1c: Identify possible reasons for inconsistent results, such as sources of error or uncontrolled conditions.

Standard 1d: Formulate explanations by using logic and evidence.

Standard 1j: Students recognize the need for controlled tests

Standard 1l: Analyze situations and solve problems that require combining and applying concepts from more than one area of science.

National Science Education Standards

LIFE SCIENCES/PHYSICAL SCIENCES/EARTH SCIENCES

Physical Science: Chemical Reactions, Catalysts

Science and Technology: Understanding about science and technology

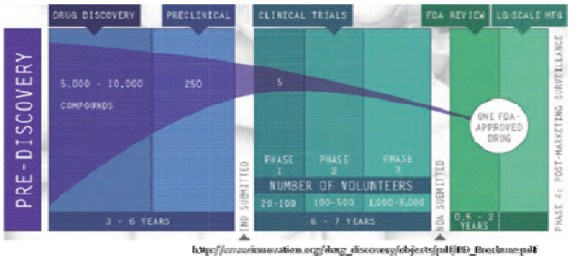
History and Nature of Science Standards: Science as a human endeavor, Historical perspectives

Science as Inquiry

BACKGROUND INFORMATION

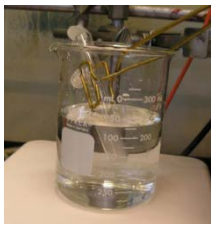
Drug Discovery

The discovery of new medicines is a process that is often not evaluated in the high school classroom; however its relevance to students is overwhelming. The discovery of new medicines is a complex process. Before a drug candidate is tested in humans, scientists scrutinize it carefully to ensure that a medicine works the way it should. First, scientists must recognize a medical problem and identify a target within the body for that medical problem, which if hit, can lead to a cure. Next, scientists design and make molecules that can hit the biological target. Often times, scientists find inspiration for their designs from molecules that are found in nature. Small changes or additions to these molecules may cause a large increase in their effectiveness to hit the target. Each newly made molecule must then be tested against the biological target to make sure that the desired effect is produced.



Chemical Synthesis

The synthesis of new molecules is an important process from which many substances essential to our everyday lives are made. Synthesis involves the making of new molecules by adding to or subtracting from

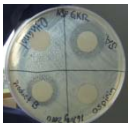
previously known ones, most of the time found in nature. Small rearrangements or adjustments to a molecule can lead to highly effective treatments for health problems, new approaches to form new technologies, or even new materials for everyday use.

Analysis of Synthesized Molecules

Every time a new molecule is synthesized it is necessary to assess the properties of the new molecule to verify that you indeed made what you wanted. One way laboratory chemists achieve this is by observing the physical properties of the new molecule. By comparing the physical properties (color, phase, odor, texture, melting point, etc.), it may be possible to confirm your starting material is indeed different from your product. If your product has a known odor, as is the case with the oil of wintergreen used in this lab activity, it may be possible to say with some confidence that you in fact made what you were trying to make. This can be further validated through other physical property identification (melting point, density, etc.). In more complex cases, high powered instruments are used to look at molecular weight (mass spectrometry) or structure (nuclear magnetic resonance) in order to confirm the success of the synthesis.

Biological Testing: Antibacterial Activity and the Zone of Inhibition

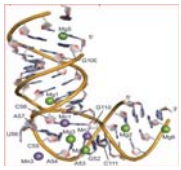
When looking for a new medicine, chemists often work with biologists to design tests, or assays, from which the scientists can get an idea of how effective the new molecule will be. One such test for antibacterial molecules is by measuring the zone of inhibition. This test involves growing bacteria in the presence of different molecules, and observing the effect each molecule has on bacterial survival. For example, in this activity, no change in growth in the presence of aspirin illustrates that this molecule is not an antibacterial, but an area around the oil of wintergreen in which bacterial growth is prevented illustrates the effectiveness of oil of wintergreen in killing bacteria. By comparing the diameters of each molecule's zone of inhibition, you can get a good idea of which molecules are more or less effective. It is also always necessary to run a positive control (inhibits bacterial growth) and a negative control (no effect on bacterial growth). It also acts as great examples of positive and negative results for the students to observe.



RESEARCH APPLICATIONS

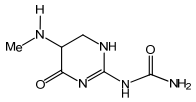
Professor Thomas Hermann Laboratory

In the Hermann Laboratory we carry out many steps of the drug discovery process. We study the Hepatitis C Virus (HCV), and have identified a specific section of its RNA genetic code as a drug target. This section,



which is known as the Internal Ribosomal Entry Site (IRES) contains a sharply bent structure which helps the virus to copy itself. Our goal is to block the virus from making copies by making a molecule that can change this bent structure. Many chemists in the lab are involved in the synthesis of libraries of molecules. Each chemist designs a series of related molecules to be tested against the HCV IRES. The central idea is that by changing small parts or functional groups of a model molecule, we can change the activity of a given molecule. Therefore, our 'libraries' of molecules are really a hunt for a molecule that has an ideal interaction with our desired target.

As a synthetic chemist in the Hermann lab, I am involved in the design and synthesis of new molecules. The design of my molecules was inspired by a natural product found in soil bacteria called TAN



1057. Each new molecule synthesized is tested to see how it affects the bend within the viral IRES. The results help us to iteratively design new and improved molecules with the ultimate goal of finding a new medicine for HCV.

Through integrating the steps of the drug discovery process used in my research into a chemistry classroom activity, students are challenged to perform the various roles necessary for the development of new medicines.

Students first identify disease-causing bacteria as the biological target. Using aspirin, a natural product, as the inspirational molecule, students are guided through a chemical synthesis where they change aspirin into a product called ‘oil of wintergreen’. Based on observations of physical properties before and after, students see evidence that a reaction has occurred and that their

molecule has changed (solid to oil, bitter smell to minty smell, white color to colorless). The students then grow bacteria in the presence of the two different molecules, and observe the effect each molecule has on bacterial survival. No change in growth in the presence of aspirin illustrates that this molecule is not an antibacterial, but an area around the oil of wintergreen in which bacterial growth is prevented illustrates the effectiveness of oil of wintergreen in killing bacteria. These results help the students to further verify that their synthesis was successful and illustrates that even a small change in the structure of a molecule can lead to a medicine. Following the activity, students convene to discuss their results and talk about the importance of those results in light of the drug discovery process.

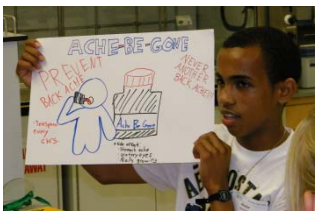
THE 5E MODEL

5Es *At-A-Glance*	Activity	Key Ideas/Questions	Timing
ENGAGE	What medical molecule would you make? Create a poster advertising this new medication and what it does.	Thinking about what drugs have not been discovered, and that 150 years ago they might make a poster for headache medicine.	20min
EXPLORE	One of these things is not like the other. Comparing molecules.	Looking at molecules to see differences of functional groups.	5 min
EXPLAIN	Powerpoint: Drug Discovery	Understanding the drug discovery process and how to synthesize a new drug. Instructions on the synthesis.	20 min
ELABORATE	Lab: Synthesis of Methyl Salicylate (an antibiotic) from Aspirin.	How do you change a molecule into another molecule?	45 min
EVALUATE	Lab: Analysis of Methyl Salicylate Physical Tests and Bacterial Plating of Product and starting material	How do you compare two molecules to ensure that a new molecule has been synthesized?	20 min setup 10 min analysis

<u>Implementation Guide</u>

ENGAGE

Activity: If you could invent one new drug, what medical molecule would you make? Create a poster advertising this new medication and what it does.



Key Questions: Thinking about what drugs have not been discovered, and that 150 years ago they might make a poster for headache medicine or antibacterial medication.

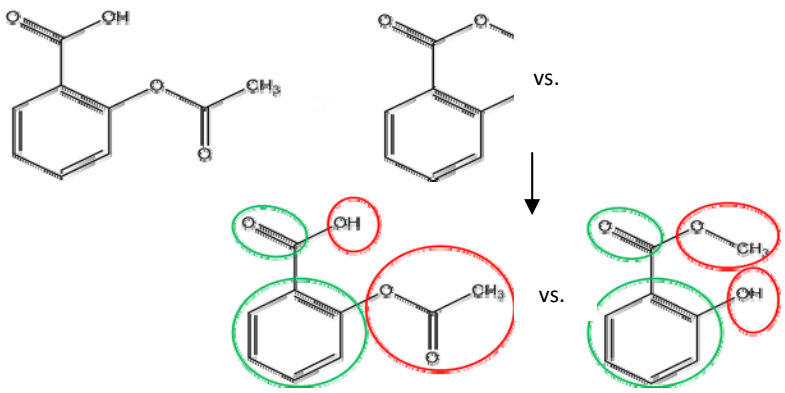
Discussion: What is the process to create a new drug?

Potential Misconceptions: We can make any drug we want.

EXPLORE

Introduce: One of these things is not like the other: Comparing molecules.

Guide: Students are invited to study the molecules as pictures and should see that the two molecules are somewhat different. We can then discuss the effect of these differences.

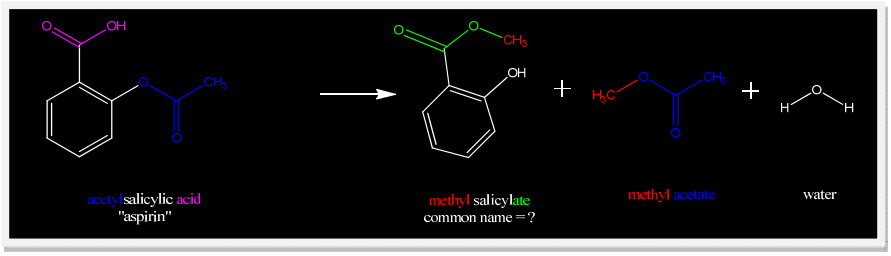


Key Questions: Looking at molecules to see differences of functional groups.

Sample Student Answers/Results: The two molecules have a similar shape, but have a few different atoms around the central ring. We can turn one into another by altering these groups.

EXPLAIN/ELABORATE

Discuss: PowerPoint on Drug Discovery and the Synthesis process.



Sample Questions: How do you change a molecule into another molecule?

Key Points: Drug Discovery takes reacts molecules to make new ones.

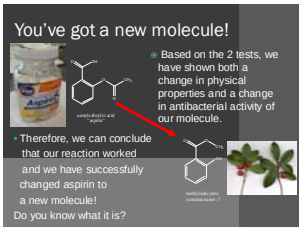
Application/Elaboration: Lab: Synthesis of Methyl Salicylate (an antibiotic) from Aspirin. When implemented, I use only the chemical name for oil of wintergreen. This makes the evaluation of the product more mysterious

and more exciting when they recognize the easily recognizable smell of wintergreen.

EVALUATE

Activity: Physical Tests and Bacterial Plating of Product and starting material

Key Questions: How do you compare two molecules to ensure that a new molecule has been synthesized?



Example Student Answers: Change in smell (wintergreen smell will appear) indicates a new molecule is present.

When plating the molecules, the aspirin will not kill the bacteria, but the Methyl Salicylate will kill the bacteria. This shows that the new molecule has different properties.

MATERIALS

MASTER LIST

Station is defined as a designated area where students go to obtain items. It is recommended to have at least 3 stations for a class of 30.

Group size is recommended to be 2 students so each student gets practice performing various techniques.

Bench is defined as 2 groups which share certain common materials.

Student is each individual.

Quantity (specify per student/station/etc.)	Material	Where to Order	CAS if applicable
2 per group	aspirin tablets	Any drugstore or supermarket	
5 milliliters per group	Methanol	Chemical supplier	67-56-1
1 per group	Filter paper (can use coffee filters)	Any drugstore or supermarket	
2 per student	Gloves	Hardware store or chemical supplier	
4 drops per group, at stations	Concentrated sulfuric acid	Chemical supplier	7664-93-9
4 per group	Paper discs (can be made from filter paper)	Any drugstore or supermarket	
1 per group	Large agar filled petri dishes	Chemical supplier	
1 per bench	Wax pencil	Office supply store	
1 per bench	Tweezers	Drugstore	
1 per group	4 compartments of an ice cube tray	Dollar store	
1 per station	Salicylic acid	Chemical supplier	69-72-7
1 per station	Sterile water	Chemical supplier	7732-18-5
1 per station	Ethanol	Chemical supplier	64-17-5
1-2 for the whole class	Pre-plated bacteria	Chemical supplier	
1 per group	Cotton swab for bacteria colony	Any drugstore or supermarket	
1 -2 next to the plated bacteria	Container with bleach solution	Any drugstore or supermarket	

TEACHER PREPARATION INSTRUCTIONS

- Agar plate with bacteria colonies should be made 1-2 days in advance of the lab. This requires an agar plate and a bacteria culture. Using the streaking technique spread the bacteria onto the agar plate to produce individual colonies for student picking. This can be done using a cotton swab and a blunt edged toothpick.
- The salicylic acid samples for the stations should be prepared no more than a day in advance to ensure the compounds to not degrade. The salicylic acid sample can be prepared by adding 0.50 g salicylic acid into a vial and dissolving it in 10 mL of ethanol. Cap it until class use.
- The aspirin samples for the stations can be prepared in two ways. The first and simplest would be placing 0.65 g acetylsalicylic acid into a vial and dissolving it in 10 mL of ethanol. *If pure acetylsalicylic acid is not available more time will be necessary to prepare the sample.* Grind up 2 aspirin tablets and dissolve in 5 mL methanol (the full tablet will not dissolve, but the acetylsalicylic acid will). Filter the sample into a vial and keep open in hood overnight to allow the methanol to evaporate. The following day dissolve the residue (acetylsalicylic acid) in 10 mL ethanol and cap until class use.

IMPLEMENTATION STRATEGIES

- Suggestion: Day 1: Intro to drug discovery and synthesis of Oil of Wintergreen from Aspirin tablets. Day 2: Setup of bacterial plates for biology assay. Day 3: Analysis of antibacterial results; group discussion of the importance of drug discovery.
- 2 students per group, 4 students per lab bench
- List of Class Shared Materials: Methanol, hot water bath, Sulfuric Acid, salicylic acid sample, aspirin sample, sterile nano water, ethanol, pre-plated bacteria, bleach water
- List of Materials at each lab bench: cotton swab (2), ruler, tweezers, wax pencil, 4 compartment ice cube tray (2), disposable pipet (2), Agar filled petri dish (2), paper discs (8), aspirin tablets (4), filter paper(2), gloves (8), test tube holder(2), test tube (2), test tube rack, funnel (2), mortar and pestle (2)
- List of Teacher Materials
    - Audio-visual materials: Powerpoint with interactive worksheet for students to follow along with powerpoint.
    - Prelab
    - Post lab questions

STUDENT GUIDE

- ✓ Lesson Overview/Learning Objectives
- ✓ Materials (see below in student protocol)
- ✓ Protocol (see below)

- ✓ Pre-Lesson Activities (Engage, will create a poster)
- ✓ Post-Lesson Activities (post-lab questions)
- ✓ Background and/or Additional Reading (“group discussion” about results, findings, and observations and uses for oil of wintergreen; supporting information found in powerpoint)

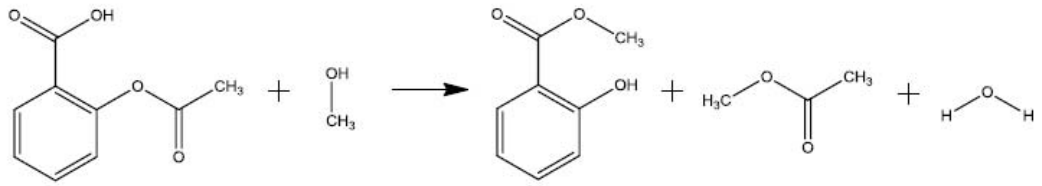
STUDENT PROTOCOL

Also included below: interactive worksheet for powerpoint introduction, pre lab questions, student protocol to guide students through lab, and post-lab questions

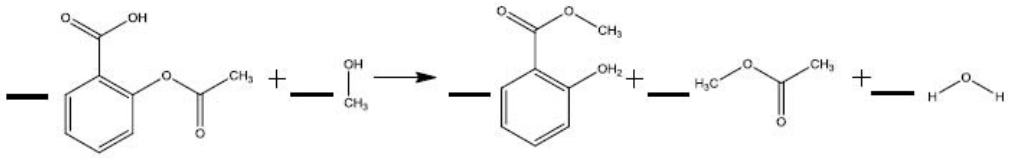
Pre-lab Worksheet

Pharma-Chemistry in the Classroom:
Synthesis and Analysis of an Antibacterial from Aspirin

Pre-lab Questions:
Use the following reaction to answer the following questions. Make sure to show all work!



acetylsalicylic acid "aspirin" | methanol | methylsalicylate common name: ? | methyl acetate | water

1. What is the chemical formula for acetylsalicylic acid:	2. What is the chemical formula for methylsalicylate?
3. What is the molecular weight of acetylsalicylic acid?	4. What is the molecular weight of methylsalicylate?
Is the equation shown above balanced? If not, balance it below (HINT: Balance carbons first). 	

Interactive Worksheet

Pharma Chem in the Classroom

Name:

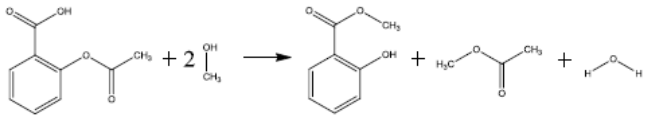
Interactive worksheet: Follow along with the powerpoint and answer the questions as you go.

Give 2 examples of the work of science in your everyday life?	Why is drug discovery important?
On average, how much does it cost to take a potential medicine through clinical trials and into the drug market?	How many main steps are there in the Drug Discovery process?
What's one way chemists try to find new drugs?	What is a physical property? List 3.
What is "wafting"? Why is it important?	What is a positive control? Why do we use one?
Follow along with the steps of the lab in your lab handout. Write notes or draw diagrams in the observation, diagram, and sources of error section next to each step.	

Student Handout

Pharma-Chemistry in the Classroom:
Synthesis and Analysis of an Antibacterial from Aspirin

Reaction:



Today you will be working with a lab partner to complete this lab. The lab is broken up into 2 parts:

A. Synthesizing methylsalicylate from acetylsalicylic acid/aspirin and analyzing physical properties of each.
B. Analyzing your new molecule through antibacterial testing of our starting material (acetylsalicylic acid/aspirin) and product (methyl salicylate)

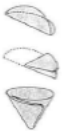
PART A: Synthesizing methylsalicylate from acetylsalicylic acid (aspirin) and analyzing physical properties

Synthesis Materials:

- 2 Tablets of aspirin
- Mortar and Pestle
- Funnel
- Filter Paper
- Gloves
- Safety glasses
- Test tube holder
- Test tube
- Test tube rack
- Beaker with room temperature tap water
- 10mL graduated cylinder

Obtain these from the hood as they are needed:

- 5mL methanol
- Hot water bath
- 4 drops H_2SO_4

Synthesis Procedure:	**Observations, Diagrams and Sources of Error**
1. Place the aspirin in the mortar and use the pestle to grind it into a powder.	1.
2. In **Data Table 1 on page 2**, list 3 physical properties to observe. for your starting material (acetylsalicylic acid/aspirin) and product (methylsalicylate)	2. Place observations from this step in Data Table 1
3. Make observations of the physical properties of the crushed up starting material (acetylsalicylic acid, common name aspirin). Place your observations in **Data Table 1**.	3. Place observations from this step in Data Table 1.
4. Add 5mL methanol from the fume hood into mortar and mix using the pestle. This should dissolve the aspirin, but the extra material in the tablet should not dissolve.	4.
5. Fold your filter paper as demonstrated to fit in the funnel. 	5.
6. Place your funnel in the top of the test tube. Slowly add the liquid from your methanol/aspirin solution. Try not to pour the solids in until the end.	6.
7. ⚠ Add 3 drops of sulfuric acid to your test tube from the fume hood.	7.

8. Place the test tube in the hot water bath in the hood and heat for 30 minutes. **Record where your sample is so you can retrieve it later.** .	8. Boat #__ Position #__ Time sample went into water bath: _____
9. While your reaction is heating, you should start setting up **Part B on page 3**. Make sure to keep an eye on the time for your reaction!	9.
10. Remove sample from hot water bath. The synthesis is complete!	10.
11. Make observations of physical qualities of your new product in **Data Table 1** below.	11.Place observations in Data table 1
12. Go back to where you left off in **Part B (step 6)** to finish preparing your antibacterial testing.	12.

Data Table 1: Physical Properties

Physical Property to Observe	Observations of Starting Material (acetylsalicylic acid/aspirin) **before Synthesis**	Observations of Product (methyl salicylate) **after Synthesis**
Example: Smell		
Which physical properties changed during the experiment? How did they change?		
Comparing the physical properties of your starting material (acetylsalicylic acid/aspirin) and product (methylsalicylate), do you think your synthesis was successful?		
Based on your observations of physical properties, do you have any ideas what the common name of your product could be?		

PART B: Analyzing your new molecule through Antibacterial Testing

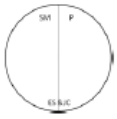
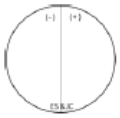
You can set this up while your synthesis experiment is heating!

Materials

- 4 paper discs
- 2 Agar filled petri dishes
- Ice cube tray
- Wax pencil
- Tweezers
- Disposable pipet

Obtain these from the designated stations as they are needed:

- Positive control (salicylic acid)
- Cotton swab with bacteria
- Sterile nano water
- Ethanol
- Ruler (mm)
- Starting material in Ethanol (acetylsalicylic acid/aspirin)

Procedure for Antibacterial Testing	Observations, Diagrams and Sources of Error
1. Obtain 4 paper discs and place them in the compartments of the ice cube tray.	1.
2. Label the rim of the tray **on the side with the agar gel** with a wax pencil for the 4 substances you will plate: a. Product (methylsalicylate) b. Starting material (acetylsalicylic acid/aspirin) c. Salicylic Acid (+) control d. Ethanol (-) control	2.
3. Add 2 drops of each substance from a designated station to the proper discs using the dropper that goes with each substance. NOTE: you cannot do the product until the synthesis is complete. Place the discs aside to allow the discs to dry while you prepare the Petri dishes.	3.
4. Before plating your samples you need to prepare the Petri dishes. Keeping your Petri dishes closed, turn it agar side up and use a wax pencil to divide each in half. **Put your team initials** on the bottom and label the halves as follows: c. Product (methylsalicylate) d. Starting material (acetylsalicylic acid/aspirin) e. Salicylic Acid (+ control) f. Ethanol (- control)  	4.
5. Using a cotton swab and help from your teacher, pick a colony from a pre-grown plate. Rub the bacteria-filled cotton swab on the Petri dish in a star fashion until you think the whole dish has been contacted as demonstrated. Repeat with your second plate.	5.
6. Once the physical properties of your product have been observed and recorded in Data Table 1 (Part A, step 11), add 3 to 5 drops of ethanol to the test tube containing your product. You only need enough to dissolve the Methyl Salicylate.	6.

7. Use your own pipet to place 2 drops of this onto the appropriate disc. Let this dry while continuing on to step 9.	7.
8. Once the paper discs have dried, place them on the matching part of the Petri dishes. Place 1 drop of sterile nano water onto each of the paper discs until they look moist so they stick to the plate.	8.
9. Place the Petri dishes in the incubator overnight to allow bacteria to grow.	9.
10. Predict whether each substance will be antibacterial or not in Data Table 2 below	10.

DAY 2: Understanding the Results

1. Find your Petri dishes.
2. Look at each disc. The bacteria should have grown everywhere except where it is near an antibacterial substance. The area where the bacteria did not grow is called the "zone of inhibition" and should be a clear area around your paper disc. If the substance has a "zone of inhibition" it is antibacterial!
3. Measure the diameter of the "zone of inhibition" which is the clear area around the paper disc.

Data Table 2: Antibacterial Testing

Substance	Prediction Will it be antibacterial? (Yes or No)	Actual Results (yes or no)	Measure zone of inhibition (mm)
Starting Material (Acetylsalicylic acid/aspirin)			
Product (Methylsalicylate)			
(+) Control (Salicylic Acid)			
(-) Control (Ethanol)			
Which substances show antibacterial activity?			
How do these results compare to your predictions?			

Post lab Worksheet

Name:

Post Lab Questions:

Do your work here if the problem includes math.	Write your answer in a full sentence here
1. How many grams of aspirin did you start with?	
2. How many moles of aspirin did you start with?	
3. Look at the reaction. What is the mole ratio of aspirin to methylsalicylate?	
4. How many moles of methylsalicylate should you end with?	
5. What is the formula to calculate % yield?	
6. Can we calculate the %yield in this reaction? Why or why not?	
7. What are **2** pieces of evidence to show the chemical reaction worked?	
8. How can you tell a substance is an antibacterial?	
9. Which substance tested was the most antibacterial?	

TEACHER SUPPLEMENTS

Powerpoint Presentation

Powerpoint includes introduction to drug discovery, virtual lab, results, and follow up.

Assessment Strategies

Grading the lab handout as well as pre-lab questions, interactive worksheet, and post-lab questions provides feedback on student understanding of the concepts used in synthesis and the drug discovery process including stoichiometry, mole, and molecular weight calculations. Teachers should also allow opportunities for Think Pair Share during discussion of results, and discussing if the synthesis was successful.

Essentials of Inquiry Reflection for our Engage Activity:

Essential features of inquiry.

Feature	More ----------Amount of Learner Self-Direction----------Less Less--------Amount of Direction from Teacher or Material--------More			
1. Learner engages in scientifically oriented questions	Learner poses a question	Learner selects among questions, poses new questions	Learner sharpens or clarifies question provided by teacher, materials, or other source	Learner engages in question provided by teacher, materials, or other source
2. Learner gives priority to evidence in responding to questions	Learner determines what constitutes evidence and collects it	Learner directed to collect certain data	Learner given data and asked to analyze	Learner given data and told how to analyze
3. Learner formulates explanations from evidence	Learner formulates explanation after summarizing evidence	Learner guided in process of formulating explanations from evidence	Learner given possible ways to use evidence to formulate explanation	Learner provided with evidence and how to use evidence to formulate explanation
4. Learner connects explanations to scientific knowledge	Learner independently examines other resources and forms the links to explanations	Learner directed toward areas and sources of scientific knowledge	Learner given possible connections	
5. Learner communicates and justifies explanations	Learner forms reasonable and logical argument to communicate explanations	Learner coached in development of communication	Learner provided broad guidelines to use sharpen communication	Learner given steps and procedures for communication

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Essentials of Inquiry Reflection for our Explore Activity:

Essential features of inquiry.

Feature	More ----------Amount of Learner Self-Direction----------Less Less--------Amount of Direction from Teacher or Material--------More			
1. Learner engages in scientifically oriented questions	Learner poses a question	Learner selects among questions, poses new questions	Learner sharpens or clarifies question provided by teacher, materials, or other source	Learner engages in question provided by teacher, materials, or other source
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Essentials of Inquiry Reflection for our Evaluate Activity:

 = Antibacterial Testing =  Analysis of Physical Properties

Essential features of inquiry.

Feature	More ----------Amount of Learner Self-Direction----------Less Less--------Amount of Direction from Teacher or Material--------More			
1. Learner engages in scientifically oriented questions	Learner poses a question	Learner selects among questions, poses new questions	Learner sharpens or clarifies question provided by teacher, materials, or other source	Learner engages in question provided by teacher, materials, or other source
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