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Synthesis of S-Ribosyl-L-homocysteine and Analogs Modified at the Homocysteine-C3 Position

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Synthesis of *S*-Ribosyl-L-homocysteine and Analogs Modified at the Homocysteine-C3 Position

A Thesis Presented to the Faculty of the Department of Chemistry at the University of San Francisco in partial fulfillment of the requirements for the Degree of Master of Science in Chemistry

Written by

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Master of Science in Chemistry

University of San Francisco

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This thesis is written under the guidance of the Faculty Advisory Committee, and approved by all its members, has been accepted in partial fulfillment of the requirements for the degree of

Master of Science in Chemistry

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Abstract

Quorum sensing (QS) is a process of bacterial cell-to-cell communication that conveys population density information in order to coordinate gene expression to produce synchronized behaviors. QS regulates the expression of virulence genes in many species of bacteria; hence, the manipulation of QS pathways may lead to treatment options against many bacterial diseases. The LuxS enzyme converts *S*-ribosyl-L-homocysteine (SRH) into homocysteine (HCys) and 4(*S*),5-dihydroxypentane-2,3-dione (DPD), which is the precursor of autoinducer-2 (AI-2). Thus, inhibitors of LuxS could prevent QS by halting the conversion of SRH to AI-2 rendering the cell "uncommunicative". This work shows the successful chemical synthesis of SRH and progress towards the synthesis of SRH analogs substituted at the HCys C3-position. The chemically-synthesized SRH can be utilized as the substrate for LuxS inhibition assays. The "HCys-C3" SRH analogs are designed to prevent the conversion of SRH to AI-2 by blocking the necessary association between the two LuxS monomers. This work also provided inspiration for the design and synthesis of other kinds of SRH analogs that could also serve as LuxS inhibitors.

Chapter 1. Introduction

Bacteria are typically considered to be unicellular, asocial, reclusive organisms. However, in recent years, it was found that bacteria operate in multicellular organization. They can talk with each other by using a chemical "language" and express collective behaviors. Bacterial cells make and secrete small signaling molecules like "words" into the environment and then recognize those "words" to perceive their population density. When bacterial cells accumulate to a certain density, they turn on group behaviors that are only successful when all of them participate in unison. This process of bacterial cell-to-cell communication is termed quorum sensing (QS).[1] It is regulated by small signaling molecules called autoinducers (AI's). Bacterial cells produce and release AI's into the environment. When the AI concentration has increased to a certain amount, they are locked down into receptors on the surface of bacteria, passing information into the bacterial cells. Thus, bacteria can acquire cell population density information and then monitor gene expression, resulting in changes to their collective behaviors including virulence, bioluminescence, biofilm formation, and motility (*Figure 1.1*).[2]

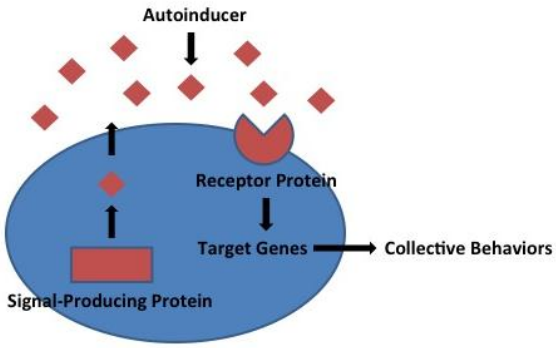


Figure 1.1. **Bacterial quorum sensing.** Autoinducer molecules are synthesized by signal-producing proteins (synthases) and detected by receptor proteins to influence collective behaviors through gene expression.

Two major QS systems exist in bacteria. System One QS mediates intra-species communication, while System Two QS mediates inter-species communication (*Figure 1.2*). System One QS allows bacteria to perceive the population density of their own species. Through the System Two QS pathway, bacteria can take a census of the population of other species in the bacterial community. Thus, by possessing both systems, a bacterium is able to detect the population density of both its own species and other species in the environment, measure which is in the majority and which is in the minority, and decide what kind of social behavior to carry out.[1]

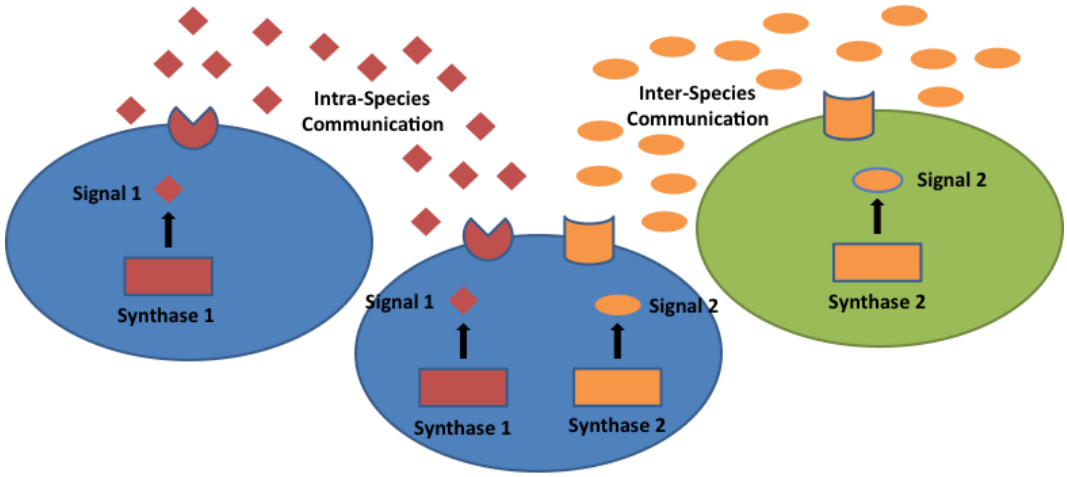


Figure 1.2. **System One and System Two QS in bacteria.** Two blue ovals represent bacterial cells of the same species; they have intra-species communication. The green cell indicates another kind of bacterial species (different from blue ones), inter-species communication happens between it and the blue cells.

1.1 System One Quorum Sensing: Intra-Species Communication

In System One QS, bacterial communication is specific to its own species. Each signaling molecule can only fit with its own receptor and no other. Hence, different bacteria

communicate with their own species by using unique AI's. By System One QS, bacteria can detect the cell number of their own species. Once the population density has reached a particular threshold, gene expression is initiated, allowing bacteria to coordinate collective behaviors. This circuit exists in both Gram-negative and Gram-positive bacteria.[1]

1.1.1 System One Quorum Sensing in Gram-Negative Bacteria

Acyl-homoserine lactones (AHLs) are the common autoinducers utilized by Gram-negative bacteria (such as *Vibrio fischeri*) for intra-species QS. The concentration of AHLs increases as the bacterial cells' population density rises. AHLs, which can freely come into and pass out of the cell, are produced by LuxI-type synthase proteins and detected by LuxR-type receptor proteins. The interaction between the LuxR proteins and the AHLs results in the specific binding of the LuxR-AHL complex to promoter DNA elements and the transcriptional activation of the target genes (*Figure 1.3*).[1]

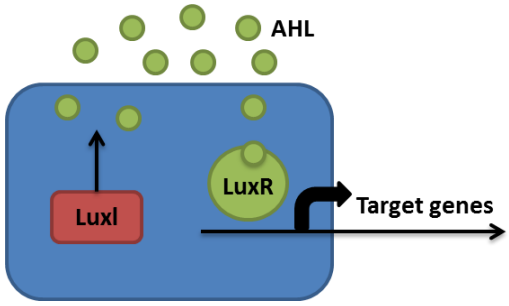


***Figure 1.3.* AHL-Based System One QS.** (Filled green circles indicate AHLs.)

Most AHLs reported to date share similar chemical structures: they all consist of an *N*-acyl homoserine lactone and a R-group side chain (*Figure 1.4* and *Table 1.1*).[3] Different bacteria produce AHLs that differ in the length of the R-group tail. Chain lengths vary

from 4 to 18 carbon atoms and in some cases include substitution of a carbonyl at the third carbon.

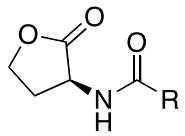
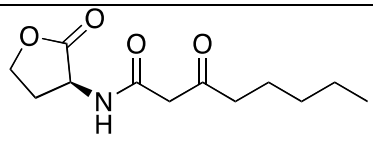
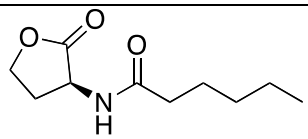
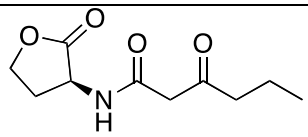
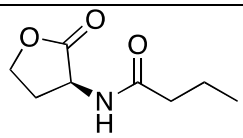
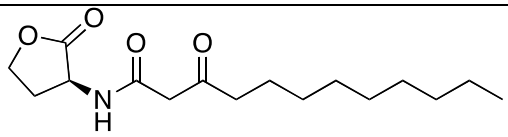


Figure 1.4. **General AHL structure.** Most AHLs share a *N*-acyl homoserine lactone head group. AHLs differ by the identity of the appended R-group tail.

Each species of bacteria accomplishes intra-species communication using a specific autoinducer. However, some species of bacteria employ more than one AI (i.e. *Pseudomonas aeruginosa*, *Table 1.1*) in System One QS. In these cases, each AI has its own pair of synthase and corresponding receptor.[4]

Table 1.1. **Selected LuxI/R QS systems with corresponding AHL structures.**[4]

Bacterium	**AHL(s)**	**LuxI/R homologs**
Agrobacterium tumefaciens		TraI/R
Chromobacterium violaceum		CviI/R
Vibrio fischeri		LuxI/R
Pseudomonas aeruginosa		RhlI/R
		LasI/R

1.1.2 System One Quorum Sensing in Gram-Positive Bacteria

To date, AHL-based QS has not been demonstrated in any Gram-positive bacteria. Instead, System One QS in Gram-positive bacteria is associated with the production of autoinducing peptides, or AIPs. Gram-positive bacteria (such as *Streptococcus pneumoniae*) synthesize peptides that are usually modified and then actively secreted. Detection occurs via a two-component signal transduction circuit, leading to the ATP-driven phosphorylation of a response regulator protein, which then binds to promoter DNA and regulates transcription of target genes (*Figure 1.5*). As with the AHL-based QS system, some bacteria utilize more than one AIP as signaling molecules, each with its own pair of synthase and receptor (*Table 1.2*).[1]

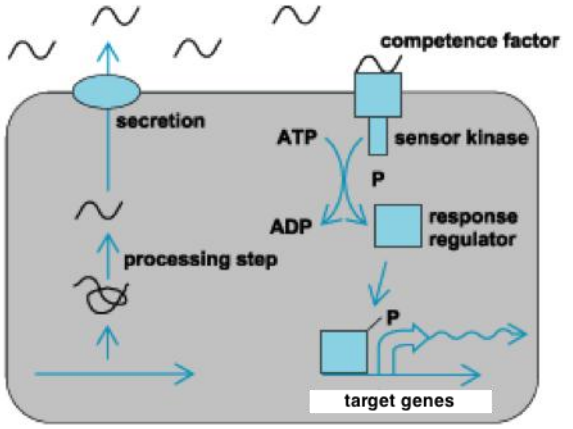
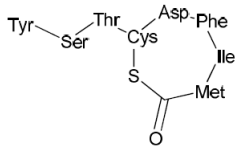
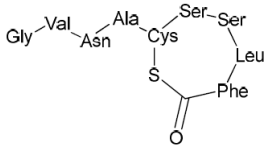
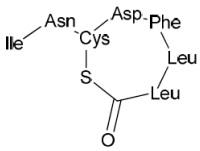
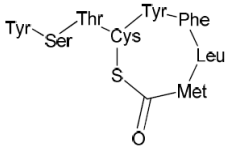


Figure 1.5. **AIP-Based System One QS.**[1] (Wavy lines indicate AIPs.)

Table 1.2. **Selected Gram-positive System One QS with corresponding AIPs.**[1](*geranyl group)

Bacterial Species	AIP	Name
Staphylococcus aureus		AIP-I
		AIP-II
		AIP-III
		AIP-IV
Bacillus subtilis	AlaAspProIleThrArgGlnTrp*GlyAsp	ComX
	GluArgGlyMetThr	CSF
Streptococcus pneumoniae	GluMetArgLeuSerLysPhePheArgAspPheIleLeuGln-	CSP
	ArgLysLys	

1.2 System Two Quorum Sensing: Inter-Species Communication

Bacteria can also communicate among different species, employing a universal signaling molecule termed autoinducer-2 (AI-2). In contrast to the AHLs and AIPs that are used as autoinducers for intra-species communication, AI-2 is a nonspecies-specific signaling molecule that mediates both intra- and inter-species communication among Gram-negative and Gram-positive bacteria.[5]

AI-2-based inter-species QS was first established in the Gram-negative bacterium *Vibrio harveyi*, in which QS regulates the gene expression of a luciferase enzyme that results in bioluminescence.[6] It was noticed by Bassler and coworkers that bioluminescence could be still observed when the System One AHL autoinducer HAI-1 (*N*-[3-(*R*)-hydroxybutanoyl]-homoserine lactone, *Figure 1.6*, left) was absent.[7] Thus, a second QS circuit was proposed in this bacterium to regulate the collective behavior of bioluminescence. Eventually, both the System Two QS circuit and its autoinducer AI-2 (*Figure 1.6*, right) were elucidated; later it was shown that this System Two QS pathway is widespread in many bacterial species.[8]

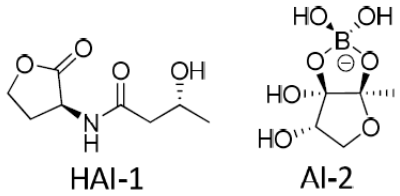


***Figure 1.6*. Autoinducer-1 and -2 in *V. harveyi*.**

System One and System Two QS circuits are parallel in *V. harveyi*. As shown in *Figure 1.7*, LuxM is responsible for HAI-1 synthesis, while LuxS is responsible for AI-2 synthesis. At high concentrations, HAI-1 and AI-2 are detected by LuxN and LuxP, respectively, leading to expression of the *lux*CDABE genes that initiate bioluminescence.[9] This well-studied System Two QS is considered to be a model for all System Two QS circuits that could be utilized for analysis of QS inhibition.[10]

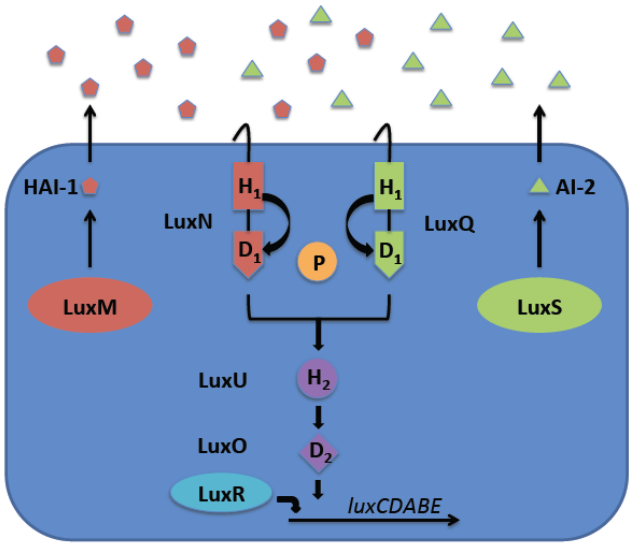


Figure 1.7. **Hybrid QS systems in *V. harveyi*.**[1]HAI-1 is the autoinducer for System One QS, while AI-2 represents the autoinducer for System Two QS. Two QS Systems are parallel in *V. harveyi*.

System Two QS has also been studied in the Gram-positive bacterium *Bacillus subtilis*. *B. subtilis* also has both System One and System Two QS circuits. It has peptide-based System One QS and also has *luxS* gene, conducting a typical LuxS catalyzed AI-2 pathway at the same time.[1]

1.2.1 AI-2 Biosynthesis

The LuxS enzyme is responsible for AI-2 biosynthesis.[8] It has a metabolic role in SAH detoxification in the activated methyl cycle in bacterial cells.[11] The metabolite *S*-adenosylmethionine (SAM), which exists ubiquitously in cells, is converted to *S*-adenosylhomocysteine (SAH) via the action of methyltransferases (Mtf). SAH is hydrolyzed to adenine (Ad) and *S*-ribosyl-L-homocysteine (SRH) by the *S*-adenosylhomocysteine nucleosidase (Pfs). Under the catalysis of *S*-ribosylhomocysteinase (LuxS), SRH is then cleaved to 4(*S*),5-dihydroxy-2,3-pentanedione (DPD) and homocysteine (HCys) (*Figure 1.8*).[12]

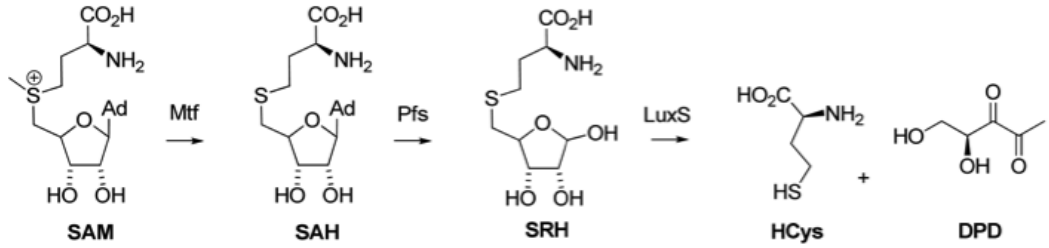


Figure 1.8. **Biosynthesis of DPD.** SRH generated from SAM converts to HCys and DPD under the catalysis of LuxS.

DPD is the precursor of AI-2. It is unstable and rearranges spontaneously to generate a variety of interconverting derivatives, each with the potential capability of mediating bacterial interspecies communication. In aqueous solution, there is spontaneous cyclization of DPD to either the *S* or *R* form of 2,4-dihydroxy-2-methyldihydro-3-furanone (DHMF), which after hydration form *S* or *R* of 2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (THMF), respectively. The *R*-THMF isomer serves as AI-2 in *Salmonella typhimurium*. If there is enough borate in solution, the *S*-THMF undergoes boronation to generate the furanosyl borate diester *S*-THMF-borate that is AI-2 in *Vibrio harveyi*.[5b, c] The interconversion of different DPD derivatives that exist in equilibrium (known as the AI-2 pool) allows bacteria to recognize the AI-2 of their own species and the AI-2 secreted by other bacterial species. Thus, AI-2 can serve as the universal "language" for bacterial interspecies communication.[5b, 13]

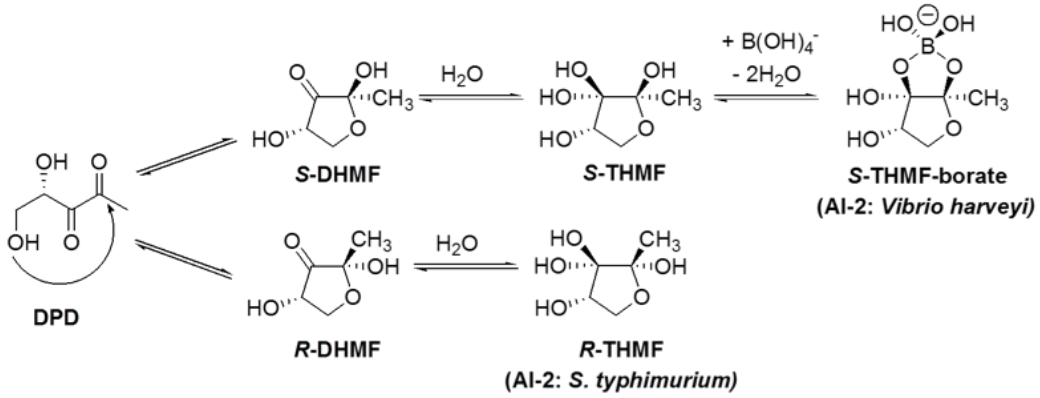


Figure 1.9. **AI-2 pool.**[5c] DPD is quite unstable and can be transformed into many forms in aqueous solution. *S*-THMF-borate is the product from boration of *S*-THMF and is the AI-2 in *Vibrio harveyi*, while the *R*-THMF isomer represents the AI-2 in *Salmonella typhimurium*. Different bacterial species own its unique AI-2 to conduct inter-species communication.

1.2.2 Catalytic Mechanism of LuxS

Pei and coworkers first reported the mechanism for the LuxS-catalyzed cleavage of SRH into HCys and DPD.[12, 14] The LuxS enzyme contains an iron (II) cation in the active site, which stabilizes various intermediates in the pathway for the LuxS-catalytic cleavage by binding to them. In the initial steps of the reaction (*Figure 1.10*, steps a-e), LuxS binds to the ring-opened hydroxyaldehyde form of SRH; the metal ion acts as a Lewis acid and amino acid residues Cys84 and Glu57 act as Lewis bases, promoting iterative isomerization and migration of the α-hydroxycarbonyl unit proceeding through intermediates **1-3** and **1-5**. Finally, elimination of homocysteine produces the enol intermediate **1-7**, which immediately rearranges to DPD (*Figure 1.10*, step f-h).

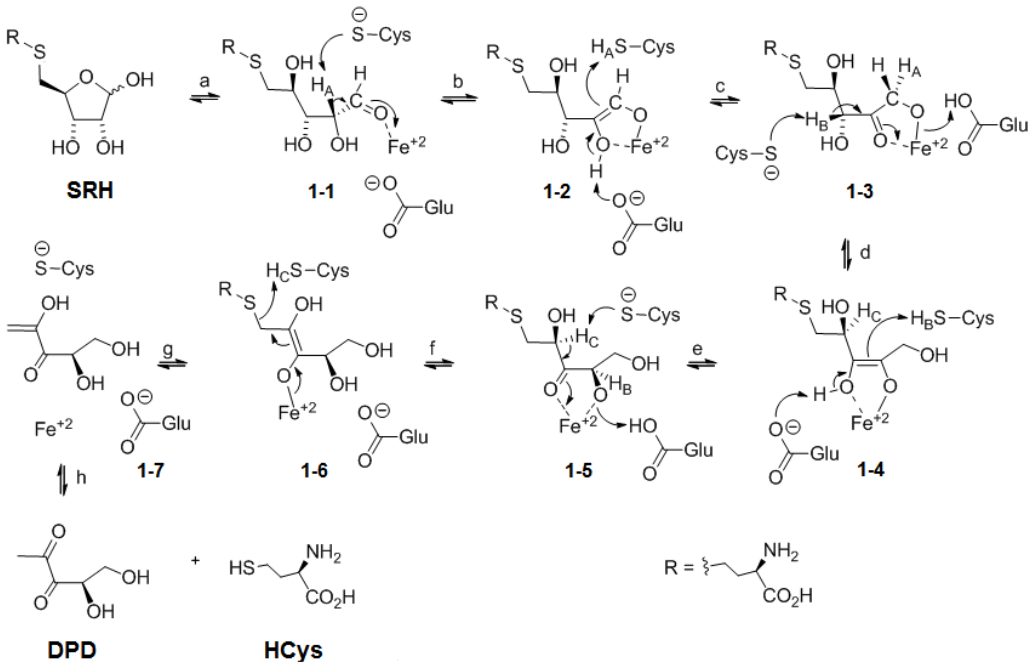


***Figure 1.10*. Catalytic mechanism of LuxS elucidated by Pei.[12]**

1.3 Modulation of AI-2-Based Quorum Sensing

As noted earlier, bacterial QS regulates group behaviors including virulence, motility, and biofilm formation in bacteria. These behaviors are correlated with pathogenicity,[15] and mutants that are deficient in QS often fail to establish infections.[5a, 10b, 15b, 16] Thus, the study of QS could lead to a new pathway towards the treatment of bacterial diseases: if communication between bacteria could be prevent, then patients will not be infecte.

Since the AI-2 system is species-nonspecific and exists in both Gram-negative and Gram-positive bacteria, System Two QS is a promising target for the development of broad-spectrum antibiotics.[15a] The *luxS* gene, which encodes for the LuxS synthase, has been found in over 70 bacterial species (*Table 1.3*).[17] The development of small molecules as potential drugs to control these pathogens through System Two QS could be a novel way

to treat bacterial infectious diseases. The particular advantage of these sorts of drugs is that they will not kill the bacteria like traditional antibiotics do. Thus the bacteria should not experience much selective pressure to develop resistance, which is a big problem with today's medicines.

***Table 1.3*. System Two QS regulated pathogenic bacteria.[17]**

Bacteria species	**Diseases**	**AI-2 QS Regulated Phenotype**
Borrelia burgdorferi	Lyme disease	Pleiotropic protein expression
Campylobacter jejuni	Food poisoning	Motility
Clostridium perfringens	Food poisoning	Toxin production
Escherichia coli	Intestinal and extra-intestinal infections	Virulence
Neisseria meningitidis	Bacterial meningitis (epidemic)	Bacteremic infection
Salmonella typhimurium	Gastroenteritis	Biofilm formation
Vibrio cholerae	Cholera	Virulence

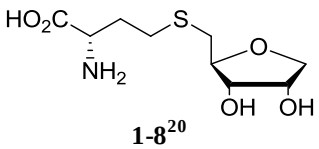
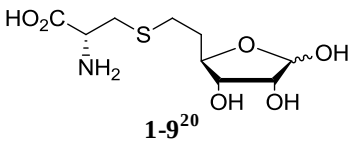
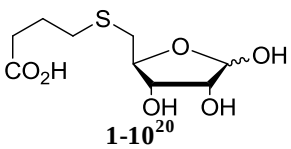
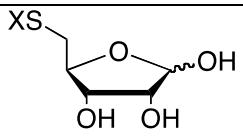
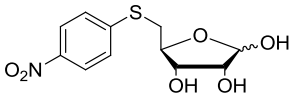
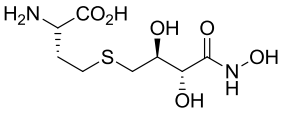
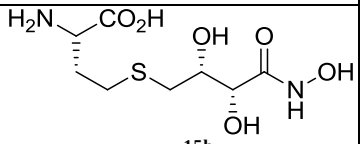
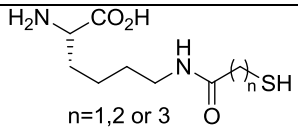
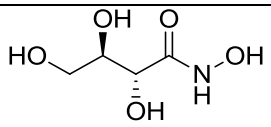
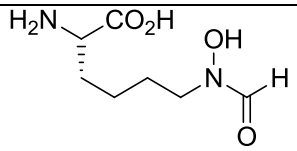
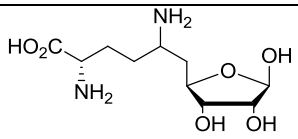
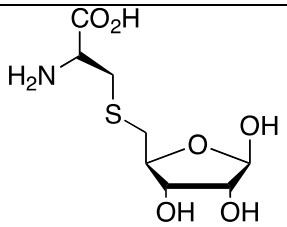
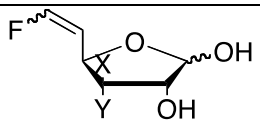
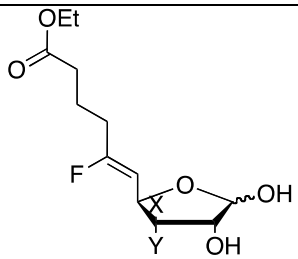
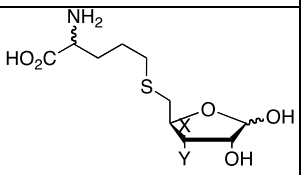
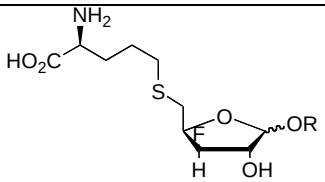
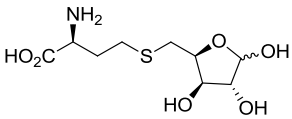
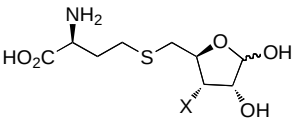
One way to block the System Two QS is to interact with AI-2 receptors. In this case, it is proposed that bacteria would not be able to communicate even when the AI-2 has accumulated to the threshold concentration in the presence of receptor inhibitors. For example, Kim Janda's research group has reported a series of design and synthesis of AI-2/ DPD analogs while some of them were found to be effective against either *V. harveyi* or *S. typhimurium*.[18] However, each bacterial species has its own AI-2, which complicates the design of small molecules for the modulation of different AI-2 receptors, and the rational design of new ligands for AI-2 receptors interaction has not really been pursued to date.[19] A better way to prevent the System Two QS pathway is by LuxS activity inhibition. SRH is converted to HCys and DPD by LuxS and DPD is the precursor molecule of AI-2. Thus, the LuxS enzyme is responsible for AI-2 biosynthesis.[13] Small

molecules that inactivate LuxS would prevent AI-2 formation and therefore the bacteria could not communicate population density information. Since LuxS is not found in humans, the inhibition of LuxS will not have effect on human bodies.

1.3.1 LuxS Inhibitors in the Chemical Literature

Several research groups have reported progress towards the synthesis of small molecules as potential LuxS inhibitors to date (*Table 1.4*).[19] All of them focus upon the modification, particularly at the ribose moiety, of the SRH molecule itself or its intermediates involved in the LuxS-catalyzed cleavage to DPD.

Table 1.4. **Summary of published SRH analogs as potential LuxS inhibitors.** Compound **1-14** and **1-15** (in red) were evaluated as the best inhibitors so far.

 **1-8**[20]	 **1-9**[20]	 **1-10**[20]
 **1-11**[21] X = H; **1-12**[21] X = CF_3.	 **1-13**[21]	 **1-14**[15b]
 **1-15**[15b]	 **1-16**[15b]	 **1-17**[15b]
 **1-18**[15b]	 **1-19**[15b]	 **1-20**[15b]
 **1-21**[22] X = OH, Y = H; **1-22**[22] X = H, Y = OH; **1-23**[22] X = H, Y = H.	 **1-24**[22] X = OH, Y = H; **1-25**[22] X = H, Y = OH.	 **1-26**[23] X = H, Y = H; **1-27**[23] X = OMe, Y = H; **1-28**[23] X = H, Y = OMe.
 **1-29**[23] R = H; **1-30**[23] R = Me.	 **1-31**[23]	 **1-32**[23] X = Br; **1-33**[23] X = F.

According with Zhou's publication in 2004, the hemiacetal of SRH was replaced by an ether group to form compound **1-8**, which could potentially bind to LuxS in a similar manner as SRH but would not be hydrolyzed to form DPD because of the lack of initial aldose-ketose isomerization in SRH hydrolysis. The position of the S-C bond of SRH was changed in compound **19**, which was expected to block the cleavage of S-C bond. However, compound **1-9** could still bind to LuxS in a similar orientation as SRH since their amino acid moieties and ribose moieties are connected by the same number of C-C and C-S bonds. Results showed that the LuxS enzyme did not break the S-C bonds in compounds **1-8** and **1-9** and therefore both of them could be potential LuxS inhibitors.[20] Considering this result with Zhao's previous report that SRH analogs **1-10** to **1-13** were not effective in LuxS inhibition[21], it may be concluded that the amino acid part in SRH plays a key role in substrate binding to LuxS.

Compounds **1-14** to **1-20** reported by Shen et al. had structures similar to those of intermediates **1-4** and **1-5** in the mechanism of LuxS-catalyzed cleavage of SRH (*Figure 1.10*). Kinetic studies showed that compounds **1-14** and **1-15** had good K_I values of 0.72 and 0.37 μM, respectively, in the evaluation of native Co(II)-substituted *B. subtilis* LuxS (Co-BsLuxS) enzyme inhibition. To explain the results, the authors stated that **1-14** and **1-15** are more stable isomers with tight binding to LuxS than **1-4** and **1-5** and therefore could slow or prevent the catalytic transformation. However, it was found that compounds **1-14** and **1-15** were less effective against Zn(II)-substituted *B. subtilis* LuxS (Zn-BsLuxS) and Co(II)-substituted *E. coli* (Co-EcLuxS) and *V. harveyi* (Co-VhLuxS) LuxS.[15b]

Wnuk et al. reported the synthesis of SRH analogs **1-21** to **1-25** with the ribose-C5 position replaced by fluoro vinyl groups. The authors hypothesized that LuxS may be

able to add water to the double bonds of these analogs, resulting in inactivation of the LuxS enzyme. One of the compounds **1-23** acted as a competitive inhibitor with moderate potency against BsLuxS.[22] In Wnuk's later report, SRH analogs (**1-26 to 1-33**) modified at the ribose-C3 position were synthesized and evaluated in the inhibition of BsLuxS and VhLuxS. The most potent compound was **1-31** with inverted stereochemistry at the ribose-C3 position. Compounds **1-32** and **1-33** were identified as time-dependent inhibitors of LuxS.[23]

Based on overall biological evaluation, the best potential LuxS inhibitors reported so far are compounds **1-14** and **1-15**. However, there is still much room for improvement on the design and synthesis of better LuxS inhibitors since all of the published SRH analogs are not functioned as LuxS inhibitors *in vivo*.

1.4 Dimerization Inhibitors

Since many enzymes are homodimers or multimers, disruption of the interactions between protein subunits provides another reasonable way for enzyme inhibition. Two primary examples are dimerization inhibitors of HIV-1 reverse transcriptase (HIV-1 RT) and of inducible nitric oxide synthase (iNOS) from the literature.[24] The first example of a small non-peptidic molecule able to interfere with the dimerization process of HIV-1 RT is TSAO-T (*tert*-butyldimethylsilyl-spiro-amino-oxathiole-dioxide, *Figure 1.11*).[24a] HIV-1 RT is an asymmetric heterodimer that is composed by two subunits named p66 and p51. The most important region of the p66-p51 dimer interface is the β7-β8 loop in p51, which builds a pathway towards the non-nucleoside reverse transcriptase inhibitor (NNRTI) binding pocket and constitutes the binding site for dimerization in the p66 subunit. This loop of the p51 subunit is required for both the catalytic function of the p66 subunit and

the RT dimerization. Based on this information of p66-p51 dimer interface and the structure of the substrate, TSAO-T was developed by Camarasa's group that has unique selectivity for HIV-1 through a specific interaction with the p51 subunit of HIV-1 RT, preventing the dimerization of p51 with p66.[24a]

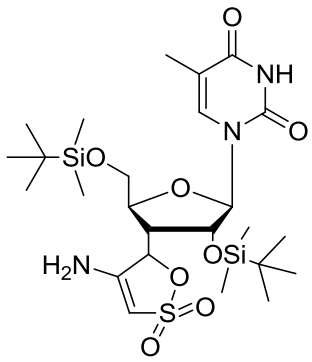
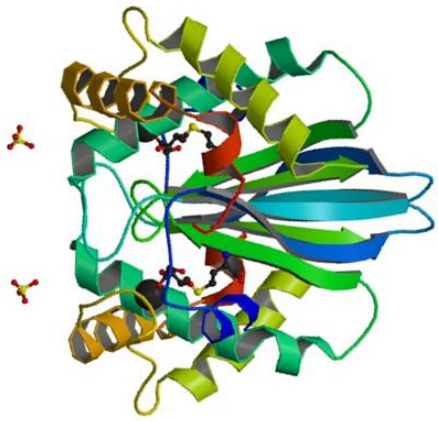


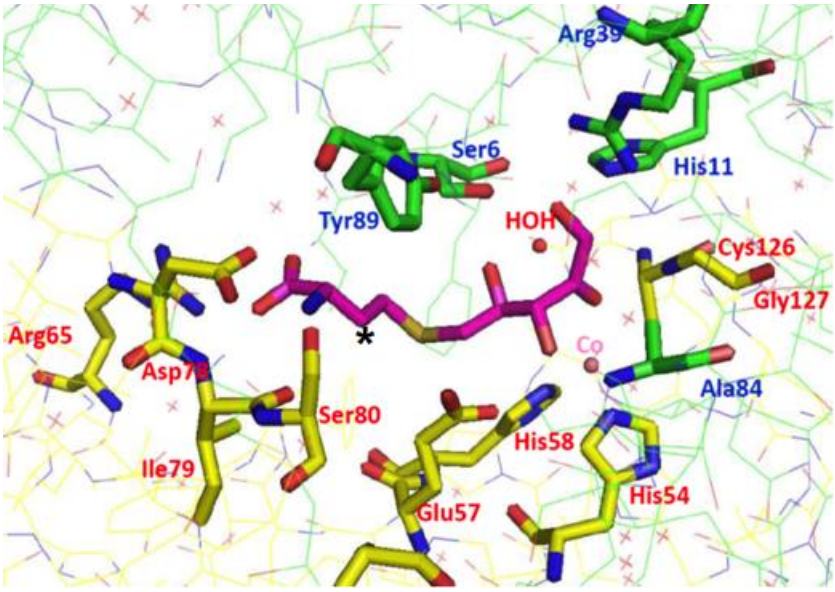
Figure 1.11. **Chemical structure of TSAO-T.** It is the first reported dimerization inhibitor for HIV-1 RT.

1.4.1 Proposed LuxS Dimerization Inhibitors

As a relatively small dimer (35 kDa) with 23% of its surface area buried at the dimer interface, the LuxS enzyme is a good candidate for dimerization inhibition. The SRH binding site is buried in the LuxS dimer and the two monomers of LuxS do not contribute to the binding equally.[25] Instead, one monomer (monomer A, *Figure 1.12*. yellow) which contains the metal ion (Fe^{2+}) contributes more substrate-binding residues, while the second monomer (monomer B, *Figure 1.12*. green) that carries the catalytic residue Cys84 contributes fewer. If a small molecule could maintain tight binding with monomer A of LuxS while preventing proper association with monomer B, which plays the key catalytic role in the cleavage of SRH to DPD, AI-2 biosynthesis could be controlled.



(a)



(b)

***Figure 1.12*. (a) X-Ray crystal structure of LuxS protein.[25] (b) LuxS Binding Site.[25]** (Yellow – Monomer A; green – Monomer B, magenta – ligand; red – residues contributed by Monomer A; blue – residues, including the catalytic parts Cys84, contributed by Monomer B; sticks – binding site residues; *****HCys-C3 position.)

Based on our detailed knowledge of the SRH binding site from the solved LuxS X-ray crystal structure, we have proposed SRH analogs with the potential to serve as LuxS inhibitors (*Figure 1.13*). By the introduction of sterically-demanding groups (alkyl or aryl) at the HCys-C3 position of SRH (*Figure 1.12b* & *1.13*. position labeled *****) these

analogs could potentially bind with monomer A (*Figure 1.12b*. yellow) while blocking the correct association of monomer B (*Figure 1.12b*. green). Thus, the activity of LuxS would be inhibited and quorum sensing would be prevented by blocking AI-2 production, rendering all cells "mute" in a bacterial population. In contrast to previously-reported LuxS inhibitors that typically focus on the modification of the ribose part of SRH (see Section 1.3.1)[20, 23], these molecules focus on the modification of the homocysteine part to modulate the activity of LuxS enzyme by acting as potential dimerization inhibitors. At this time, no one has reported the synthesis of such kind of SRH analogs. The chemical synthesis of these HCys-C3 analogs, and of the SRH substrate itself, is detailed in the chapters that follow.

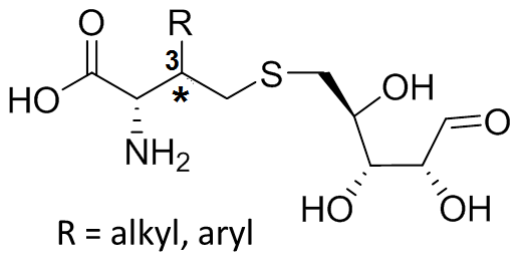


***Figure 1.13*. Proposed LuxS inhibitors.**

Chapter 2. Chemical Synthesis of *S*-Ribosyl-L-homocysteine (SRH)

The first task was to chemically synthesize SRH. The motivation was twofold: (i) to acquire sufficient quantities of SRH to serve as the substrate for LuxS activity/inhibition assays and (ii) to develop the skills necessary to synthesize analogs of SRH later on. The general idea behind the synthesis of SRH is to obtain both an amino acid moiety (*Figure 2.1.* red) and a ribose moiety (*Figure 2.1.* blue), and then build a S-C bond (*Figure 2.1.* **black bold**) between them through a coupling reaction.

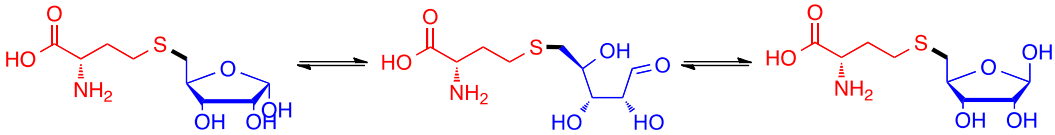
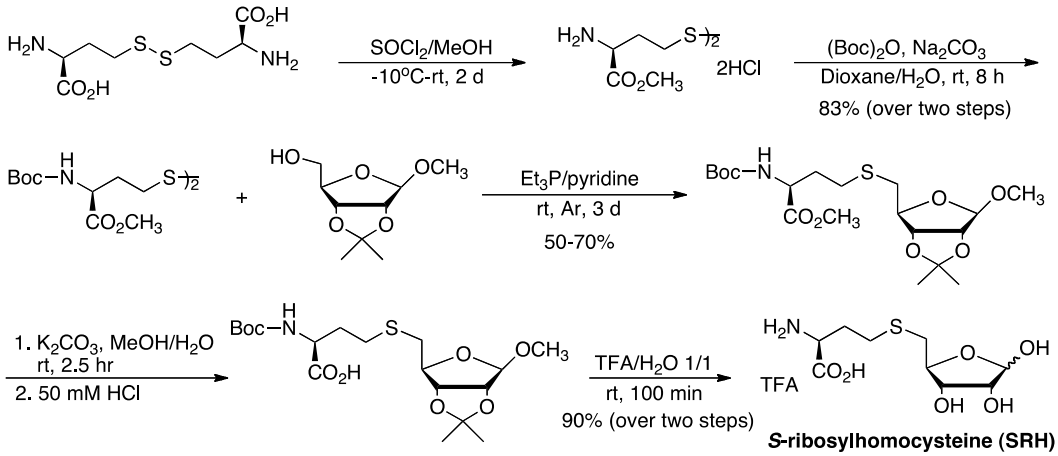


***Figure 2.1.* Cyclic and ring-opened forms of SRH.** The cyclic form of SRH can have either of two stereochemical arrangements: α or β. Left to right: α-cyclic form, ring-opened form, β-cyclic form. (Red – amino acid moiety, **black bold** – S-C bond, blue – ribose moiety.)

2.1 First Attempt – Unsuccessful Implementation of Literature Precedent

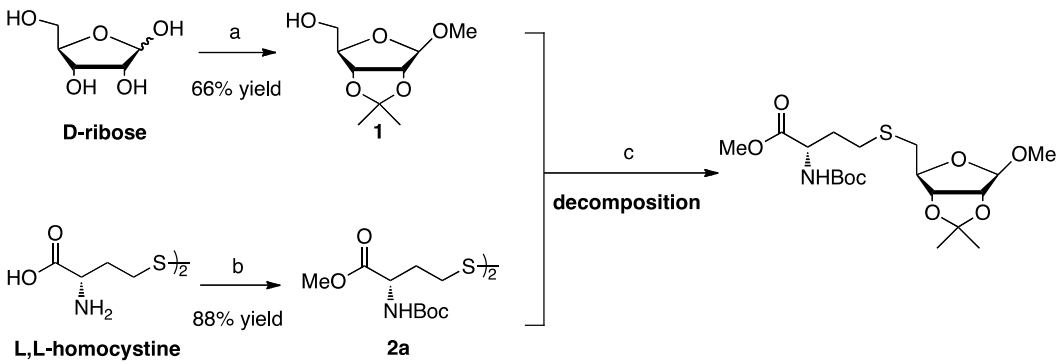
The first chemical synthesis of SRH was reported from Guillerm et al. in 1991 with a low yield of ~5% over eight linear steps.[26] This chemically synthesized SRH was specifically tritiated at the ribose-C5 position and used to assay SRH hydrolase. Their key step was the nucleophilic substitution of C5-ribosyltosylate with the disodium salt of L – homocysteine to form a S–C bond. Later on Zhao laboratory published another more straightforward synthetic method with only five linear steps with a higher overall yield of ~52%.[21] In this straightforward method (*Scheme 2.1*), the amino and carboxyl groups in (L, L)-homocystine were protected in two steps as *tert*-butyl carbamate (Boc) and methyl ester, respectively. The key step was the S–C bond formation, effected by a modified

Mitsunobu coupling between the fully protected homocystine and the D-ribose derivative.[27] Fully-protected *S*-ribosylhomocysteine was then deprotected by standard procedures resulting in the trifluoroacetic acid **(**TFA) salt of SRH.



***Scheme 2.1*. SRH synthesis reported by Zhao et al.[21]** Mitsunobu-type coupling of protected homocystine to ribose moiety effected the S-C bond (the third step above).

Our attempt to implement this synthetic procedure (shown in *Scheme 2.2*) began with commercial D-ribose and (L,L)-homocystine. Ribose derivative **1** and the fully protected homocystine **2a** were successfully obtained in 66% and 88% yields, respectively. However, in our hands, the Mitsunobu-type coupling of compound **1** with compound **2a** failed after repeated attempts, either under the presence of tri-ethylphosphine (Et_3P) as in Zhao's protocol or tri-*n*-butylphosphine (*n*-Bu_3P) that also serves as a catalyst for this type of reaction.[27] Even after modification of the experimental procedure (i.e. adjusting the reagent equivalents, prolonging or reducing reaction time, and lowering the reaction temperature), S-C bond formation was still unsuccessful.

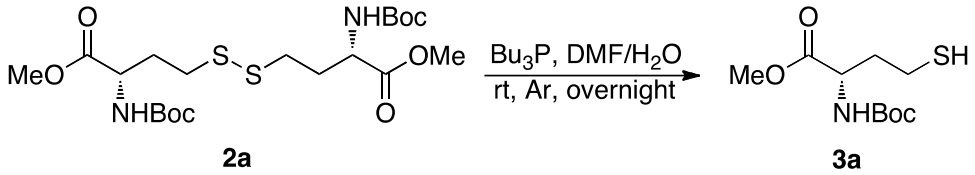


Scheme 2.2. **Unsuccessful attempt of HCys-ribose coupling.** Reaction conditions: a. MeOH/acetone, conc. HCl, reflux, 1.5 h; b. (i) MeOH, $SOCl_2$, 0 ^{o}C-rt, Ar, 3 d; (ii) $(Boc)_2O$, dioxane/Na_2CO_3(aq), 0 ^{o}C-rt, overnight; c. Et_3P (or *n*-Bu_3P), pyridine, rt, Ar, 3 d.

We utilized both thin layer chromatography (TLC) and nuclear magnetic resonance (NMR) methods to analyze the crude product of the attempted coupling reaction. Our TLC plate for the crude product of coupling reaction showed several new spots with different R_f values from compound **1** and **2a**, which suggested a decomposition happened (see Appendix 7.1). Additionally, neither any proton peaks that would indicate the existence of starting materials (compound **1** and **2a**) nor new proton peaks that would suggest the S-C bond formation were observed in the ^{1}H NMR spectrum of the crude product, indicating that the coupling reaction failed. Thus, decomposition was observed after purification rather than desired fully protected ribosylhomocysteine product.

The Mitsunobu-type reaction used to create the S-C bond in Zhao's synthesis can be broken down into two steps: (1) the cleavage of the S-S bond in protected disulfide **2a** and then (2) S_N2-type coupling with ribose moiety **1**. At first, it was hypothesized that the failure of the Mitsunobu reaction might be attributable to the lack of initial cleavage of the S-S bond. To test this hypothesis, we ran a single-step reaction to determine if disulfide **2a** could convert to thiol (-SH) under the Mitsunobu conditions. The protected disulfide **2a** transformed to thiol **3a** under the presence of tri-*n*-butylphosphine

successfully in nearly quantitative yield after flash column chromatography purification (followed by ^{1}H NMR characterization). [a] Thus our initial hypothesis was disproved by this result.



2a **3a**

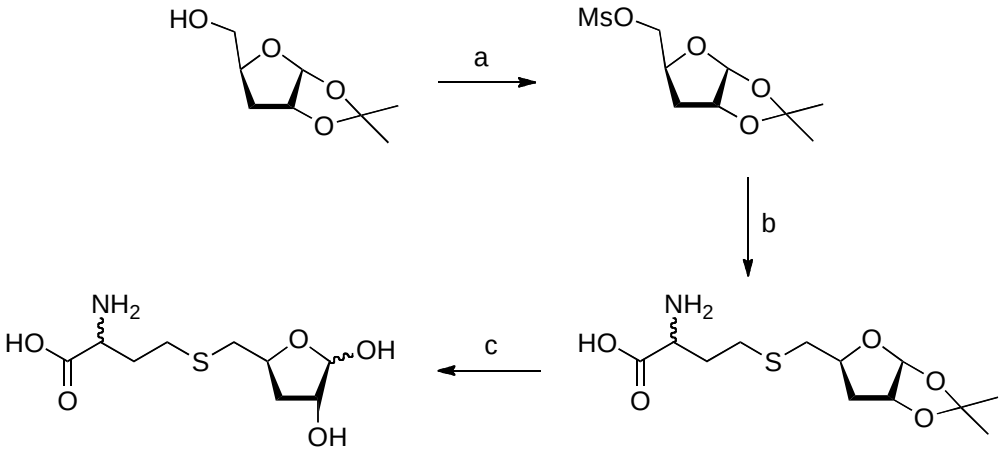
***Scheme 2.3*. Initial step of modified Mitsunobu reaction – cleavage of the S-S bond.**

Therefore, it was concluded that the reason for the failure of the Mitsunobu reaction must be unsuccessful nucleophilic substitution between the 5-hydroxyl group of the ribose and the thiol/thiolate of fully protected homocysteine. To overcome this problem, one or both better coupling partners (either in place of the ribose moiety **1** and/or amino acid moiety **2a**) would be required to be utilized in the nucleophilic substitution.

2.2 Second Attempt – Successful Synthesis of SRH (without Quantification)

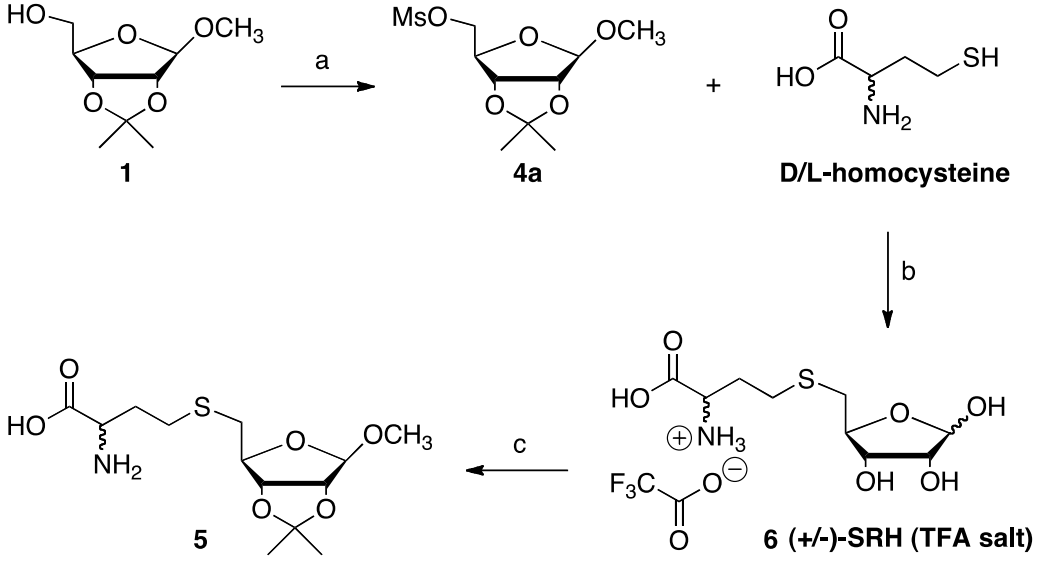
A second attempt at the synthesis of SRH was inspired by the work of Wnuk et al (2009)[23], who reported the synthesis of several modified SRH analogs as potential LuxS inhibitors, including 3-deoxy-SRH (*Scheme 2.4*). Mesylation at the 5-free hydroxyl group of the ribose, displacement with the thiolate derived from D/L-homocysteine under basic (NaOH) conditions, and treatment with aqueous trifluoroacetic acid (TFA) gave the desired 3-deoxy-SRH analog that lacks a hydroxyl group at the ribose C-3 position.

a. The nearly quantitative yield was determined by weight after column chromatography purification and then ^{1}H NMR was utilized to characterize the purity of thiol **3a**.



***Scheme 2.4*. Wnuk's synthesis of SRH analog modified at the ribosyl C-3 position.**[23] Reagents: (a) MsCl/Et_3N, 97%; (b) D/L-HCys/NaOH/MeOH/H_2O, 31%; (c) TFA/H_2O, 90%.

In accordance with this publication, we applied synthesized ribosyl mesylate **4a** to couple with commercial D/L-homocysteine (D/L-HCys) that effected the S-C bond formation successfully. After a standard deprotection procedure by TFA, the desired product SRH•TFA salt was obtained (*Scheme 2.5*).



***Scheme 2.5*. Successful coupling of HCys and activated ribose moiety**. Reaction conditions: a. MsCl, Et_3N, DCM, 0 ^oC–rt, Ar, 30 min, 99%; b. 1 M NaOH/H_2O, 60 ^oC, Ar, overnight; c. TFA/H_2O, 0 ^oC–rt, 3 h.

In comparison to Zhao's method (shown in *Scheme 2.1*), this synthetic route has several advantages. Firstly, the free 5-hydroxyl group of the ribose is as converted into the mesylate, which was a better leaving group than the hydroxyl group in substitution reactions. Secondly, the native D/L-HCys, with its free thiol group, was utilized here instead of protected homocystine, avoiding the S-S bond cleavage step. Apparently, in our hands the pair of ribosyl mesylate **4a** and unprotected HCys were better coupling partners than ribose moiety **1** and fully protected homocystine **2a** utilized by Zhao. Finally, this synthetic method needed fewer steps to obtain the final product SRH, which could possibly improve the overall yield of the reaction sequence.

However, this synthetic method posed insurmountable disadvantages as well. Firstly, the coupling reaction utilized aqueous solution as solvent. Since the protected SRH **5** is a water-soluble compound, this meant the product could contain some residual water from the solvent, [b] which is undetectable by routine analysis (i.e. ^{1}H NMR). Secondly, the coupling of key compounds **4a** and HCys using NaOH produced an inseparable salt byproduct (NaCl) in addition to the protected SRH **5**. Even though the mixture produced the desired (+/-)-SRH•TFA salt (as characterized by ^{1}H NMR, see Appendix 7.2) in the next deprotection step, the byproduct salt still persisted in the final product since our yield of (+/-)-SRH•TFA was far over 100%. It was difficult to isolate this byproduct from the final product (+/-)-SRH•TFA salt because both salts are water-soluble.[c] In Wnuk's report, a high performance liquid chromatography (HPLC) method was applied to purify the SRH. However, due to a limitation of facilities in our department, the HPLC purification method was impractical; in order to obtain sufficient quantities of SRH, several days of continuous work and a prep-scale column would be required.

b. Separation funnel technique (solvent system CH_2Cl_2/H_2O) failed to extract compound **5**.
c. Recrystallization from MeOH attempted but failed.

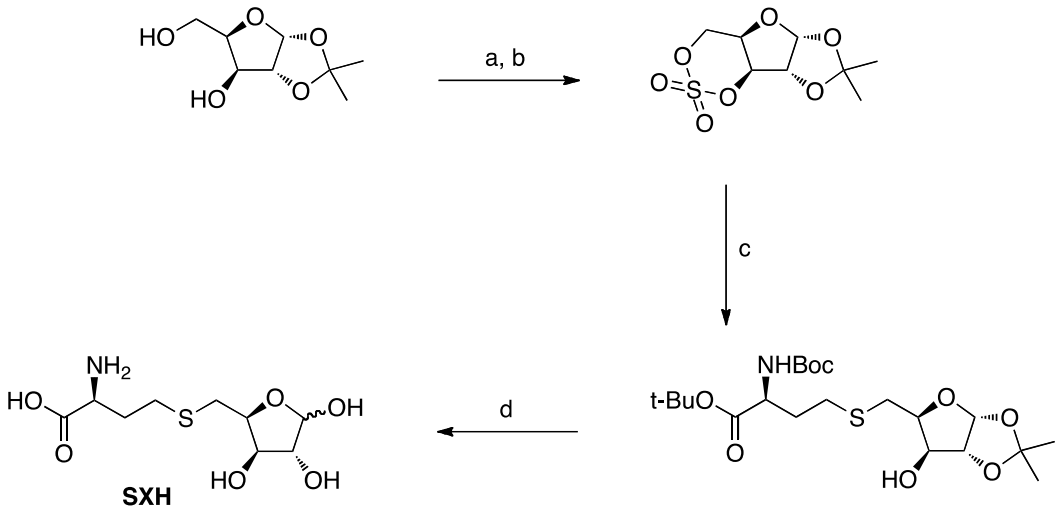
Instead, we introduced C18 SEP-PAK cartridges (Water Associates, Inc.) here to purify the SRH. The sorbent of C18 cartridge is made of a silica-based bonded phase with strong hydrophobicity. It can adsorb analytes of weak hydrophobicity from aqueous solutions and becomes a convenient method in the solid phase extraction. It has similar behavior with reversed-phase HPLC columns, but much smaller and without the need for specialized equipment.[46] To purify SRH with C18 cartridges, first an aqueous solution of the mixture containing SRH and byproduct salt was made. It was expected that the SRH would be absorbed by the cartridges while the byproduct salt would elute with the residual aqueous solution. Then the SRH could be washed from the cartridges in a separate step using a less polar solvent (i.e. MeOH or acetonitrile) and concentrated by rotary evaporation. After purification by C18 cartridges (initial weight of crude product ~ 0.8 g) in this manner, we obtained a product with fewer impurities, as indicated by its reduced weight (~ 0.3 g, see Appendix 7.3). However, it was inferred that the byproduct salt still persisted in the product since the yield of SRH was still over 100% of theoretical. As this byproduct salt was undetectable by NMR, we could not determine the exact yield of the SRH through this method.

The fact that these (+/-)-SRH•TFA crystals might contain some amount of residual water and byproduct salt meant that we could not accurately determine the exact amount of (+/-)-SRH produced by our synthetic protocol by weight. This was problematic, as SRH is meant for use in a biochemical assay to determine the catalytic activity of LuxS in the presence and absence of putative inhibitors and therefore needs to be quantified exactly. To address this issue, a fluorescamine quantification method for (+/-)-SRH in aqueous solution was attempted in the lab by Richa Gupta.[45] However, this biochemical assay proved unreliable, with non-reproducible results and unacceptably high error when used to quantify known standards. Therefore, an alternate synthetic method, one that would

produce SRH cleanly without inseparable, undetectable byproducts was necessary.

2.3 Third Attempt – Successful Synthesis and Quantification of SRH

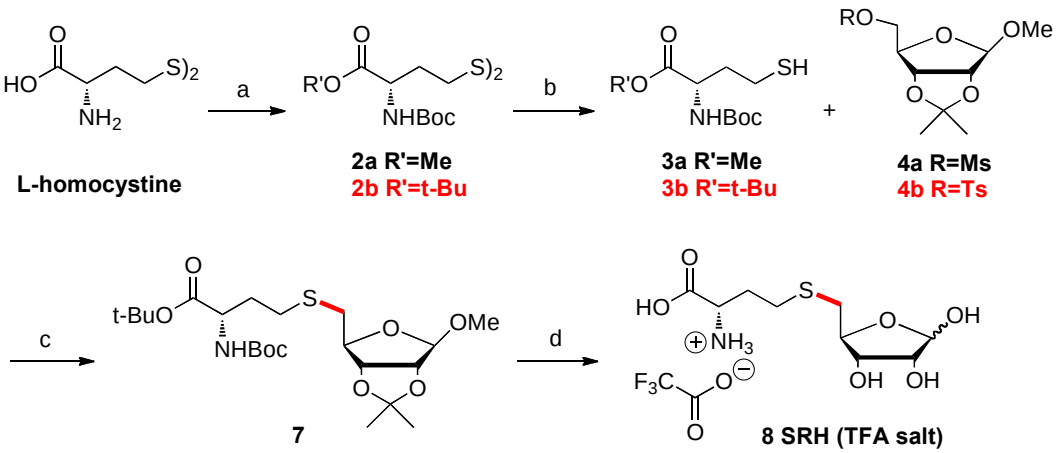
An alternate synthetic approach to the formation of the S-C bond of SRH was also reported by Wnuk for the synthesis of a different SRH analog, *S*-(5-deoxy-D-xylofuranos-5-yl)-L-homocysteine (SXH) (*Scheme 2.6*).[23] Here, a protected homocysteine (BocNH–CH(CH_2CH_2SH)–CO_2t-Bu) and an activated ribose moiety were used in the coupling reaction under the presence of *n*-BuLi as the base for S_N2 substitution.



Scheme 2.6. **Wnuk's synthesis of SXH.[23]** Reagents and conditions: (a) $SOCl_2/Et_3N$/DCM/-78 °C to rt, yield 74%; (b) $RuCl_3/NaIO_4/CCl_4/CH_3CN/H_2O$/0 °C to rt, yield 67%; (c) (i) BocNH–CH(CH_2CH_2SH)–CO_2t-Bu/BuLi/DMF/0 °C to rt, (ii) THF/H_2SO_4/H_2O/0 °C; (d) TFA/H_2O/0 °C to rt, yield 61%.

Inspired by Wnuk's synthesis of SXH, a sequence was initiated to synthesize SRH utilizing protected homocysteine derivatives **3** and activated ribose moieties **4** in a coupling reaction with *n*-BuLi (*Scheme 2.7*). For the amino acid moiety (refer to *Figure 2.1*), a fully protected homocysteine **2** was utilized as in Zhao's synthesis but converted to

the free thiol **3** as in Wnuk's application of free HCys to the synthesis of 3-deoxy-SRH. For the ribose moiety **4** (refer to *Figure 2.1*), a good leaving group was introduced as in Wnuk's synthesis of SXH. According with Wnuk's report, the use of anisole in addition to the mixture of trifluoroacetic acid (TFA) and water at the final deprotection step also proved a beneficial modification.[23] Gratifyingly, compound **7** was satisfactorily purified and quantified before the near-quantitative conversion to the TFA salt of SRH **8**, which could be lyophilized to a pure, powdery solid that could be easily measured by weight. Another benefit of this synthetic method is its use of enantiomerically pure materials to produce the single desired isomer of SRH in very good overall yield (~60% over five linear steps).



Scheme 2.7. **Successful hybrid method of SRH synthesis.** Reaction conditions: (a) (i) 10% Na_2CO_3 (aq)/dioxane, $(Boc)_2O$, 0 °C-rt, overnight, (ii) *tert*- butyl- 2,2,2- trichloroacetimidate, DCM, Ar, rt, overnight, 90%; (b) *n*-Bu_3P, DMF/ H_2O, Ar, rt, overnight, without purification used for next step; (c) *n*-BuLi, DMF, Ar, 0 °C–rt, overnight, 70%; (d) TFA/anisole/H_2O, 0 °C–rt, 6 h, 94%.

In a separate effort towards the synthesis of SRH HCys-C3 analogs (see Section 3.1), we had observed that free thiol **14b** was prone to be oxidized into disulfide **14a** when exposed to air (see Appendix 7.4). Thus, in later implementations of the hybrid method of

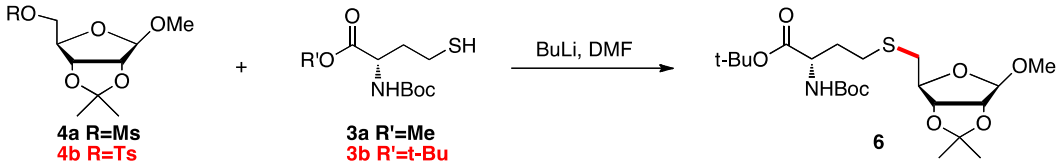
SRH synthesis shown in *Scheme 2.7*, purification of thiol **3b** was skipped in order to prevent contact with air (see experimental procedure of compound **7** in Chapter 5.). The coupling reaction was still successful, and with a good yield of 70%, which means the existence of tri-*n*-butylphosphine does not effect the success of coupling reaction. Thus, the cleavage of disulfide and the coupling reaction could be combined as one step, which is corresponding with the Mitsunobu-type coupling implemented by Zhao originally (*Scheme 2.1*).

2.3.1 Impact and Future Applications of Revised Synthesis

To evaluate our synthesized SRH obtained by the third synthetic method, it was utilized as the substrate in an Ellman's assay for LuxS activity (*Figure 2.4*).[45] This chemically synthesized SRH was effective to be used as the substrate in the determination of LuxS activity (see Appendix 7.5).[d]

In addition, a general idea was concluded towards the synthesis of SRH or its analogs: (i) obtaining a homocysteine moiety and a ribose moiety; (ii) then building a S-C bond between the two moieties. Particularly, considering that the S-C bond formation is the key step for the synthesis of SRH, it is interesting to note that the identity of both the protecting group **R'** on the carboxylate **3** and the leaving group **R** on the ribose moiety **4** had a significant effect on the success of this coupling reaction. In no combination did the use of methyl protection on **3** or mesylate activation on **4** result in successful formation of the S-C bond (*Scheme 2.9*).

d. Biological assays were performed by Richa Gupta.



	Mesylate (Ms) **4a**	Tosylate (Ts) **4b**
Methyl (Me) **3a**	✗	✗
Tert-butyl (tBu) **3b**	✗	✓

Scheme 2.9. **Coupling results of combinations of protecting groups and leaving groups.**

This information, obtained after extensive troubleshooting (note that the original synthesis of Zhao utilized **2a** rather than **2b**, and that of Wnuk, which was successful in our hands, utilized **4a** rather than **4b**), could be used to inform the synthetic pathways towards SRH analogs (Chapter 3).

Chapter 3. Synthesis of SRH Analogs Modified at the Homocysteine-C3 Position

As LuxS is responsible for the biosynthesis of AI-2, small molecules that inhibit LuxS dimerization could prevent quorum sensing by blocking AI-2 production. Since it is known that SRH binds in the middle of LuxS dimer, this protein is a good candidate for dimerization inhibition. The introduction of a bulky steric group at the HCys-C3 position of SRH could potentially prevent the correct association of the two monomers of LuxS. Therefore, these SRH analogs modified at the HCys-C3 position are potential inhibitors of LuxS.

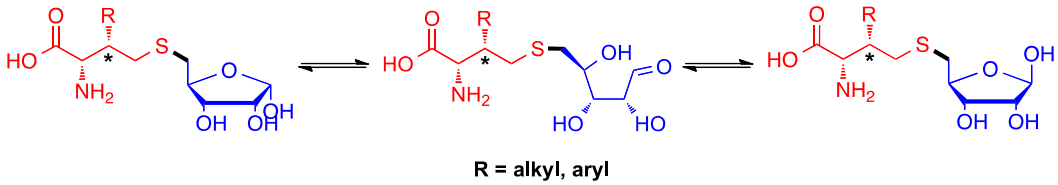


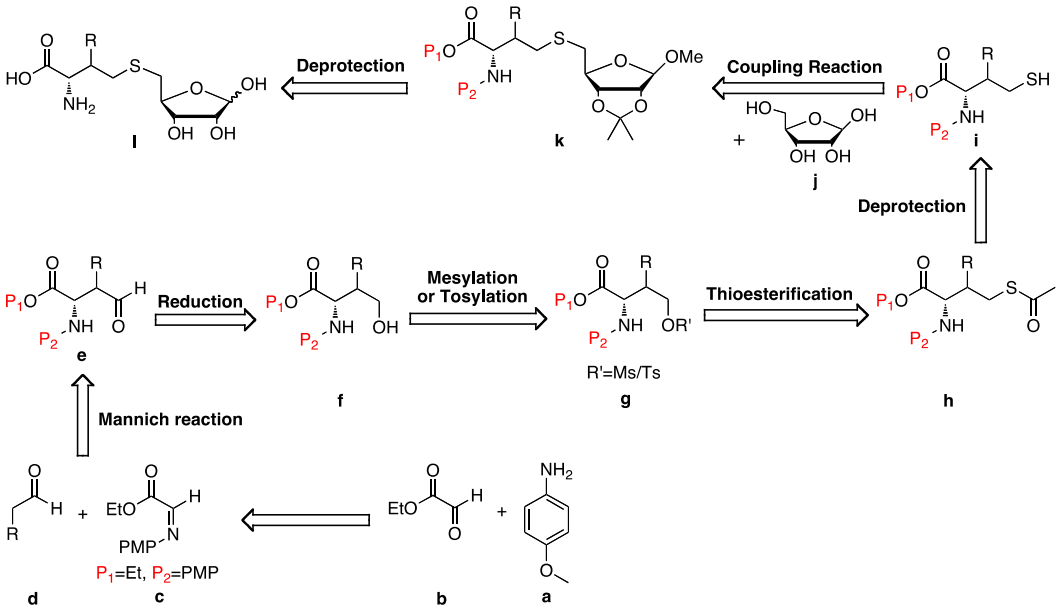
Figure 3.1. **Cyclic and ring-opened forms of SRH HCys-C3 analogs.** The cyclic form of SRH analogs modified at the HCys-C3 position can have either of two stereochemical arrangements: α or β. Left to right: α-cyclic form, ring-opened form, β-cyclic form. (Red – modified homocysteine moiety, **black bold** – S-C bond, blue – ribose moiety, *HCys-C3 position.)

Similar to the synthesis of SRH, the synthesis of these HCys-C3 derivatives can be broken down into two phases: the synthesis of a modified homocysteine moiety containing an additional "R" group at the HCys-C3 position* (*Figure 3.1.* red) and the coupling of this novel amino acid moiety with the ribose moiety as in SRH synthesis (*Figure 3.1.* blue).

3.1 First Attempt – Mannich Reaction Approach

It was initially proposed that SRH analogs with substitution at the HCys-C3 position

could be synthesized from commercial compounds ethyl glyoxylate, *p*-anisidine, and ribose according to the retrosynthesis shown in *Scheme 3.1*.

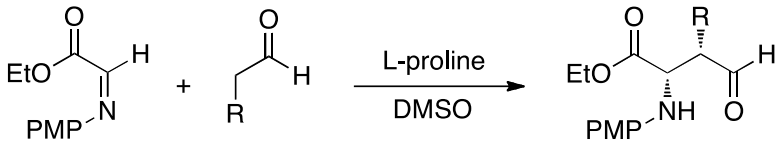


Scheme 3.1. **Retrosynthetic analysis of SRH HCys-C3 analogs.** (P_1, P_2 = protecting groups)

Imine **c**, which could be obtained from the reaction between commercial ethyl glyoxylate **b** and *p*-anisidine **a**, could be coupled with aldehyde **d** in the next step of Mannich reaction. Then the obtained Mannich-type aldehyde product **e** could be reduced to alcohol **f** through a standard reduction procedure. The free hydroxyl group of the alcohol could be protected into either mesylate or tosylate **g**, both of which could be transformed into thioesters **h** later. Then the thioester **h** could be deprotected into thiol **i**, which is the desired HCys moiety with a substitution at the C3 position. Then the HCys-C3 moiety **i** could be coupled with ribose **j** to obtain the protected SRH **k**. After a standard deprotection procedure, the SRH HCys-C3 analog **l** could be obtained.

The Mannich reaction is the key step in this reaction sequence, completing the substitution of the HCys-C3 position with the new "R" group (*Scheme 3.2*). Barbas et al reported an asymmetric Mannich reaction coupling the imine derived from ethyl

glyoxylate and *p*-anisidine[28] with unmodified aldehydes, such as propionaldehyde (R = CH_3), butyraldehyde (R = CH_2CH_3), etc., catalyzed by L-proline in DMSO at room temperature. This L-proline catalyzed Mannich reaction mechanism is stereoselective with a preference for the product having (*S*, *S*) configuration (*Figure 3.2*).[29] It is proposed that the imine can react with different aldehyde donors to produce a series of Mannich products with different "R" groups.



Scheme 3.2. **Mannich reaction.** (Proposed substituents: R = Me, Et, *i*-Pr, *t*-Bu, Ph.)

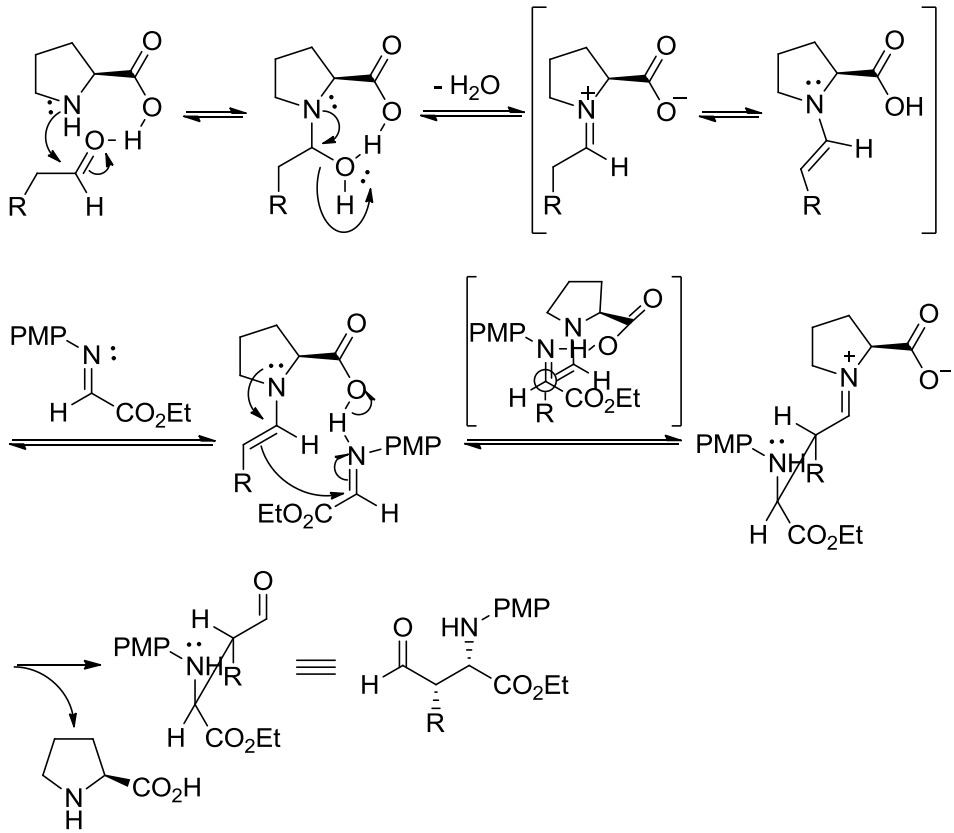
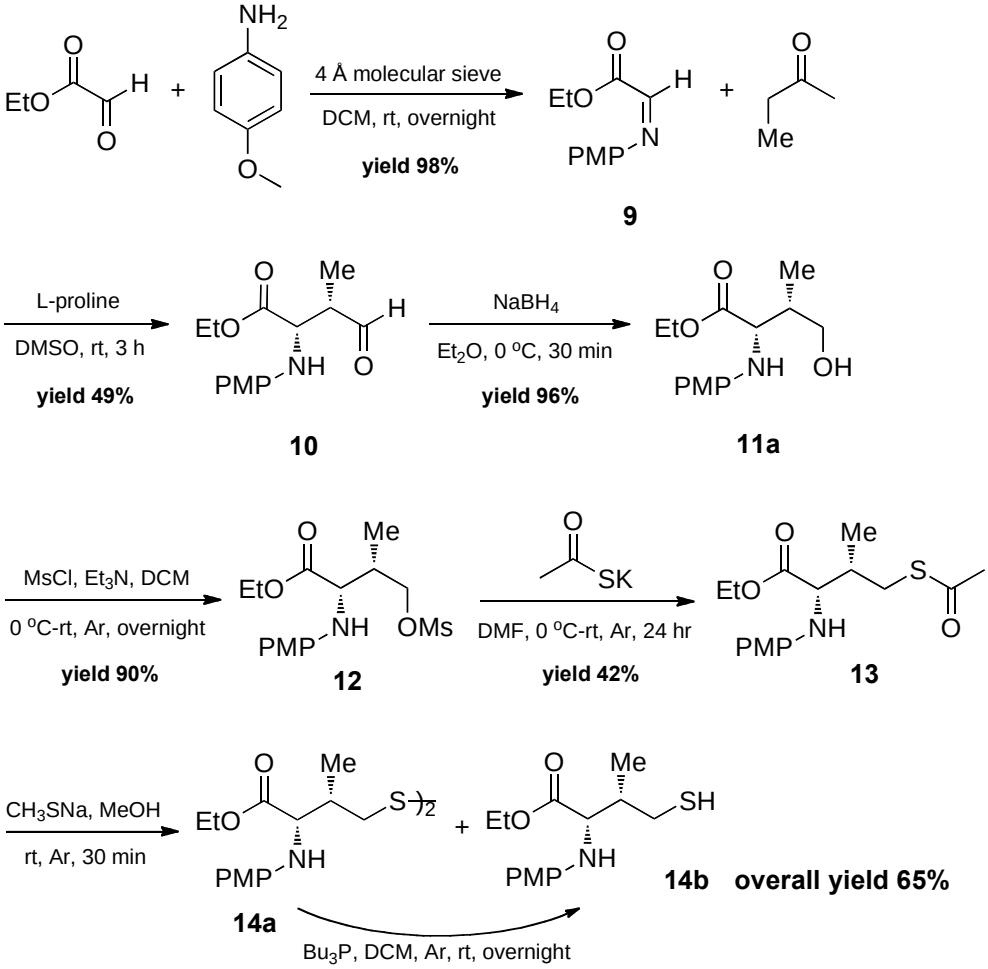


Figure 3.2. **Mechanism of L-proline catalyzed asymmetric Mannich reaction.** It is prone to form (*S*, *S*) confirguration product.[30]

Our initial approach to the synthesis of SRH HCys-C3 analogs began with the synthesis

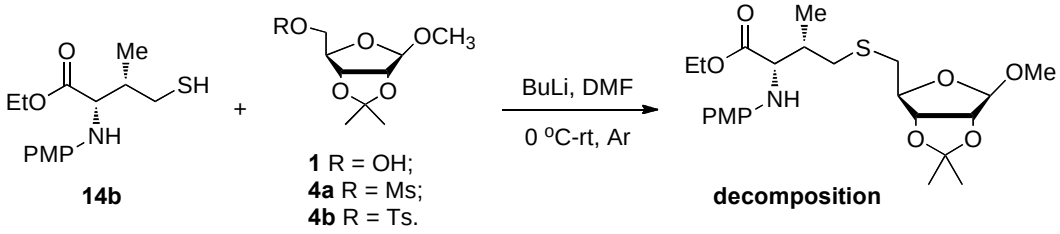
of the modified homocysteine moiety (*Figure 3.1*. red) to be later coupled with an appropriate ribose moiety (*Figure 3.1*. blue), according to the protocol established in Chapter 2.

The synthetic pathway towards the synthesis of modified homocysteine moiety with a methyl substituent (R = Me) at the C3 position is shown in *Scheme 3.3*. Imine **9** was derived straightforwardly from commercial ethyl glyoxylate and *p*-anisidine and was of sufficient purity to be used for next step without purification.[28] The asymmetric Mannich reaction between imine **9** and propionaldehyde was carried out using L-proline as the catalyst, which gave the key methyl-substituted β-amino aldehyde **10** {with presumed stereochemistry of (*S*, *S*)} in 49% yield after silica gel column chromatography purification.[29] After standard reduction by sodium borohydride, alcohol **11a** was obtained and then converted to mesylate **12** in 87% yield over two steps. The conversion of **11a** to tosylate (OH➔OTs) was unsuccessful; decomposition was observed after repeated attempts. According with the standard procedures from published references, mesylate **12** was converted in two steps into a mixture of disulfide **14a** and thiol **14b**.[31, 32] Since only thiol **14b** was required for the subsequent coupling reaction, disulfide **14a** was transformed into thiol by a standard cleavage under the presence of tri-*n*-butylphosphine.[33] After this standard conversion, the overall yield of **14b** from **13** was improved substantially (from 15% to 65% over 2 steps).



Scheme 3.3. **Successful synthesis of HCys-C3 substituted moiety.**

Next we attempted to utilize ribose moieties **1**, **4a** and **4b**, as used in the synthesis of SRH to couple with C3-methyl homocysteine moiety **14b**, in which compound **4b** had successfully worked in the SRH synthesis in our hands. However, the coupling reaction was unsuccessful, resulting in decomposition of compound **14b** and all ribose moieties.



***Scheme 3.4*. Unsuccessful coupling between the C3 substituted homocysteine moiety with three ribose moieties utilized in the synthesis of SRH or analogs.** Additional deprotection steps would be required to produce the final SRH C3 analog.

Our prior studies into the synthesis of SRH (see Chapter 2) demonstrated that the identity of protecting groups on the HCys moiety could have a significant effect upon the success of the coupling reaction. The possible reason for the failure of this initial approach to synthesis of SRH HCys-C3 analogs could be the different protecting groups of the modified homocysteine moiety **14b** (Et and PMP) as compared to those of compound **3b** (*t*-Bu and Boc) as shown in *Figure 3.2*.

3.2 Second Attempt – Protecting Group Modifications of C3-Substituted HCys

Based on our acknowledgement that the protecting groups of the amino acid moiety likely have a great effect on the coupling reaction, our next efforts were focused on the synthesis of the target compound **15** shown in *Figure 3.3*. It has a similar structure to compound **3b**, which was previously successfully coupled with ribose derivative **4b** except for the substitution at the C3 position.

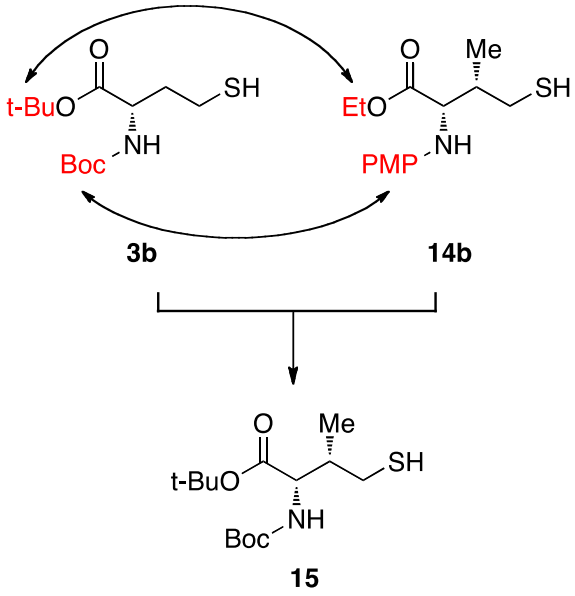
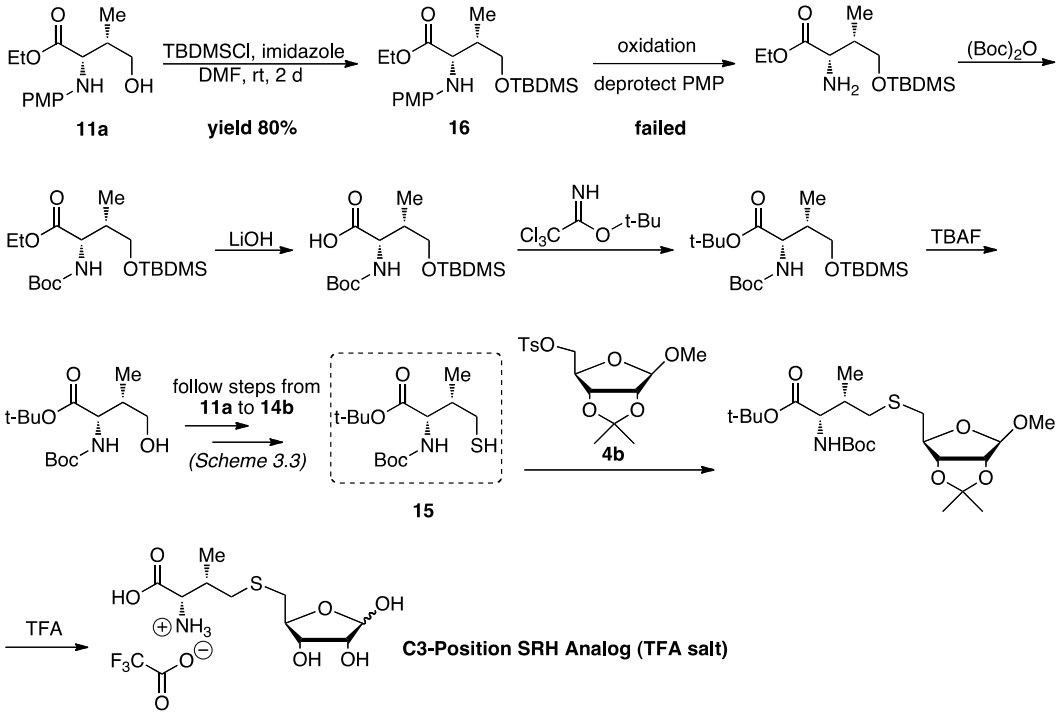


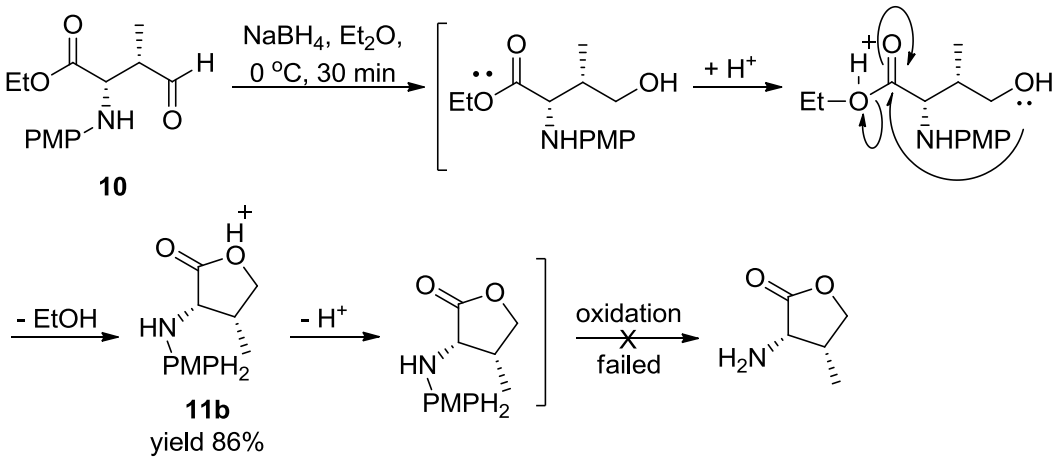
Figure 3.3. **Target C3 position substituted homocysteine moiety 15.** Desired compound **15** has the same protecting groups as compound **3**, which is the homocysteine moiety used in the successful synthesis of SRH (see *Scheme 2.9*).

To obtain target compound **15**, we returned to earlier stages in the synthesis of original target **14b** (*Scheme 3.3*) and initially planned to complete two conversions: (1) PMP (of **14b**) to Boc (as in **3b**) and (2) EtO- (also of **14b**) to tBuO- (also as in **3b**) through several steps (*Scheme 3.4*). The first conversion (PMP➔Boc) began at intermediate **11a** (*Scheme 3.5*). Protection of the free hydroxyl group of **11a** with TBDMS was necessary due to the highly oxidative conditions required for the removal of the PMP protection. Silyl ether **16** was obtained successfully with a good yield of 80%. Although this protection proceeded easily, the deprotection of the PMP group was unsuccessful using numerous oxidizing reagents, including ceric ammonium nitrate (CAN)[34], phenyl iodoacetate ($PhI(OAc)_2$)[35], trichloroisocyanuric acid (TCCA) and periodic acid (H_5IO_6)[36]. Attempts to remove the PMP from intermediate **11a** prior to silyl protection by the same oxidative conditions had also failed. Thus, the sequence reported in *Scheme 3.5* was abandoned.



***Scheme 3.5*. Proposed approach to the replacement of two protecting groups.** The following oxidizing reagents were utilized for the deprotection of PMP group: CAN, $PhI(OAc)_2$, TCCA and H_5IO_6.[34-36]

The synthesis of **11a** by reduction of **10** (*Scheme 3.3*), as already established in the synthesis of modified HCys **14b**, interestingly gave a different product here under the exact same reduction conditions. Whereas **11a** was an oil in the original synthesis, this new product was a solid. Compound **11a** was not observed in this product by ^{1}H NMR. The structure for this new product **11b** (as characterized by ^{1}H and ^{13}C NMR and IR) and a possible explanation of formation are shown in *Scheme 3.5*. We also attempted to deprotect the PMP group from **11b** by the same oxidative conditions mentioned above, with the hope that we could later run a ring-opening reaction and reutilize this unexpected compound **11b** to continue the synthesis. However, the removal of PMP group from **11b** was likewise unsuccessful.

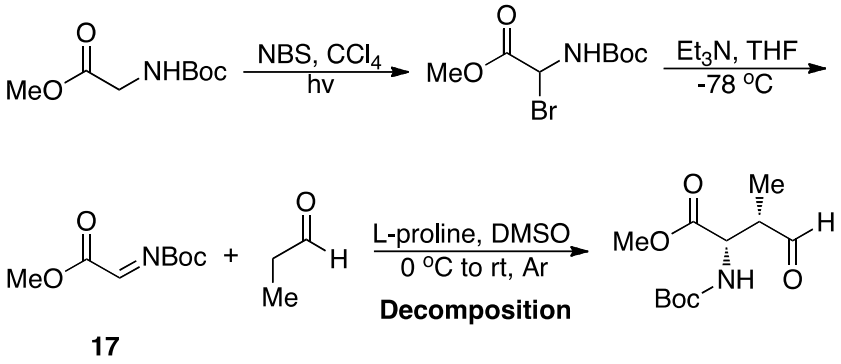


***Scheme 3.6*. Mechanism of the formation of unexpected 5-membered ring product 11b.**

Since the removal of the PMP protecting group proved an insurmountable problem, the use of an alternate imine (in place of compound **9**) for the Mannich reaction, ideally having the Boc protecting group in place of PMP from the very beginning, was proposed.

3.3 Third Attempt – An Alternate Imine Synthetic Pathway

Realizing the difficulty of removal of the PMP protecting group, another synthetic pathway was proposed to utilize an alternate imine (to replace compound **9**) having the desirable Boc protecting group in place of PMP from the start. The synthesis of imine **17** with Boc protect on was approached by Armstrong's report in 2005 as shown in *Scheme 3.6*.[37] It was anticipated that this new imine could react stereospecifically with simple aldehyde donors to produce the desired Mannich products under the presence of L-proline, as demonstrated previously.[29]



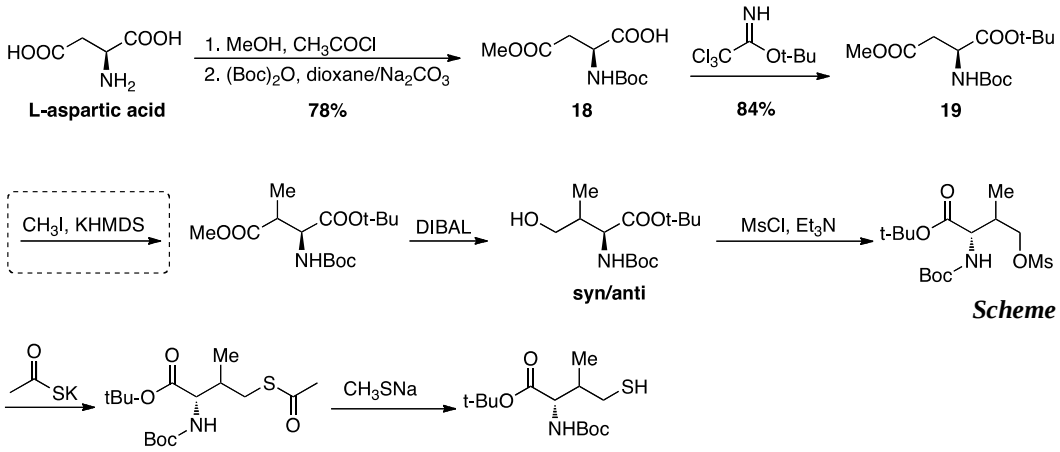
Scheme 3.7. **Mannich reaction carried with an alternate imine.**

However, imine **17** was reported to be quite unstable even at low temperature of -78°C and therefore needed to be used for the next step immediately, without purification.[37] It was difficult to characterize compound **17** from the ^{1}H NMR spectrum of the crude product. Next, a Mannich reaction with propionaldehyde employing similar reaction conditions as applied previously was unsuccessful. Since we could not determine whether we obtained desired imine **17** or not in the earlier step, we cannot identify which step was the reason for the failure of the Mannich reaction under these conditions.

3.4 Fourth Attempt – Alkyl Iodine Pathway

At this point, an alternative approach the stereochemical product bearing the novel "R" group substituent was needed. The reasons are as follows: (1) The product from the Mannich reaction is unpredictable; we could obtain two different products (**11a** and/or **11b**) through the same reaction. (2) The PMP group of the imine **9** that is utilized in common asymmetric Mannich reactions is intransigent to deprotection. Both cases prevent the later steps required for the synthesis of SRH HCys-C3 analogs. Therefore, another synthetic method avoiding using the Mannich reaction to produce the compound substituted at the C3 position was required.

In light of Wolf's report on a general synthetic methodology for preparation of lactams[38], we proposed the following synthetic pathway towards the synthesis of C3-substituted HCys (shown in *Scheme 3.8*). Beginning with the commercially available L-aspartic acid, selective protection of the two carboxyl groups into β-methyl ester and α-*tert*-butyl ester, and then amino group to *tert*-butyl carbamate would produce the fully protected aspartic acid **19**. The next step is key step in this proposed synthesis: reaction with methyl iodine to accomplish methyl substitution at the C3 position. Then, standard reduction with DIBAL would afford an alcohol that is similar to compound **11a**. By following previously-successful steps, that alcohol could be converted to a thiol having the same protecting groups as the homocysteine moiety used in the successful synthesis of SRH (*Scheme 2.7*).



3.8. **Synthesis of modified homocysteine moiety inspired by Wolf et al.[37]** This plan failed at the key step (third step above) of reaction with methyl iodine.

The L-aspartic acid was fully protected as compound **19** through 2 steps. Compounds **18** and **19** were successfully characterized by NMR with the excellent yields of 78% and 84%, respectively. However, the reaction between the fully protected L-aspartic acid **19** and the methyl iodine was unsuccessful resulted in decomposition. The possible reason for this failure may be protecting groups Boc and *t*-Bu, which perhaps shield the C3

position of compound **19** from substitution. Also, different bases (i.e. KH, LDA) could be tried for the crucial reaction with methyl iodine, in place of KHMDS.

Overall, all attempts tried towards the synthesis of SRH C3 analogs were unsuccessful due to different reasons. However, the information obtained could be helpful for the future synthesis of HCys-C4 analogs or HCys-C3/C4 analogs proposed in our lab. These SRH analogs are also modified at the homocysteine moiety and designed to maintain as many binding contacts between SRH and monomer A of LuxS as possible, yet prevent the association with monomer B that carries the catalytic Cys84 residue.

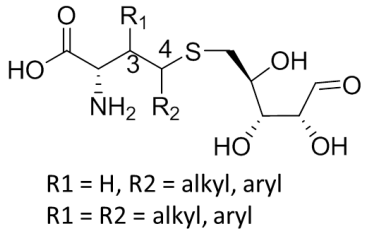


Figure 3.4. **Other proposed SRH analogs as potential LuxS inhibitors in our lab.** Introduction of steric bulk at positions 3 and/or 4 of SRH as shown may impede the approach of monomer B while maintaining tight binding with monomer A.

Chapter 4. Appendices

4.1 TLC Analysis

In the TLC analysis of the crude product from a reaction (shown in *Figure 4.1.a*), it was expected if there was only one new spot shown on the TLC plate, that would mean coupling reaction likely succeeded. Alternatively, if there were still two separated spots with same R_f values as starting materials Reagent **1** (**R1**) and Reagent **2** (**R2**), that would mean the two starting materials had not reacted with each other. Finally, if several new spots other than starting materials **R1** and **R2** showed on the TLC plate, then the reaction was possibly decomposed. Our TLC plate (*Figure 4.1.b*) for the crude product of Mitsunobu-type coupling reaction (*Scheme 2.2*) showed several new spots with different R_f values from compound **1** and **2a**, which suggested a decomposition happened.

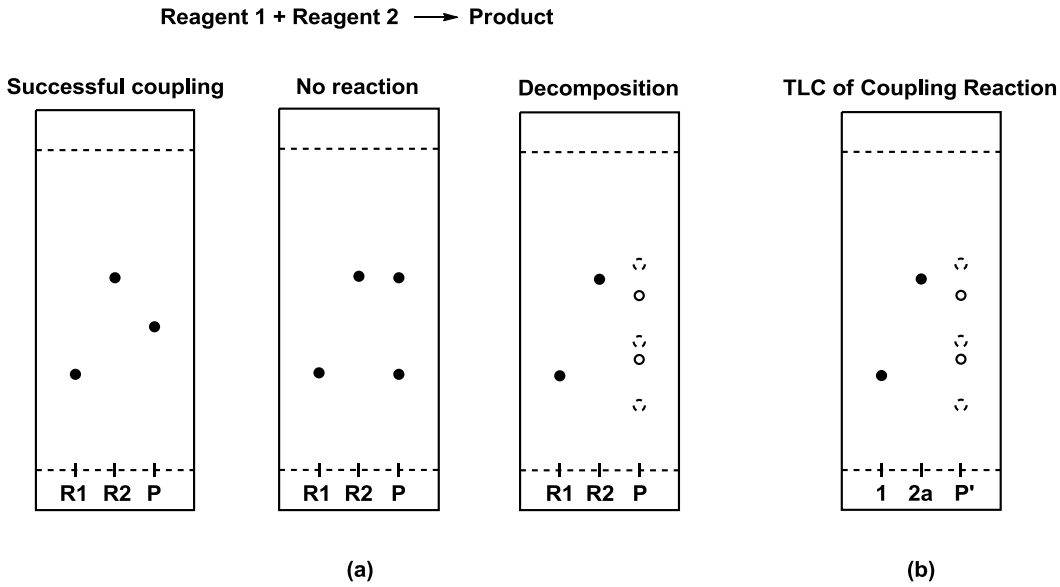


Figure 4.1. **(a) Three possible results of TLC analysis (under same solvent system)**. R1 – Reagent 1; R2 – Reagent 2; P – Product. **(b) TLC results of Mitsunobu-type coupling reaction.** 1 – Compound 1; 2a – Compound 2a; P' – Prodouct from attempted coupling reaction.

4.2 ^{1}H NMR Characterization of (+/-) SRH•TFA Salt 6

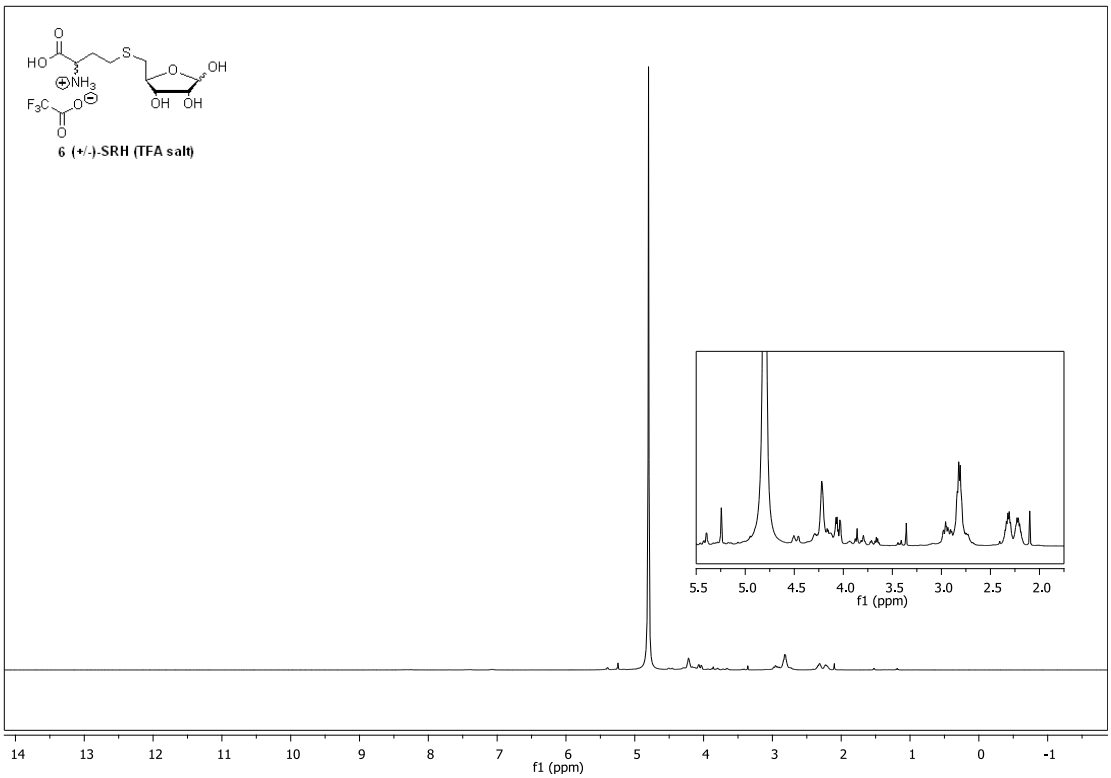


***Figure 4.2*. ^{1}H NMR Characterization of (+/-) SRH•TFA salt 6.** The new peaks between δ 2.5 ~ 3.0 ppm suggested the formation of S-C bond.

4.3 Re-characterization of (+/-) SRH•TFA Salt 6 after C18 Column Purification

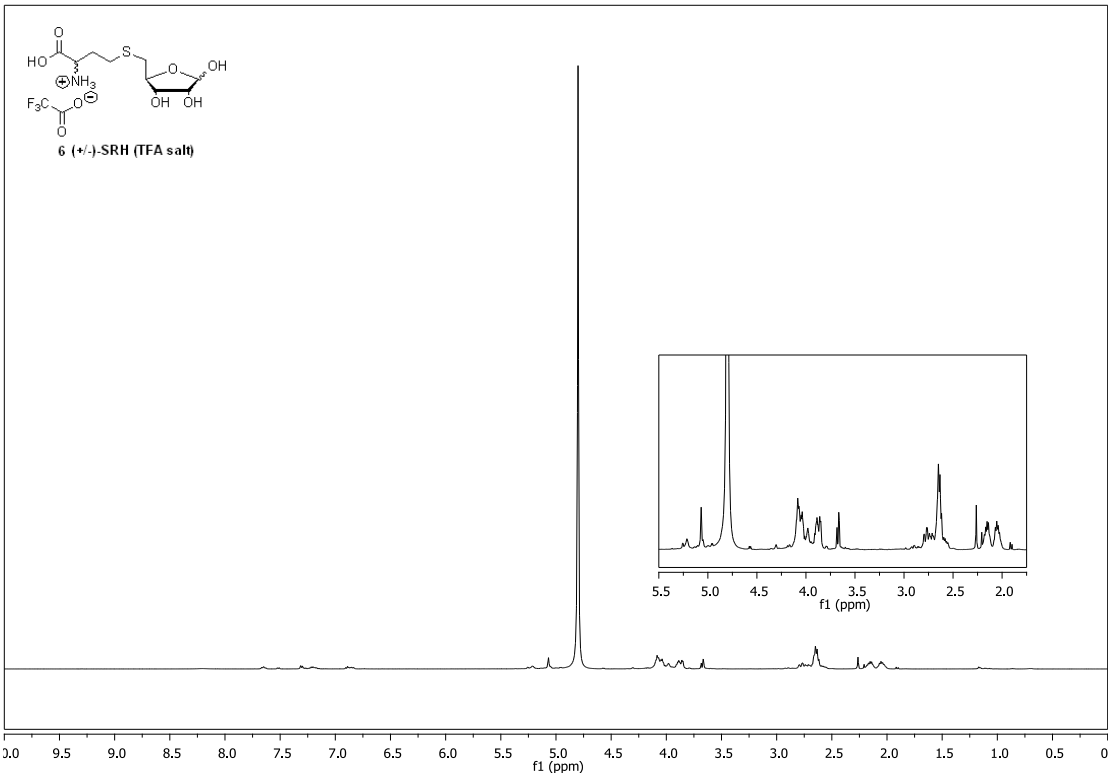
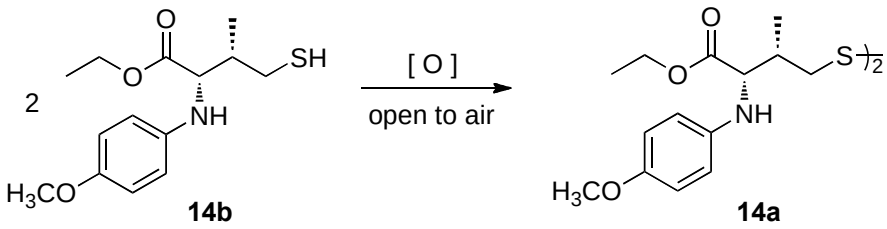


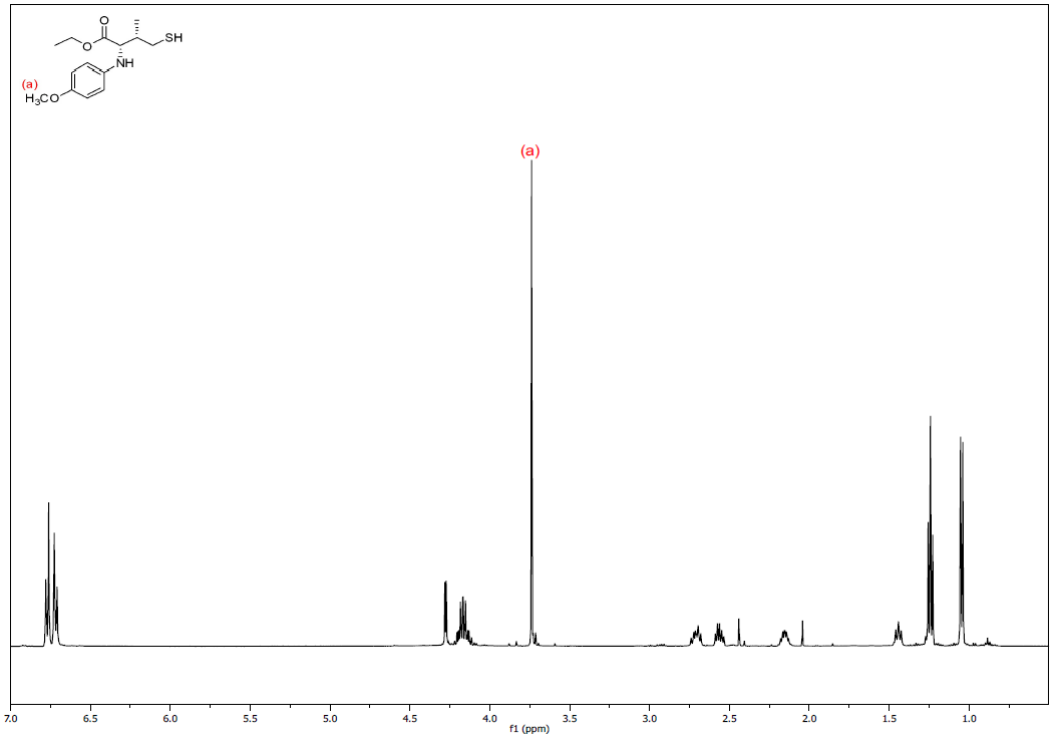
Figure 4.3. **Re-characterization of (+/-) SRH•TFA salt 6.** After C18 column purification, the compound has been re-characterized showing same chemical shifts with previously-characterized.

4.4 ^{1}H NMR Characterization of Thiol to Disulfide Conversion

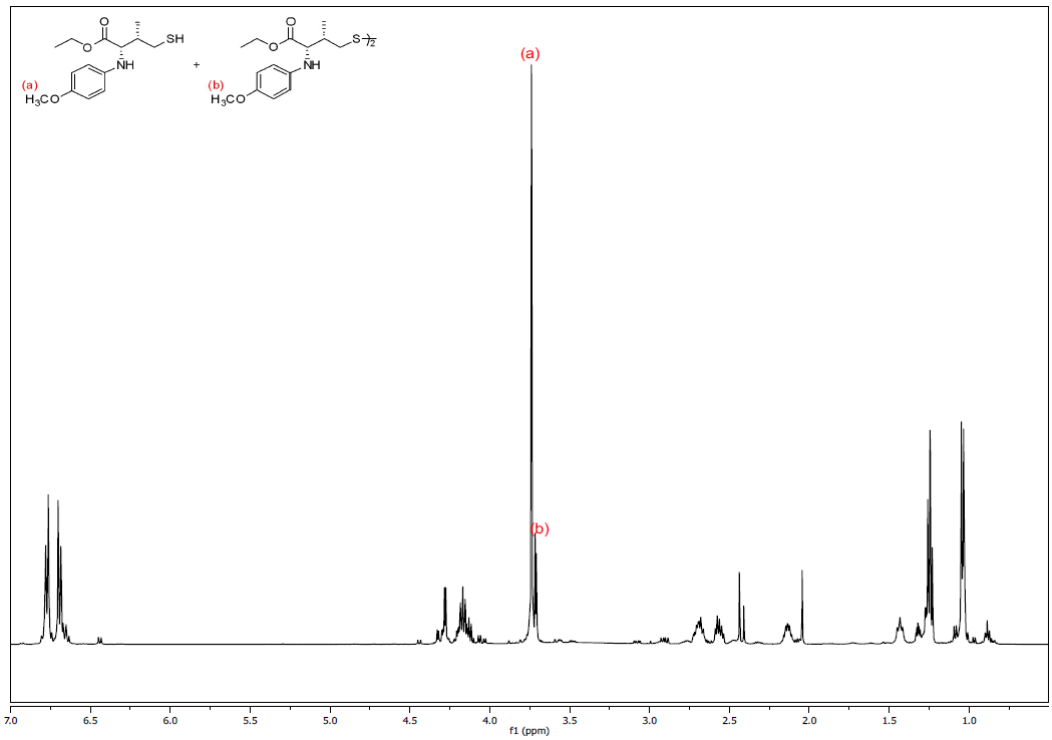


Scheme 4.1. **Oxidation of free thiol to disulfide.**

The purified thiol **14b** from silica gel column chromatography was prone to be oxidized into disulfide. This conversion from free thiol **14b** to disulfide **14a** was characterized by ^{1}H NMR as shown in *Figure 7*. First, a sample of pure free thiol **14b** was examined by ^{1}H NMR (*Figure 2.3*.A. proton peak (a) indicates the –OCH_3 group). After being exposed to air for 3 ~ 4 days, the same thiol sample in the same NMR tube was re-characterized by ^{1}H NMR. *Figure 2.3*.B shows the splitting of -OCH_3 peak into (a) and (b). Peak (a) indicates the existence of free thiol, while the peak (b) suggests the formation of disulfide. Thus, it was concluded that this free thiol is not stable.



(A)



(B)

Figure 4.4. **(A) ^{1}H NMR of thiol; (B) ^{1}H NMR shows the conversion of free thiol to disulfide.**

4.5 Biological Evaluation of Chemically – Synthesized SRH

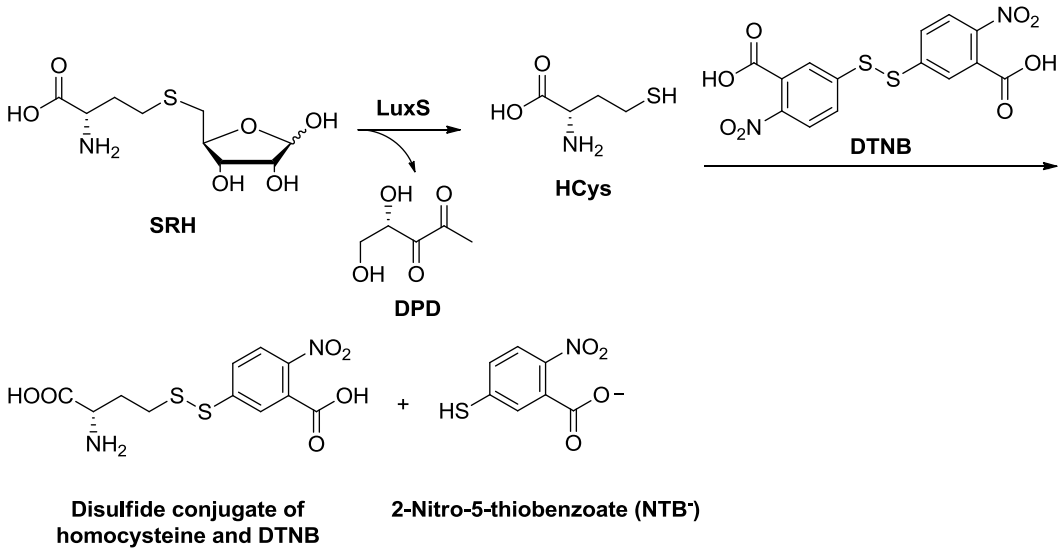
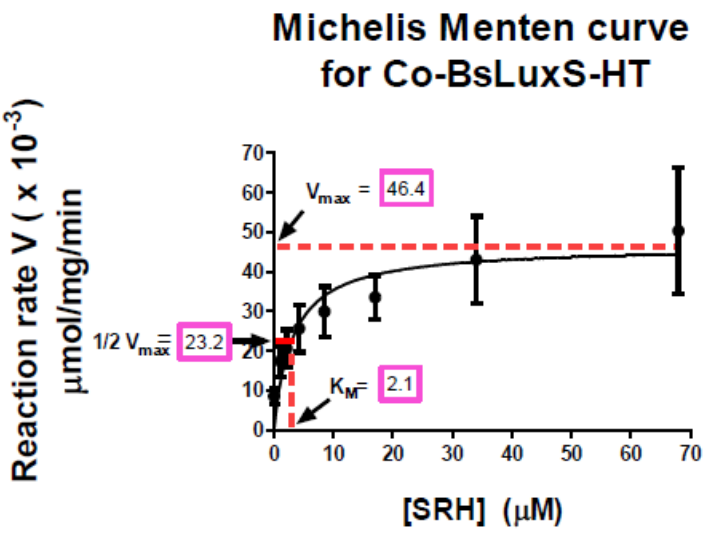


Figure 4.5. **Ellman's assay for LuxS activity.** Catalyzed by the LuxS enzyme, SRH converts to DPD and HCys. HCys reacts with DTNB to give a disulfide conjugate and a byproduct 2-nitro-5-thiobenzoate (NTB^-). NTB^- could ionize to NTB^{2-} in aqueous solution. This yellow NTB^{2-} ion can be measured at λ_{max} = 412 nm to determine the activity of LuxS.



(A)

Enzyme	K_M(µM)	V_{max} (µmol/s)	k_{cat}(s^{-1})
Co-Bs-LuxS-HT House-purified	2.1±1.4	1.1×10^{-5}	0.014±0.002
Co-Bs-LuxS-HT (by Pei et al.)[47]	2.3±0.5	2.8×10^{-5}	0.035±0.003

(B)

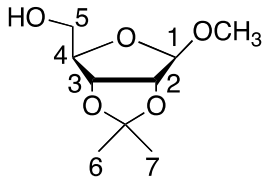
Figure 4.6. **Michaelis Menten Curve and Comparison of Kinetic Constants.** (A) Michaelis-Menten curve for Co-BsLuxS-HT enzyme purified in our lab conditions using chemically-synthesized SRH as substrate. Red dotted lines show the values of kinetic constants on the curve. (B) Kinetic constants comparing in-house LuxS activity assay to reported values of the two Co-BsLuxS-HT enzymes. The K_M values for both enzymes are almost same confirming that our enzyme is equally active.[45](Graph and data by courtesy of Richa Gupta.)

Chapter 5. Experimental Section

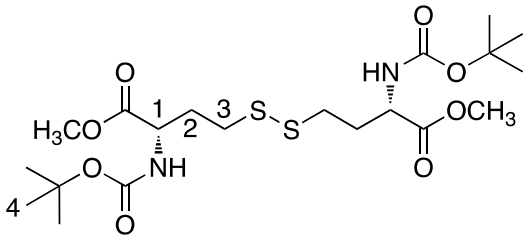
5.1 General Information

Unless otherwise indicated, all reagents were of research grade or finer and were used without further purification. Methanol was distilled at reflux from magnesium and iodine. Anhydrous solvents dichloromethane, ethyl ether, toluene and tetrahydrofuran (THF) were obtained from a glass contour solvent system (SG Water USA, LLC). Air- and water-sensitive reactions were carried out in oven-dried glassware under a dry argon atmosphere. Flash column chromatography was carried out with Sorbent Technologies 60 Å (500-600 Mesh) silica gel, applying the chromatographic technique reported by W. Clark Still (1978).[39] Thin Layer Chromatography (TLC) was performed using Sorbent Technologies silica gel UV254 (200 μm layer) plates. NMR spectra were acquired on a Varian – NMR 500 MHz instrument. Chemical shift values are expressed in ppm and residual solvent signals were used as references: $CDCl_3$ (7.26 ppm for ^1H and 77.16 ppm for ^{13}C), D_2O (4.79 ppm for ^1H). IR data was obtained through PerkinElmer Frontier IR Spectrometer.

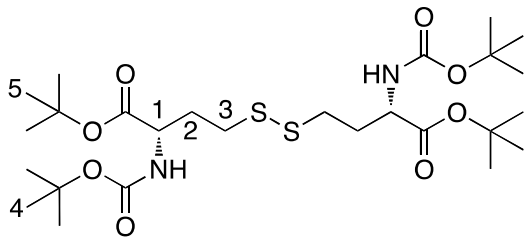
5.2 Experimental Procedures and Data



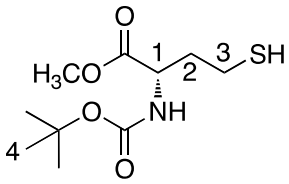
Methyl 2,3-O-isopropylidene-D-ribofuranoside (1) Modification of published procedure.[40] D-ribose (3.49 g, 23.02 mmol) was dissolved in 1:1 MeOH : acetone (30 mL) and allowed to stir at room temperature. Concentrated HCl (12.1 M, 0.5 mL) was added in one portion via pipet. The reaction mixture was heated at reflux for 1.5 h. The solution was cooled to room temperature and pyridine was added dropwise until the mixture was neutral by pH paper. The solution was concentrated by rotary evaporation to a thick yellow oil and then redissolved in a mixture of EtOAc (20 mL) and water (50 mL). The product was extracted with EtOAc (3 × 25 mL). The organic fractions were combined and washed with saturated aqueous copper sulfate (50 mL), then water (50 mL), and finally with brine (50 mL). The organic fraction was dried over $MgSO_4$, filtered and concentrated to a pale blue oil. Silica gel column chromatography with 2:1 EtOAc : hexanes afforded **1** as a thick colorless oil (3.10 g, 15.19 mmol, 66%). TLC: R_f= 0.54 in 2:1 EtOAc : hexanes. ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.97 (s, H_1), 4.83 (d, J = 6.0 Hz, H_2), 4.58 (d, J = 6.0 Hz, H_3), 4.43 (t, J = 2.3 Hz, H_4), 3.70 (m, H_5), 3.61 (m, $H_{5'}$), 3.43 (s, OCH_3), 3.23 (dd, J = 10.6, 2.6 Hz, OH, disappeared upon D_2O exchange), 1.48 (s, H_7), 1.32 (s, H_6). ^{13}C NMR (126 MHz, $CDCl_3$): δ 24.87, 26.51, 55.67, 64.18, 81.64, 85.99, 88.55, 110.16, 112.27. According with the GC-analysis from previous report, the product is a mixture of the anomeric methyl 2,3-*O*-isopropylidene-D-ribofuranoside, in which the β-anomer predominates.[40]



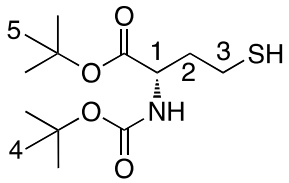
N,N'-Bis(tert-butoxycarbonyl)-L-homocystine methyl ester (2a) Modification of published procedure.[40] $SOCl_2$ (1 mL) was added dropwise to methanol (10 mL, distilled) at 0 ℃. Upon complete addition, the mixture was stirred for 10 min. L-homocystine (268 mg, 1.00 mmol) was then added to the solution at 0 ℃. The reaction mixture was stirred at room temperature for 72 h under argon and then concentrated by rotary evaporation at 40 ℃. Dioxane (2 mL) and water (1 mL) were added to the residue. The pH of the reaction mixture was adjusted to approximately 9 with 10% aqueous Na_2CO_3 solution. After the reaction mixture was cooled to 0 ℃, di-*tert*-butyldicarbonate (1.00 g, 4.58 mmol) was added. The reaction mixture was stirred overnight at room temperature and the pH of the reaction mixture was maintained around 9 by adding 10% aqueous Na_2CO_3 solution periodically. Then the reaction mixture was extracted with $CHCl_3$ (10 mL × 3) and concentrated by rotary evaporation. The residue was redissolved in EtOAc (50 mL), washed with saturated aqueous $NaHCO_3$ solution (15 mL × 3) and brine (30 mL), dried over $MgSO_4$, filtered and concentrated. Silica gel column chromatography with 2:1 hexanes : EtOAc afforded **2a** as a white crystal (437 mg, 0.88 mmol, 88%). TLC: R_f = 0.34 in 2:1 hexanes : EtOAc. ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.20 (d, J = 8.7 Hz, NH), 4.33 (m, H_1), 3.69 (s, OCH_3), 2.65 (t, J = 7.4 Hz, H_3), 2.18 (m, H_2), 1.94 (m, $H_{2'}$), 1.37 (s, H_4). ^{13}C NMR (126 MHz, $CDCl_3$): δ 28.30, 32.47, 34.52, 52.44, 52.53, 80.00, 155.34, 172.62. Previously characterized.[40]



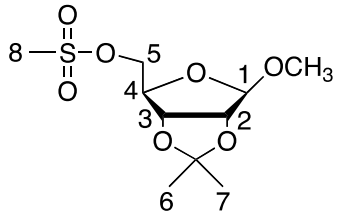
N,N'-Bis(tert-butoxycarbonyl)-L-homocystine di(tert-butyl) ester (2b) Modification of published procedure.[33,41] To a solution of L-homocystine (268 mg, 1.00 mmol) in the mixture of 10% aqueous Na_2CO_3 solution (9 mL) and dioxane (8 mL) was added $(Boc)_2O$ (0.48 g, 2.20 mmol) at 0 ℃. The mixture was stirred overnight at room temperature. The solution was adjusted to pH 4 with 10% aqueous citric acid solution and extracted with EtOAc (3 × 10 mL). The organic fractions were combined, washed with brine (20 mL), dried over $MgSO_4$, and concentrated by rotary evaporation. The residual solid was redissolved in dichloromethane (10 mL) followed by the addition of *tert*-butyl-2,2,2-trichloroacetimidate (1.15 g, 5.00 mmol) under argon. After overnight stirring at room temperature, the solvent was removed by rotary evaporation. Silica gel column chromatography with 4:1 hexanes : EtOAc afforded **2b** as a colorless oil (523 mg, 0.90 mmol, 90%, the oil phase converted to white solid when temperature was down to 8 ℃). TLC: R_f = 0.33 in 4:1 hexanes : EtOAc. ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.10 (d, J = 7.6 Hz, NH), 4.24 (m, H_1), 2.69 (m, H_3), 2.20 (m, H_2), 1.98 (m, $H_{2'}$), 1.47 (s, H_4), 1.44 (s, H_5). ^{13}C NMR (126 MHz, $CDCl_3$): δ 28.16, 28.47, 33.05, 34.69, 53.36, 79.98, 82.41, 155.48, 171.34. Previously characterized.[33,41]



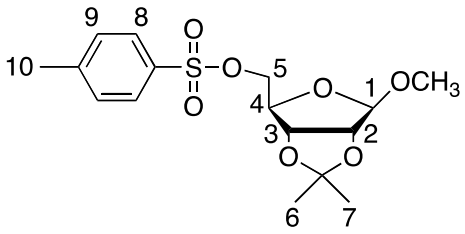
N-tert-Butoxycarbonyl-L-homocysteine methyl ester (3a) Modification of published procedure.[33,41] To a solution of **2a** (0.51 g, 1.03 mmol) in DMF (10 mL) was added water (1 mL) and tri-*n*-butylphosphine (292 µL, 236 mg, 1.13 mmol) and the mixture was stirred at room temperature overnight under argon (TLC [3:1 hexanes : EtOAc] showed the conversion of disulfide **2a** [R_f 0.31] into thiol **3a** [R_f 0.46]). The reaction mixture was quenched with water (100 mL) and thoroughly extracted with EtOAc (3 × 50 mL). The organic fractions were combined and washed with brine (2 × 50 mL), dried over $MgSO_4$, filtered and concentrated by rotary evaporation. Silica gel column chromatography with 3:1 hexanes : EtOAc afforded **3a** as a thick colorless oil (513 mg, 2.06 mmol, quantitative yield). TLC: R_f = 0.46 in 3:1 hexanes : EtOAc. ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.07 (d, *J* = 6.9 Hz, NH), 4.45 (m, H_1), 3.75 (s, OCH_3), 2.59 (m, H_3), 2.10 (t, *J* = 6.7 Hz, H_2), 1.93 (m, $H_{2'}$), 1.56 (t, *J* = 8.0 Hz, SH), 1.44 (s, H_4). ^{13}C NMR (126 MHz, $CDCl_3$): δ 20.85, 28.44, 37.39, 52.42, 52.59, 80.32, 155.94, 173.07. Previously characterized.[42]



N-tert-Butoxycarbonyl-L-homocysteine tert-butyl ester (3b) Modification of published procedure.[33,41] Treatment of **2b** (639 mg, 1.10 mmol) with tri-*n*-butylphosphine (313 μL, 253 mg, 1.21 mmol) in the mixture of DMF (10 mL) and water (1 mL), as described for **3a**, followed by silica gel column chromatography with 4:1 hexanes : EtOAc afforded **3b** as a thick colorless oil (641 mg, 2.20 mmol, quantitative yield). TLC (4:1 hexanes : EtOAc) showed the conversion of disulfide **2b** (R_f 0.33) into thiol **3b** (R_f 0.50). ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.05 (d, J = 6.7 Hz, NH), 4.31 (m, H_1), 2.58 (m, H_3), 2.06 (m, H_2), 1.95 (m, $H_{2'}$), 1.58 (t, J = 4.9 Hz, SH), 1.47 (s, H_4), 1.45 (s, H_5). ^{13}C NMR (126 MHz, $CDCl_3$): δ 20.86, 28.10, 28.44, 37.69, 53.04, 79.90, 82.28, 155.47, 171.46. Previously characterized.[33,41]

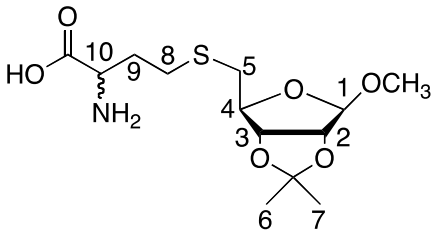


Methyl 2,3-O-isopropylidene-5-O-methylsulfonyl-D-ribofuranoside (4a) Modification of published procedure.[23] To a stirred solution of **1** (0.61 g, 3.00 mmol) in anhydrous CH_2Cl_2 (100 mL) was added triethylamine (1.25 mL, 0.91 g, 9.00 mmol) and mesyl chloride (350 μL, 0.51 g, 4.50 mmol) at 0 °C under argon. After stirring for 30 min, the reaction mixture was partitioned ($NaHCO_3/H_2O/CHCl_3$), and the organic layer was washed by brine, dried over $MgSO_4$, filtered and concentrated by rotary evaporation to yield **4a** as a white crystal (0.84 g, 2.97 mmol, 99%) of sufficient purity to be used for the formation of compound **5**. ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.97 (s, H_1), 4.68 (d, J = 5.9 Hz, H_2), 4.59 (d, J = 5.9 Hz, H_3), 4.39 (t, J = 2.8 Hz, H_4), 4.18 (dd, J = 7.2, 1.7 Hz, H_5), 3.33 (s, OCH_3), 3.05 (s, H_8), 1.46 (s, H_7), 1.30 (s, H_6). ^{13}C NMR (126 MHz, $CDCl_3$): δ 25.01, 26.49, 37.83, 55.33, 68.61, 81.45, 83.90, 85.02, 109.68, 112.93.

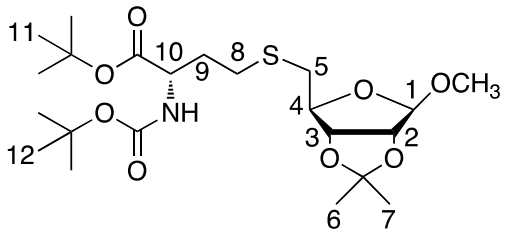


Methyl 2,3-O-isopropylidene-5-O-p-toluenesulfonyl-D-ribofuranoside (4b)

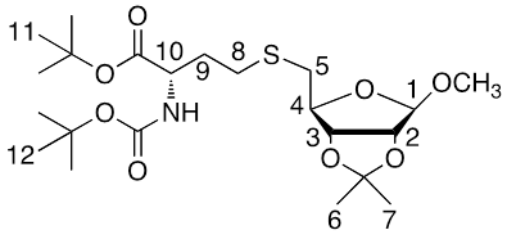
Modification of published procedure.[23] TsCl (191 mg, 1.00 mmol) was added to a stirred solution of **1** (102 mg, 0.50 mmol) in pyridine (2 mL) at 0 °C under argon. After stirring for 24 h at room temperature, pyridine was evaporated/coevaporated with toluene (3 × 2 mL) and the residue was dissolved in EtOAc (20 mL) and washed with saturated aqueous $NaHCO_3$ solution (2 × 10 mL) and brine (20 mL). The organic layer was dried over $MgSO_4$, filtered, concentrated and recrystallized by EtOH (30 mL) to give **4b** (176 mg, 0.49 mmol, 98%) as a white crystal. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.81 (d, J = 8.3 Hz, H_8), 7.36 (d, J = 7.9 Hz, H_9), 4.93 (s, H_1), 4.60 (d, J = 5.9 Hz, H_2), 4.53 (d, J = 5.9 Hz, H_3), 4.31 (t, J = 7.2 Hz, H_4), 4.02 (dd, J = 7.2, 2.8 Hz, H_5), 3.24 (s, OCH_3), 2.46 (s, H_{10}), 1.45 (s, H_7), 1.29 (s, H_6). ^{13}C NMR (126 MHz, $CDCl_3$): δ 21.61, 24.85, 26.32, 54.98, 69.34, 81.33, 83.60, 84.86, 109.44, 112.66, 127.94, 129.94, 132.74, 145.12.



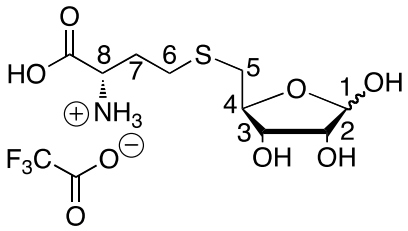
S-(Methyl-2,3-O-isopropylidene-D-ribofuranoside) homocysteine (5) Modification of published procedure.[23] Compound **4a** (51 mg, 0.18 mmol) was added to 1 M NaOH/ H_2O (5 mL) and the reaction mixture was stirred under argon. D/L-Homocysteine (37 mg, 0.27 mmol) was then added, and the suspension was heated to 60 ^{o}C. After 12 h, the reaction mixture was cooled to room temperature, neutralized with dilute HCl (0.5 M) to pH ~ 7 and washed with EtOAc (3 × 5 mL). The aqueous layer was evaporated by rotary evaporation at 50 ^{o}C to give **5** as a white solid. (Yield was not determined since it was not purified.) ^{1}H NMR (500 MHz, D_2O): δ 5.21 (s, H_1), 5.00 (dd, J = 5.9, 2.8 Hz, H_2), 4.90 (d, J = 5.9 Hz, H_3), 4.43 (d, J = 1.4 Hz, H_4), 3.97 (m, H_{10}), 3.51 (s, OCH_3), 3.00 – 2.82 (m, $H_{5,8}$), 2.35 – 2.19 (m, H_9), 1.62 (s, H_7), 1.48 (s, H_6). ^{13}C NMR (126 MHz, D_2O): δ 23.85, 25.40, 27.03, 30.56, 34.68, 53.88, 55.11, 82.96, 84.45, 85.36, 108.85, 113.17, 174.07.



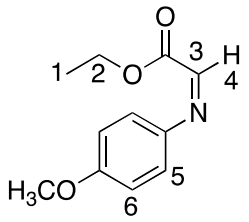
S-(Methyl-2,3-O-isopropylidene-D-ribofuranoside)-N-(tert-butoxycarbonyl)-L-homocysteine tert-butyl ester (7) Modification of published procedure.[23] Method **A** (used purified **3b**): To a solution of purified **3b** (1.08 g, 3.70 mmol) in DMF (15 mL) was added BuLi (1.6 M in hexane, 2.32 mL, 3.71 mmol) at 0 °C under argon. After stirring for 10 min, a solution of tosylate **4b** (0.66 g, 1.85 mmol) was dissolved in DMF (10 mL) was added. The mixture was stirred for overnight at room temperature, diluted with EtOAc (75 mL), washed with H_2O (50 mL) and brine (25 mL). The organic layer was dried over $MgSO_4$ and concentrated by rotary evaporation. The residue was dissolved in THF (3 mL), concentrated H_2SO_4 (18.0 M, 2 μL) and H_2O (2 μL) were added at 0 °C. The mixture was stirred for 1 h at 0 °C and then partitioned between EtOAc (100 mL) and 5% $NaHCO_3$ solution (40 mL). The organic layer was collected, washed with H_2O (30 mL) and brine (30 mL), dried over $MgSO_4$ and concentrated. Silica gel chromatography with 1:3 EtOAc : hexane afforded protected homocysteine **7** (0.51 g, 1.06 mmol, 70%) as a colorless oil. TLC: R_f = 0.34 in 1:3 EtOAc : hexane. ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.09 (d, *J* = 8.4 Hz, NH), 4.95 (s, H_1), 4.68 (d, *J* = 6.0 Hz, H_2), 4.58 (d, *J* = 5.9 Hz, H_3), 4.29 – 4.18 (m, $H_{10,4}$), 3.33 (s, OCH_3), 2.74 (dd, *J* = 13.5, 6.1 Hz, H_5), 2.60 – 2.52 (m, $H_{5',8}$), 2.06 (s, H_9), 1.91 – 1.83 (m, $H_{9'}$), 1.46 (s, $H_{7,12}$), 1.43 (s, H_{11}), 1.30 (s, H_6). ^{13}C NMR (126 MHz, $CDCl_3$): δ 25.09, 26.56, 27.87, 28.13, 28.45, 33.25, 35.72, 53.51, 55.07, 55.08, 82.33, 83.45, 85.39, 85.89, 109.71, 112.57, 155.45, 171.35.



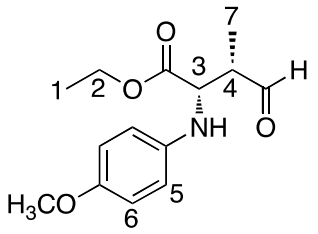
S-(Methyl-2,3-O-isopropylidene-D-ribofuranoside)-N-(tert-butoxycarbonyl)-L-homocysteine tert-butyl ester (7) Modification of published procedure.[23, 33, 41] Method **B** (used crude **3b**): To a solution of **2b** (581 mg, 1.00 mmol) in DMF (10 mL) was added water (1 mL) and tri-*n*-butylphosphine (285 µL, 230 mg, 1.10 mmol) and the mixture was stirred at room temperature overnight under argon (TLC [4:1 hexanes : EtOAc] showed the completed conversion of disulfide **2b** [R_f 0.33] into thiol **3b** [R_f0.50]). The reaction mixture was quenched with water (100 mL) and thoroughly extracted with EtOAc (3 × 50 mL). The organic fractions were combined and washed with brine (2 × 50 mL), dried over $MgSO_4$, filtered and concentrated by rotary evaporation. Without further purification, the residue was redissolved in DMF (8 mL) was added BuLi (1.6 M in hexane, 1.25 mL, 2.01 mmol) at 0 °C under argon. After stirring for 10 min, a solution of tosylate **4b** (0.36 g, 1.00 mmol) was dissolved in DMF (5.5 mL) was added. The mixture was stirred for overnight at room temperature, diluted with EtOAc (50 mL), washed with H_2O (30 mL) and brine (20 mL). The organic layer was dried over $MgSO_4$ and concentrated by rotary evaporation. The residue was dissolved in THF (1.5 mL), concentrated H_2SO_4 (18.0 M, 1 µL) and H_2O (1 µL) were added at 0 °C. The mixture was stirred for 1 h at 0 °C and then partitioned between EtOAc (50 mL) and 5% $NaHCO_3$ solution (20 mL). The organic layer was collected, washed with H_2O (15 mL) and brine (15 mL), dried over $MgSO_4$ and concentrated. Silica gel chromatography with 1:3 EtOAc : hexane afforded protected homocysteine **7** (0.31 g, 0.64 mmol, 64%) as a colorless oil. TLC and NMR data were same as previously characterized in Method **A**.



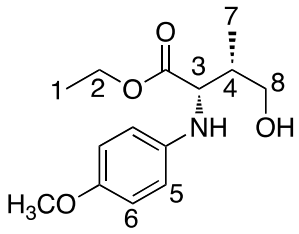
S-Ribosyl-L-homocysteine (8) Modification of published procedure.[23] Compound **7** (148 mg, 0.31 mmol) was added into the mixture of TFA (4 mL), water (0.5 mL) and anisole (0.5 mL) at 0 °C. After stirring for 6 h at room temperature, the volatile solvents were removed by rotary evaporation, and the residue was dissolved in H_2O (3 mL) and washed with CH_2Cl_2 (3 × 2 mL). The aqueous solution was lyophilized to give the desired SRH **8** as a trifluoroacetate salt (111 mg, 94%). ^{1}H NMR (500 MHz, D_2O): δ 5.23 (d, J = 4.1 Hz, H_1), 5.08 (d, J = 1.2Hz, H_1), 4.11 – 3.84 (m, $H_{2,3,4,8}$), 2.84 – 2.58 (m, $H_{5,6}$), 2.23 – 2.00 (m, H_7). Previously characterized.[40] The ratio of α-form and β-form was 1 to 2, determined from ^{1}H NMR integration of the anomeric proton peaks (α-form at 5.23 ppm, J = 4.1 Hz; and β-form at 5.08 ppm, J = 1.2Hz).



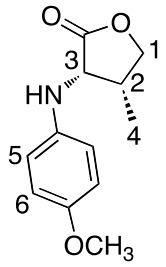
Ethyl (E)-2-(p-methoxyphenylimino) acetate (9) Modification of published procedure.[28] A mixture of ethyl glyoxylate (polymer form 45-50% in toluene, 1 mL, ~ 4.5 mmol), *p*-anisidine (554 mg, 4.50 mmol), and molecular sieves 4 Å (5 g) in anhydrous CH_2Cl_2 (25 mL) was stirred at room temperature overnight. After filtration through celite, the solution was concentrated by rotary evaporation to afford the imine **9** as thick dark brown oil (914 mg, 4.40 mmol, 98%) of sufficient purity to be used for the Mannich reaction. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.93 (s, H_4), 7.36 (d, J = 8.9 Hz, H_6), 6.93 (d, J = 8.9 Hz, H_5), 4.41 (q, J = 7.1 Hz, H_2), 3.84 (s, OCH_3), 1.40 (t, J = 7.1 Hz, H_1). ^{13}C NMR (126 MHz, $CDCl_3$): δ 14.38, 55.67, 62.06, 114.69, 123.74, 141.55, 148.05, 160.66, 163.77. Previously characterized.[28]



Ethyl (2S,3S)-3-formyl-2-(p-methoxyphenylamino)-butanoate (10) Modification of published procedure.[29] To a stirred solution of imine **9** (104 mg, 0.50 mmol) in DMSO (1 mL) was added L-proline (17 mg, 0.15 mmol) followed by the addition of propionaldehyde (58 mg, 72 μL, 1.00 mmol) in one portion. The reaction mixture was stirred at room temperature for 3 h (TLC showed the completion of reaction). The saturated aqueous NH_4Cl solution (10 mL) and EtOAc (10 mL) were added with vigorous stirring, the layers were separated and the organic phase was washed with water, dried over $MgSO_4$, concentrated by rotary evaporation. Silica gel column chromatography with 3:1 hexanes : EtOAc afforded **10** as a thick yellow oil (65 mg, 0.25 mmol, 49%). TLC: R_f = 0.26 in 3:1 hexanes : EtOAc. ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.73 (s, CHO), 6.78 (m, H_6), 6.68 (m, H_5), 4.45 (dd, J = 10.0, 4.4 Hz, H_3), 4.20 (q, J = 7.0 Hz, H_2), 3.91 (d, J = 10.2 Hz, NH), 3.74 (s, OCH_3), 2.87 (m, H_4), 1.28 – 1.22 (m, $H_{1,7}$). ^{13}C NMR (126 MHz, $CDCl_3$): δ 9.27, 14.33, 48.41, 55.81, 58.73, 61.73, 114.99, 116.53, 140.64, 153.64, 172.48, 201.91. Previously characterized.[30]

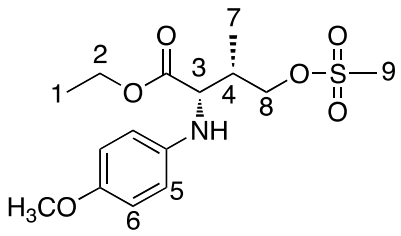


Ethyl (2S,3S)-4-hydroxy-2-(p-methoxyphenylamino)-3-methyl-butanoate (11a) Modification of published procedure.[30] To a solution of compound **10** (60 mg, 0.23 mmol) in anhydrous ethyl ether (2 mL) was added $NaBH_4$ (44 mg, 1.15 mmol) at 0 °C. After 30 min, the saturated aqueous NH_4Cl solution was added until no air bubbles formed. The reaction mixture was extracted with EtOAc (3 × 10 mL). The organic fractions were combined and washed with brine, dried over $MgSO_4$ and concentrated by rotary evaporation. Silica gel column chromatography with 2:1 hexanes : EtOAc afforded **11a** as a thick yellow oil (59 mg, 0.22 mmol, 96%). TLC: R_f = 0.29 in 2:1 hexanes : EtOAc. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.78 (m, H_6), 6.72 (m, H_5), 4.18 (m, $H_{2,3}$), 4.02 (s, NH), 3.74 (s, OCH_3), 3.72 – 3.62 (m, H_8), 2.39 (s, OH), 2.24 (m, H_4), 1.24 (t, *J* = 7.1 Hz, H_1), 1.00 (d, *J* = 7.1 Hz, H_7). ^{13}C NMR (126 MHz, $CDCl_3$): δ 11.90, 14.40, 38.28, 55.84, 61.21, 61.30, 65.97, 114.99, 116.62, 141.53, 153.49, 174.01.



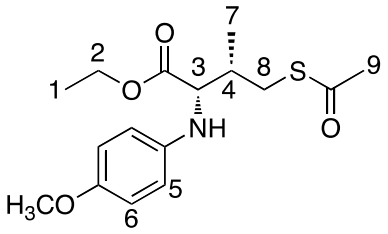
(3S,4R)-3-(p-methoxyphenylamino)-4-methyl-dihydro-2(3H)-furanone (11b)

Modification of published procedure.[30] Treatment of **10** (1.34 g, 5.05 mmol) with $NaBH_4$ (0.96 g, 25.25 mmol) in ethyl ether (40 mL), as described for **11a**, followed by silica gel column chromatography with 2:1 hexanes : EtOAc afforded another product **11b** as a yellow solid (0.96 g, 4.34 mmol, 86%). TLC: R_f = 0.33 in 2:1 hexanes : EtOAc. Compound **11a** was not observed in this manner. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.77 (m, H_6), 6.68 (m, H_5), 4.40 (dd, *J* = 9.1, 7.6 Hz, H_3), 3.84 (m, NH, H_1), 3.74 (s, OCH_3), 3.72 (d, *J* = 2.4 Hz, $H_{1'}$), 2.48 (tt, *J* = 10.5, 6.8 Hz, H_2), 1.24 (d, *J* = 6.7 Hz, H_4). ^{13}C NMR (126 MHz, $CDCl_3$): δ 15.34, 38.94, 55.78, 61.03, 71.26, 114.90, 115.73, 140.78, 153.28, 176.79. IR (ATR): 3391 (NH), 2963, 1763 (C=O), 1513, 1234, 1030, 801 cm^{-1}.

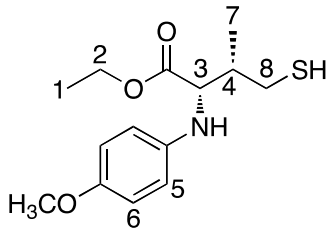


Ethyl (2S,3S)-2-(p-methoxyphenylamino)-3-methyl-4-methylsulfonyl-butanoate (12)

Modification of published procedure.[43] To a stirred solution of **11a** (99 mg, 0.37 mmol) in anhydrous CH_2Cl_2 (10 mL) was added triethylamine (154 μL, 112 mg, 1.11 mmol) and mesyl chloride (44 μL, 64 mg, 0.56 mmol) at 0 °C under argon. After stirring overnight, the reaction mixture was partitioned ($NaHCO_3/H_2O/CH_2Cl_2$), and the organic layer was washed by brine, dried over $MgSO_4$, filtered and concentrated by rotary evaporation to yield **12** as a dark yellow oil (120 mg, 0.35 mmol, 94%) of sufficient purity to be used for the formation of compound **13**. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.76 (d, J = 8.7 Hz, H_6), 6.67 (d, J = 8.8 Hz, H_5), 4.25 (dd, J = 9.7, 8.5 Hz, H_3), 4.22 – 4.12 (m, $H_{2,8}$), 3.73 (s, OCH_3), 2.97 (s, H_9), 2.53 (m, H_4), 1.25 (t, J = 7.1 Hz, H_1), 0.99 (d, J = 7.0 Hz, H_7). ^{13}C NMR (126 MHz, $CDCl_3$): δ 11.40, 14.32, 36.15, 37.27, 55.77, 58.87, 61.55, 71.32, 114.99, 116.32, 141.30, 153.43, 173.18.

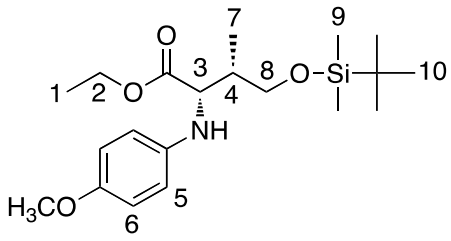


Ethyl (2S,3S)-2-(p-methoxyphenylamino)-3-methyl-4-thioacetyl-butanoate (13) Modification of published procedure.[31] To a solution of compound **12** (0.35 g, 1.01 mmol) in DMF (10 mL) was added potassium thioacetate (0.51 g, 4.40 mmol) at 0 °C under argon. The reaction mixture was stirred for 1 d at room temperature. The solvent was removed by rotary evaporation. The residue was dissolved in the mixture of EtOAc (30 mL) and water (30 mL). The organic layer was collected and washed by water, dried over $MgSO_4$ and concentrated by rotary evaporation. Silica gel column chromatography with 3:1 hexanes : EtOAc afforded **13** as a brown oil (166 mg, 0.51 mmol, 50%). TLC: R_f = 0.46 in 3:1 hexanes : EtOAc. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.77 (d, J = 8.9 Hz, H_6), 6.65 (d, J = 8.9 Hz, H_5), 4.20 – 4.10 (m, H_2), 4.04 (d, J = 4.1 Hz, H_3), 3.74 (s, OCH_3), 3.06 (m, H_8), 2.93 (m, $H_{8'}$), 2.36 (s, H_9), 2.22 (qd, J = 6.9, 3.9 Hz, H_4), 1.24 (t, J = 7.1 Hz, H_1), 1.03 (d, J = 7.0 Hz, H_7). ^{13}C NMR (126 MHz, $CDCl_3$): δ 195.50, 173.56, 153.22, 141.58, 116.16, 114.97, 61.48, 61.29, 55.82, 36.99, 32.91, 30.80, 14.86, 14.38.

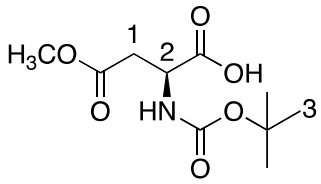


Ethyl (2S,3S)-2-(p-methoxyphenylamino)-3-methyl-4-thiol-butanoate (14b)

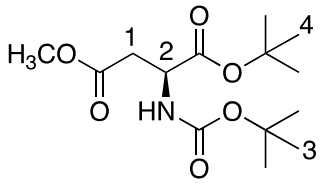
Modification of published procedure.[32] To a stirred solution of Compound **13** (0.12 g, 0.37 mmol) in distilled MeOH (10 mL) was added sodium thiomethoxide (0.41 g, 0.56 mmol) at room temperature under argon. After 30 min, the reaction was quenched with 0.1 M HCl (10 mL), extracted with CH_2Cl_2 (3 × 10 mL). The organic fractions were combined and washed with brine, dried over $MgSO_4$, filtered and concentrated by rotary evaporation. Then treatment of the residue with tri-*n*-butylphosphine (96 µL, 77 mg, 0.37 mmol) in the mixture of DMF (3 mL) and water (0.3 mL), as described for compound **3a**, followed by silica gel column chromatography with 2:1 hexanes : EtOAc afforded **14b** as a light yellow oil (68 mg, 0.24 mmol, 65%). TLC: R_f = 0.56 in 2:1 hexanes : EtOAc. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.77 (d, *J* = 8.9 Hz, H_6), 6.72 (d, *J* = 8.9 Hz, H_5), 4.28 (d, *J* = 4.2 Hz, H_3), 4.21 – 4.12 (m, H_2), 3.74 (s, OCH_3), 2.71 (m, H_8), 2.56 (dt, *J* = 13.4, 6.5 Hz, $H_{8'}$), 2.15 (dd, *J* = 6.7, 4.4 Hz, H_4), 1.44 (t, *J* = 9.4, 7.4 Hz, SH), 1.24 (t, *J* = 7.1 Hz, H_1), 1.05 (d, *J* = 6.8 Hz, H_7). ^{13}C NMR (126 MHz, $CDCl_3$): δ 14.40, 14.70, 28.47, 39.97, 55.83, 60.82, 61.31, 114.99, 116.54, 141.22, 153.46, 173.79.



Ethyl (2S,3S)-4-(tert-butyldimethylsilyloxy)-2-(p-methoxyphenylamino)-3-methyl-butanoate (16) Modification of published procedure.[44] To a solution of compound **11a** (0.27 g, 1.01 mmol) in DMF (5 mL) was added *tert*-butyldimethylsilyl chloride (0.78 g, 5.00 mmol) and imidazole (0.68 g, 10 mmol) at room temperature under argon. After stirring for 2 days, the reaction mixture was quenched with water (30 mL). The aqueous solution was extracted with EtOAc (3 × 15 mL). The combined organic fractions were washed with brine, dried over $MgSO_4$ and concentrated by rotary evaporation. Silica gel column chromatography with 5:1 hexanes : EtOAc afforded **16** as a colorless oil (0.33 g, 0.87 mmol, 86%). TLC: R_f= 0.67 in 2:1 hexanes : EtOAc. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.75 (m, H_6), 6.68 (m, H_5), 4.22 (d, J = 3.4 Hz, H_3), 4.16 (dd, J = 8.8, 7.2 Hz, H_2), 3.73 (s, OCH_3), 3.63 (dd, J = 10.0, 8.8 Hz, H_8), 3.53 (m, $H_{8'}$), 2.28 – 2.18 (m, H_4), 1.24 (t, J = 7.1 Hz, H_1), 0.94 (s, H_{10}), 0.90 (d, J = 7.1 Hz, H_7), 0.07 (d, J = 4.5 Hz, H_9). ^{13}C NMR (126 MHz, $CDCl_3$): δ 0.03, 11.70, 14.39, 18.39, 26.04, 39.06, 55.84, 59.59, 60.96, 65.16, 114.87, 115.78, 142.42, 152.81, 174.47.



4-Methyl N-(tert-butyloxycarbonyl)-L-aspartate (18) Modification of published procedure.[38] To a stirred suspension of L-aspartic acid (4.00 g, 0.03 mol) in distilled MeOH (10 mL) was added acetyl chloride (3.34 g, 0.04 mol) in distilled MeOH (10 mL) at 0 °C. The reaction mixture was stirred for 1 day from 0 °C to room temperature under argon. Then the solution was poured into Et_2O (60 mL) and cooled for 1 h. The precipitate was collected and was dissolved in the mixture of dioxane (60 mL) and water (30 mL). Sodium carbonate (6.36 g, 0.06 mmol) was added in one portion at 0 °C followed by the addition of $(Boc)_2O$ (10.90 g, 0.05 mmol). The reaction mixture was stirred overnight from 0 °C to room temperature. The dioxane was removed and the residue was poured into ice-water (60 mL), washed with Et_2O (30 mL) to remove nonacidic impurities. The aqueous solution was adjusted to pH = 2.5 with saturated $NaHSO_4$ (aq) and extracted with Et_2O (3 × 45 mL). The combined organic fractions was washed with water, dried over $MgSO_4$, filtered and concentrated to afford **18** as a colorless oil (5.78 g, 23.40 mmol, 78%) with sufficient purity to be used for the formation of compound **19**. ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.44 (s, COOH), 5.57 (d, J = 8.6 Hz, NH), 4.71 – 4.52 (m, H_2), 3.70 (s, OCH_3), 3.02 (dd, J = 17.1, 4.6 Hz, H_1), 2.84 (dd, J = 17.1, 4.8 Hz, $H_{1'}$), 1.44 (s, H_3). ^{13}C NMR (126 MHz, $CDCl_3$): δ 175.71, 171.70, 155.76, 67.14, 52.25, 49.90, 36.53, 28.40.



α-tert-Butyl β-methyl N-(tert-butyloxycarbonyl)-L-aspartate (19) Modification of published procedure.[39] Compound **18** (1.12 g, 4.51 mmol) was dissolved in dichloromethane (50 mL) following by the addition of *tert*-butyl-2,2,2-trichloroacetimidate (3.95 g, 18.04 mmol) under argon. After overnight stirring at room temperature, the solvent was removed by rotary evaporation. Silica gel column chromatography with 4:1 hexanes : EtOAc afforded **19** as a colorless oil (1.15 g, 3.79 mmol, 84%, the oil phase converted to white solid when temperature was down to ~ 8 ℃). TLC: R_f= 0.43 in 3:1 hexanes : EtOAc. ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.42 (d, *J* = 8.6 Hz, NH), 4.47 – 4.36 (m, H_2), 3.66 (s, OCH_3), 2.91 (dd, *J* = 16.6, 4.7 Hz, H_1), 2.74 (dd, *J* = 16.6, 5.0 Hz, $H_{1'}$), 1.42 (d, *J* = 5.7 Hz, $H_{3,4}$). ^{13}C NMR (126 MHz, $CDCl_3$): δ 171.43, 170.05, 155.51, 82.40, 79.95, 51.91, 50.68, 37.08, 28.42, 27.98.

Chapter 6. References

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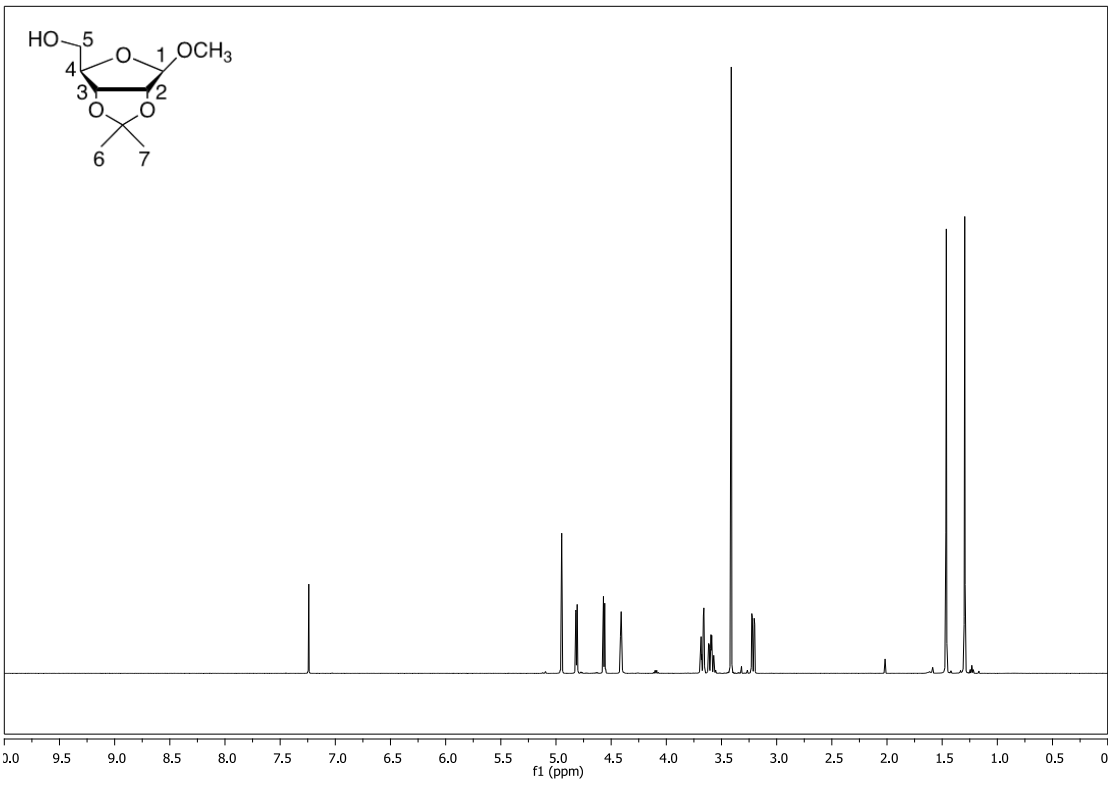
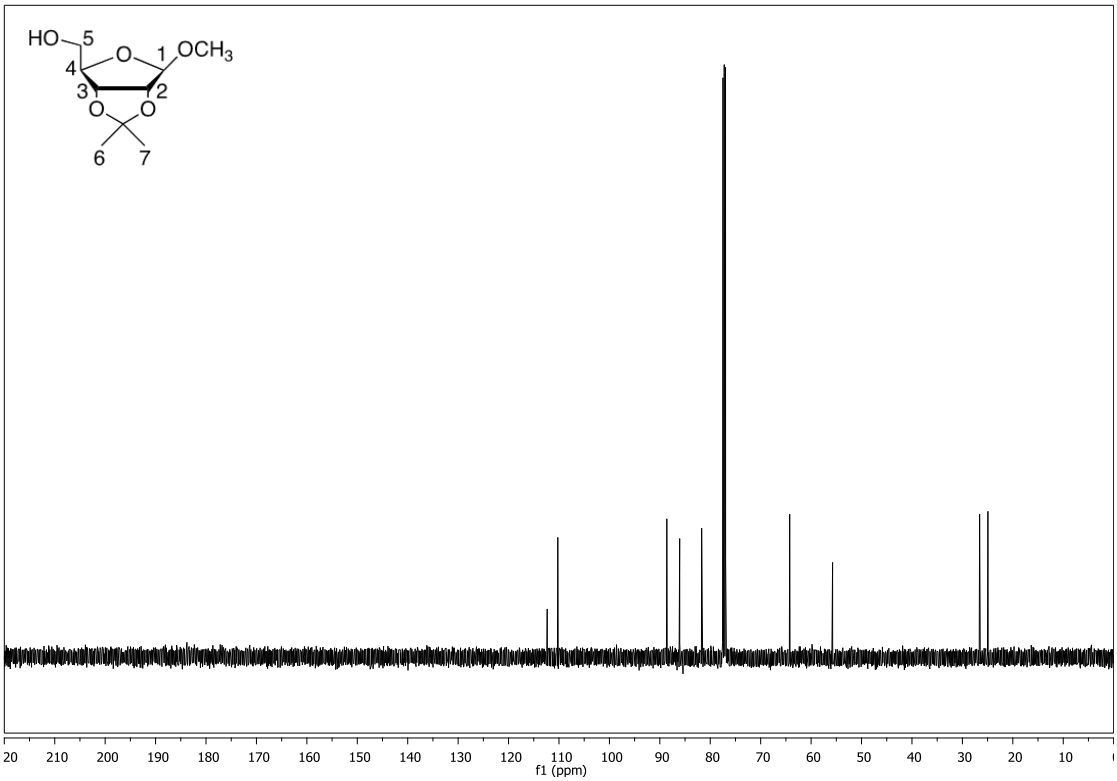
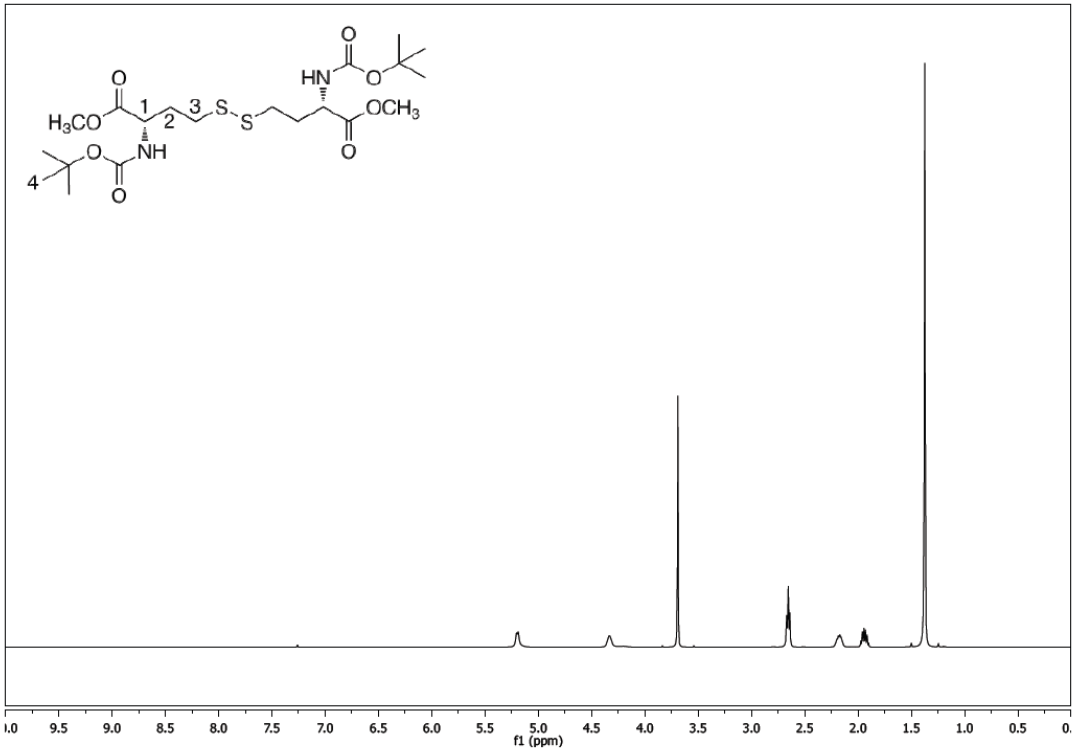
Chapter 7. Supporting Information

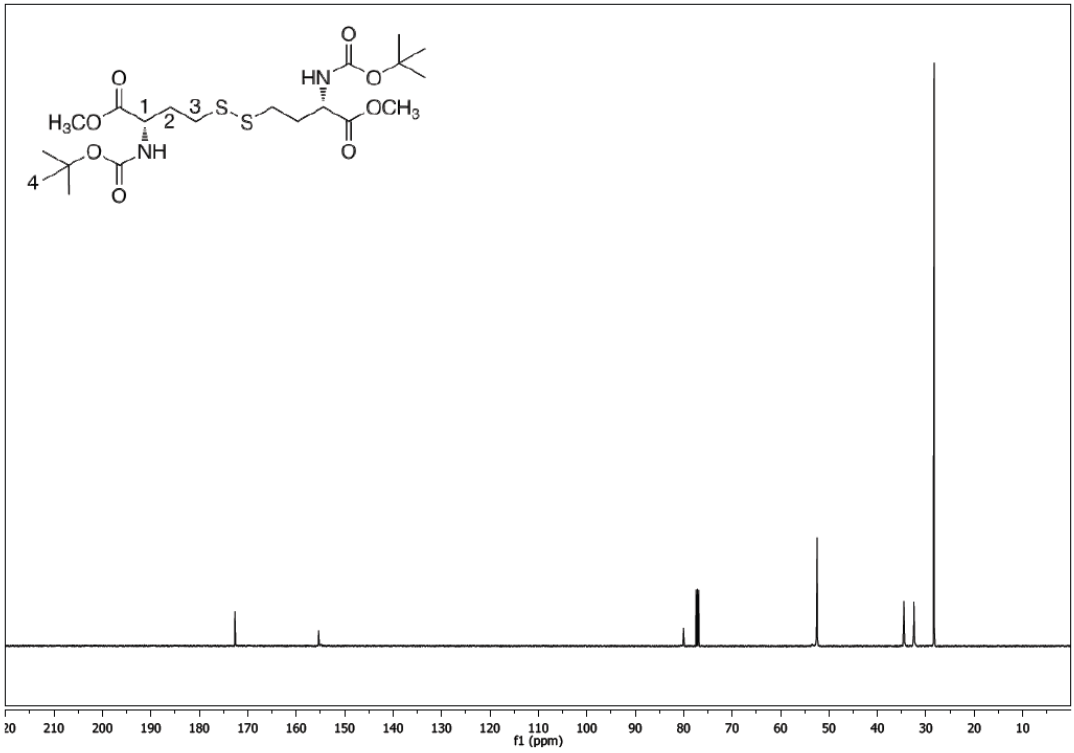
^{1}H and ^{13}C NMR Spectra



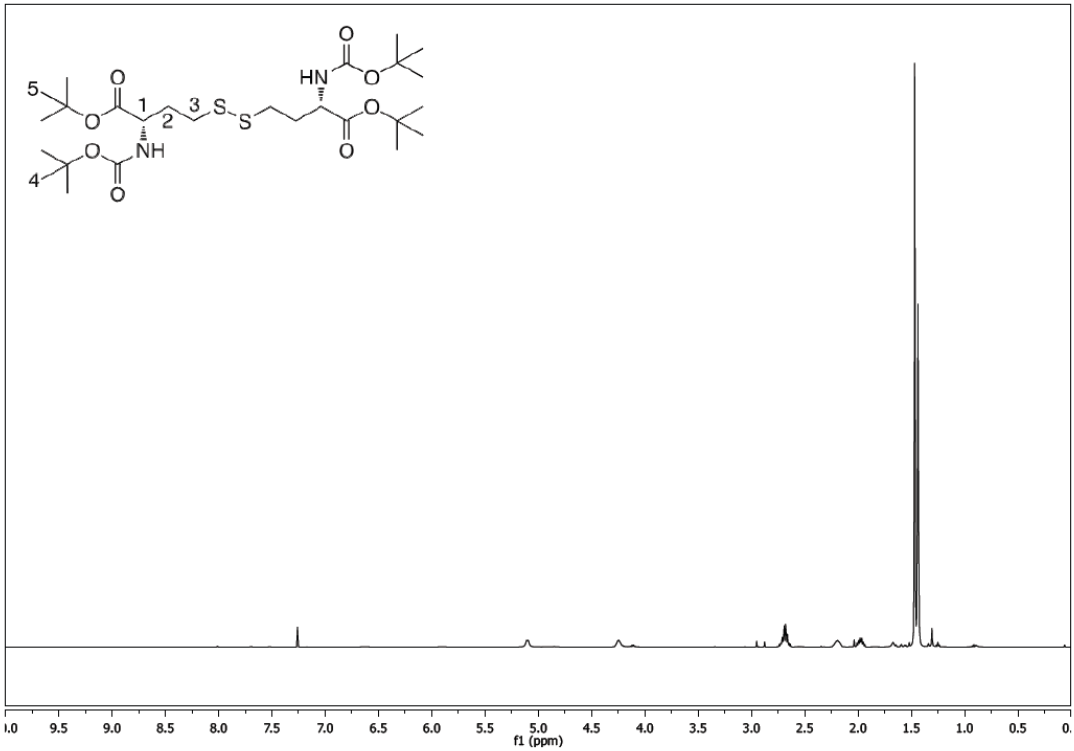


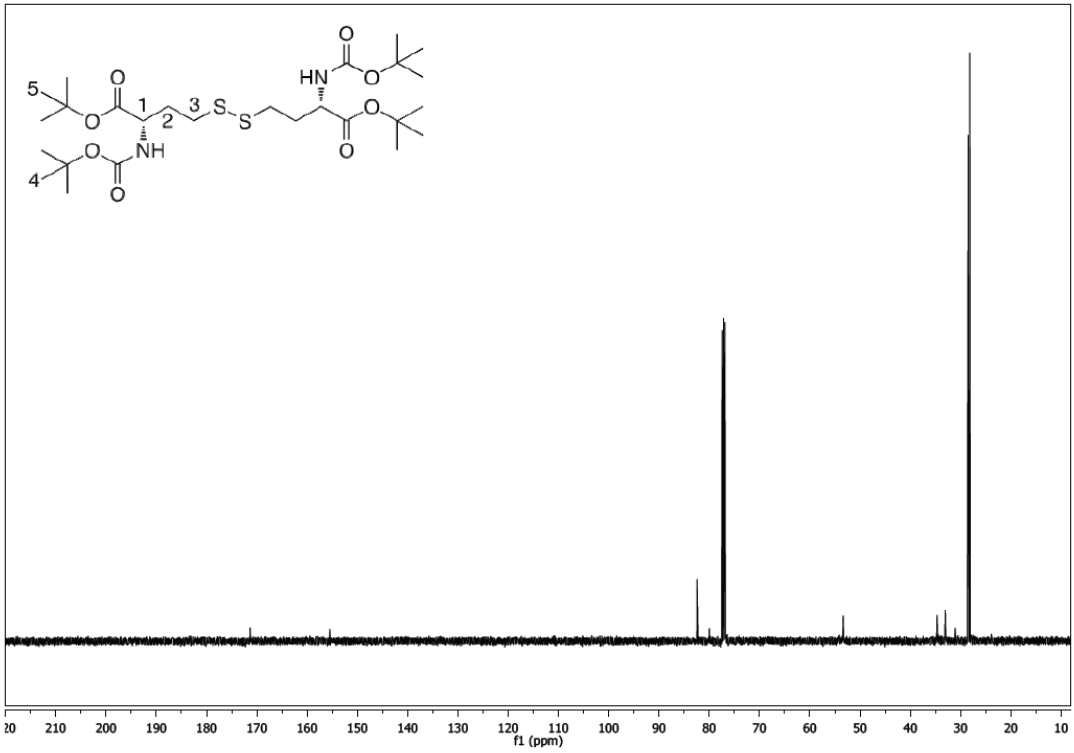
Compound (1)


HN
O
O
OCH3
H3CO
1
2
3
S
S
NH
4
f1 (ppm)


HN
O
O
OCH3
H3CO
1
2
3
S
S
NH
4
f1 (ppm)

Compound (2a)





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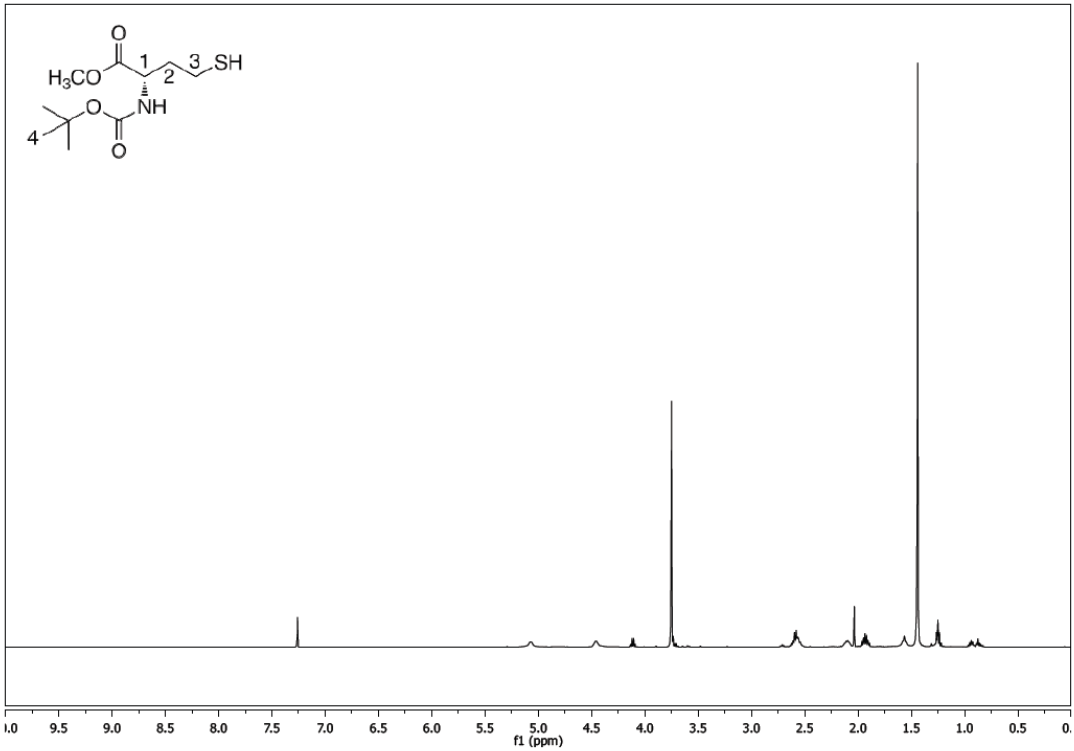
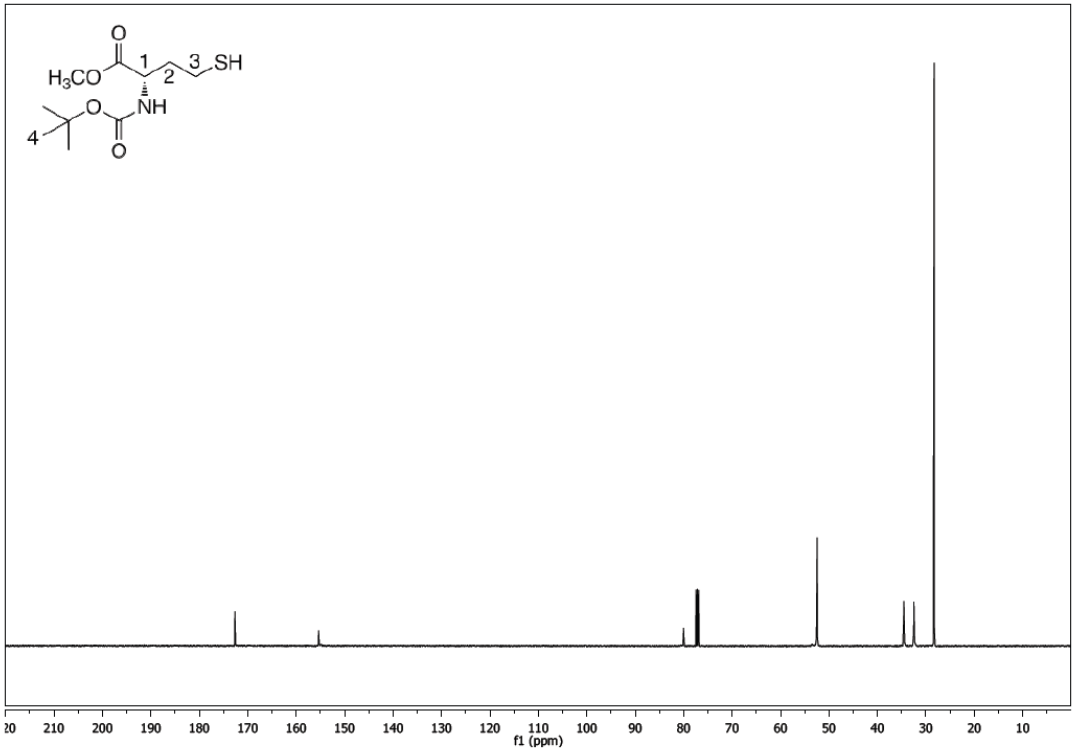
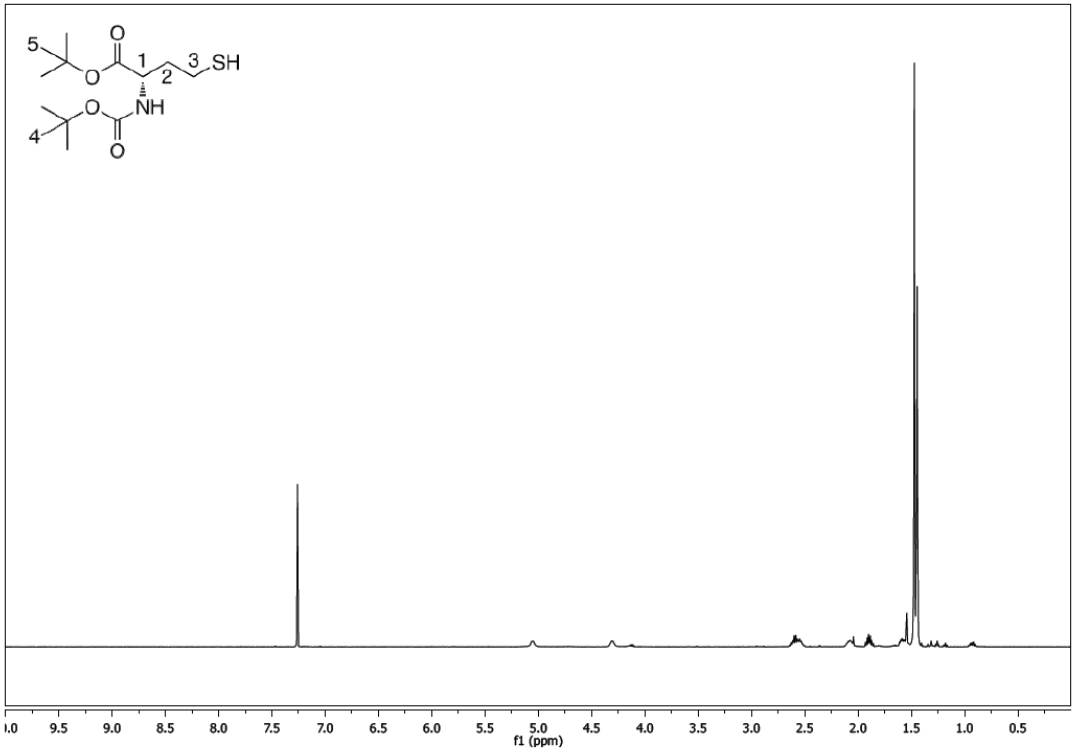
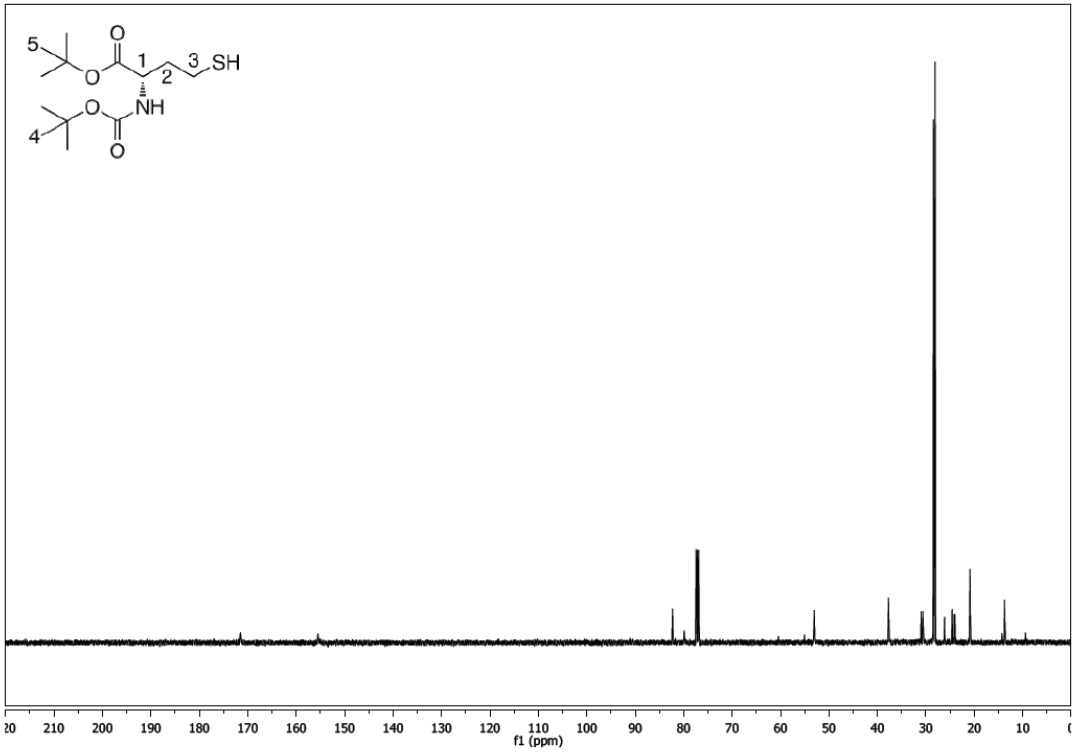

O
H3CO
1
2
3
SH
NH
O
4
O
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f1 (ppm)

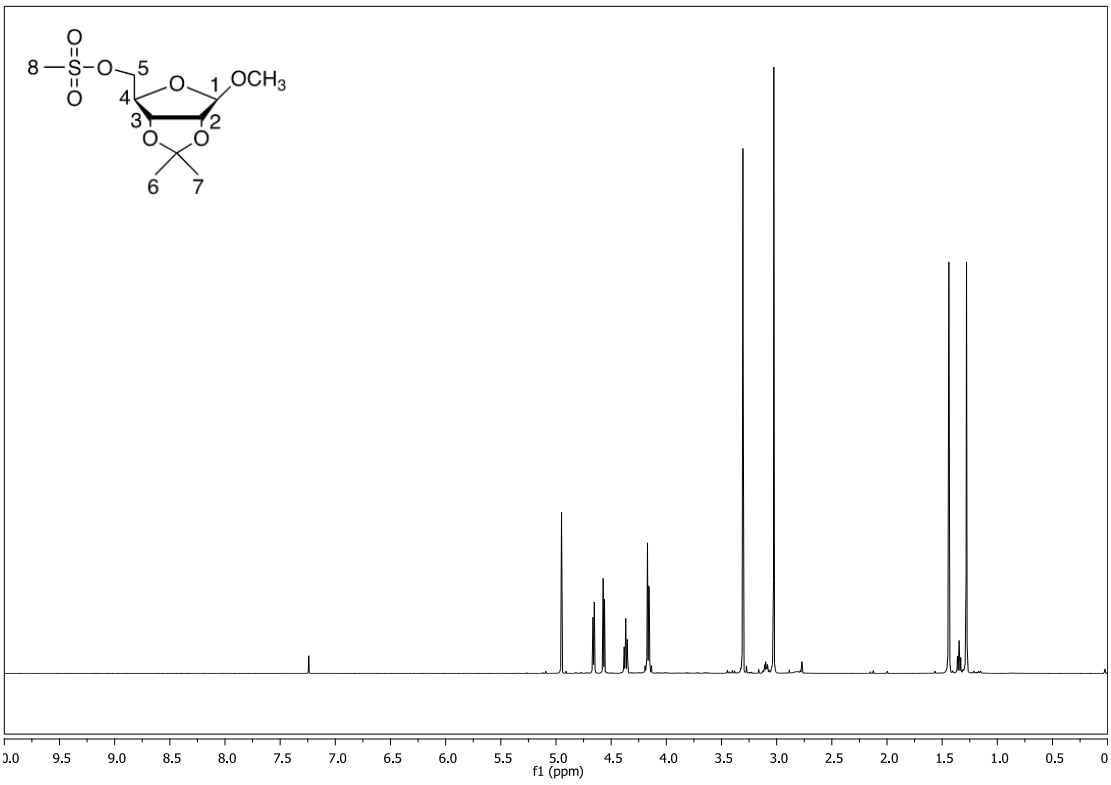

O
H3CO
1
2
3
SH
NH
O
4
O
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

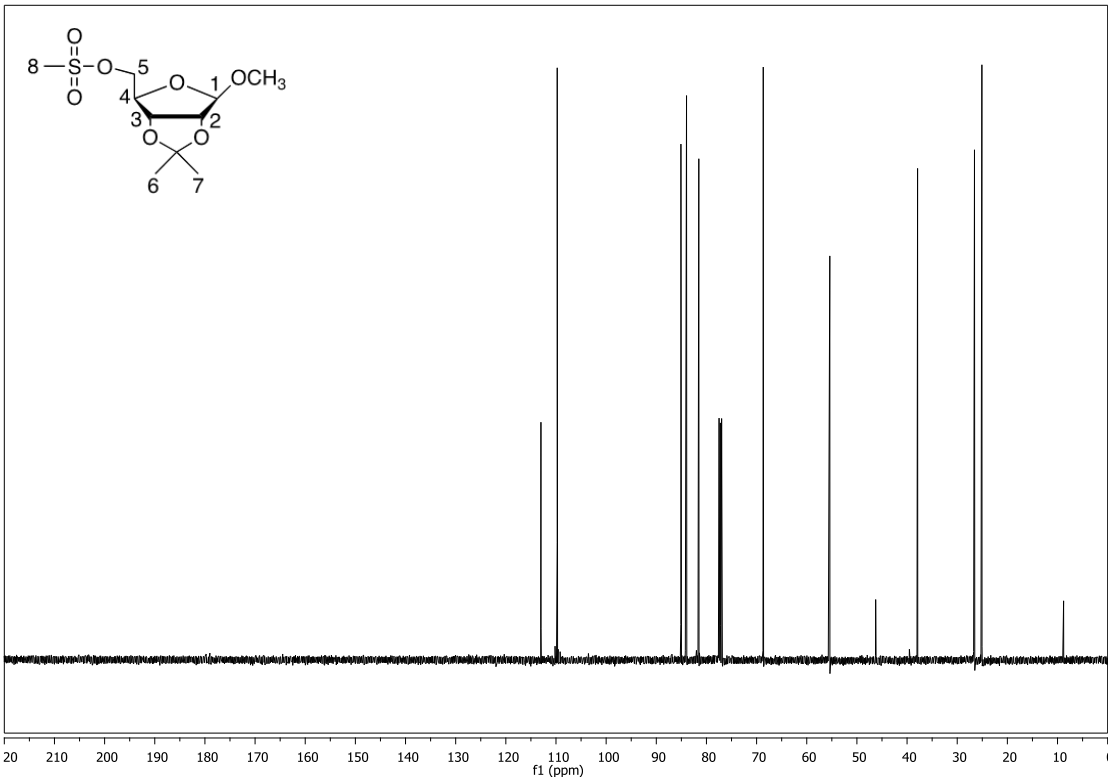
Compound (3a)



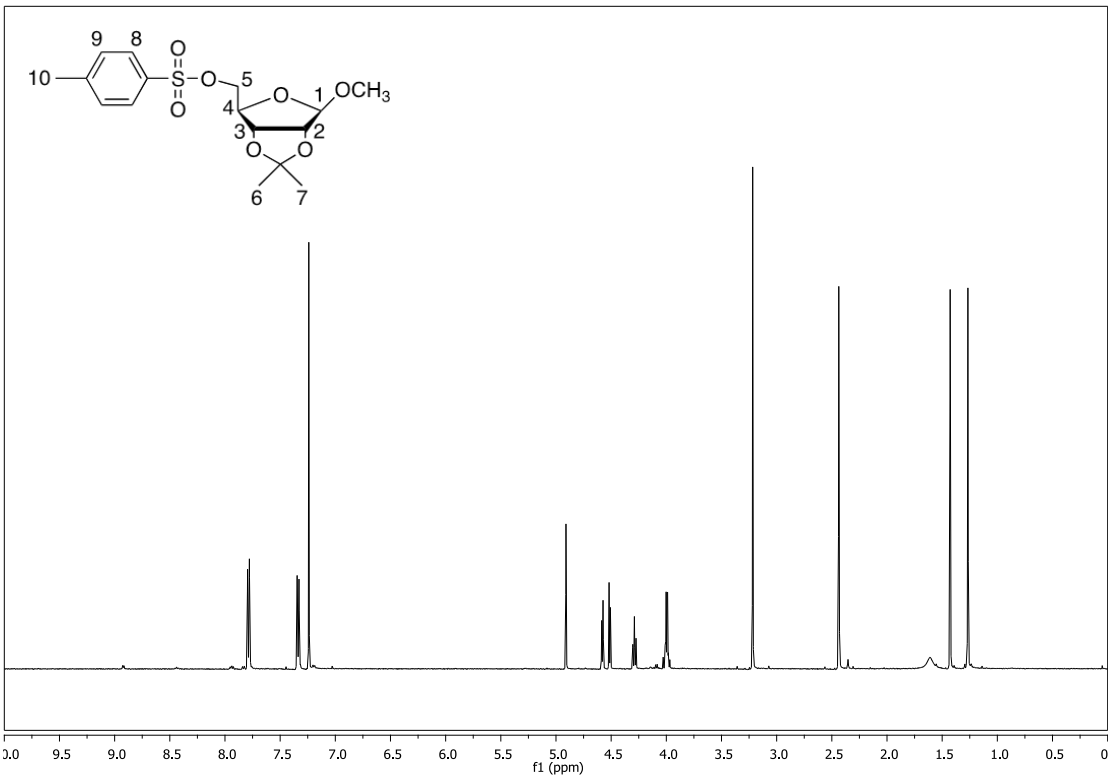


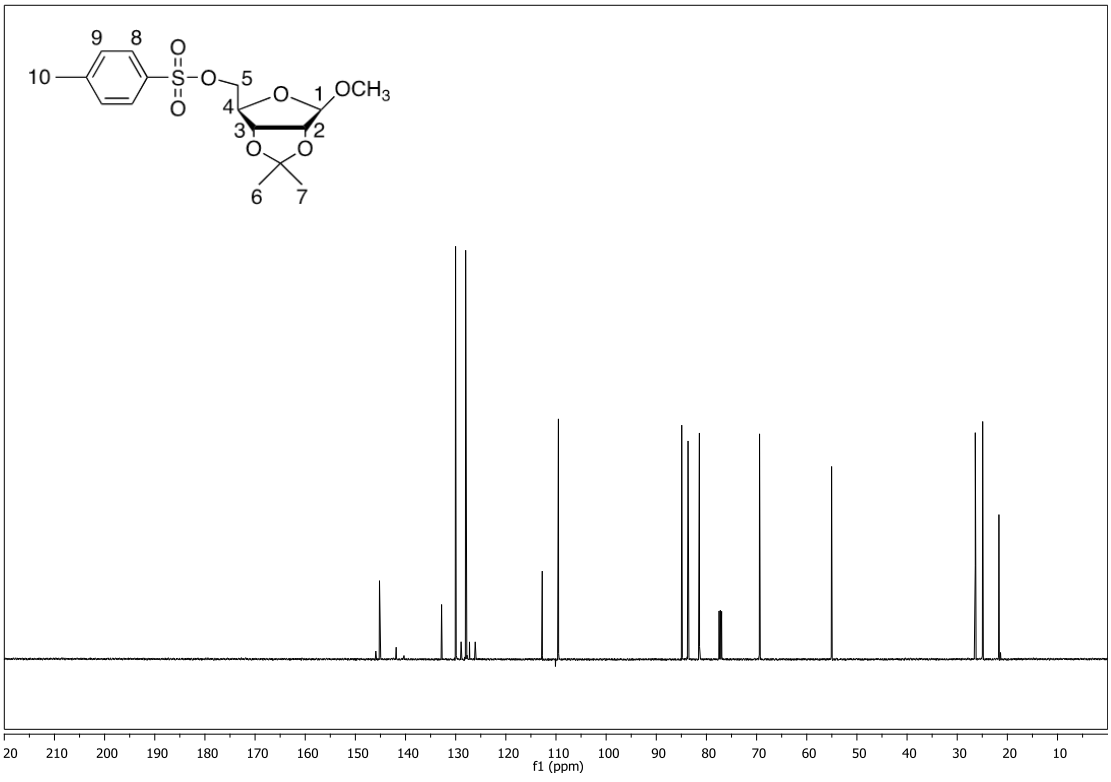
Compound (3b)



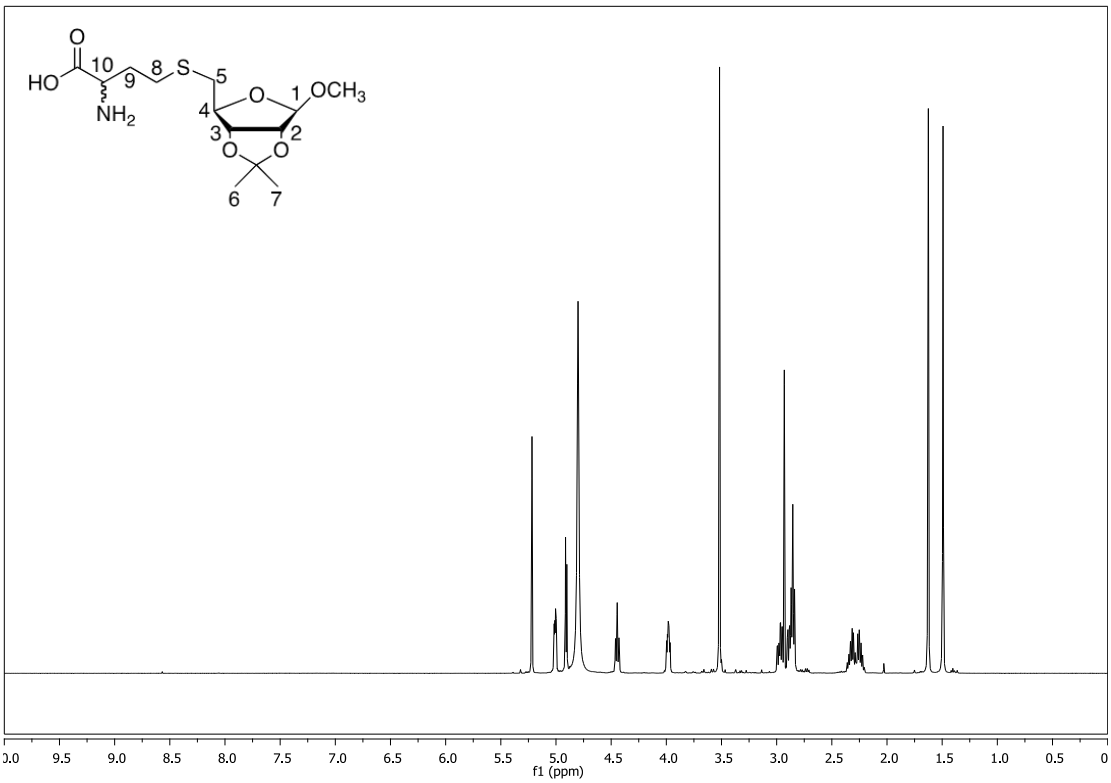


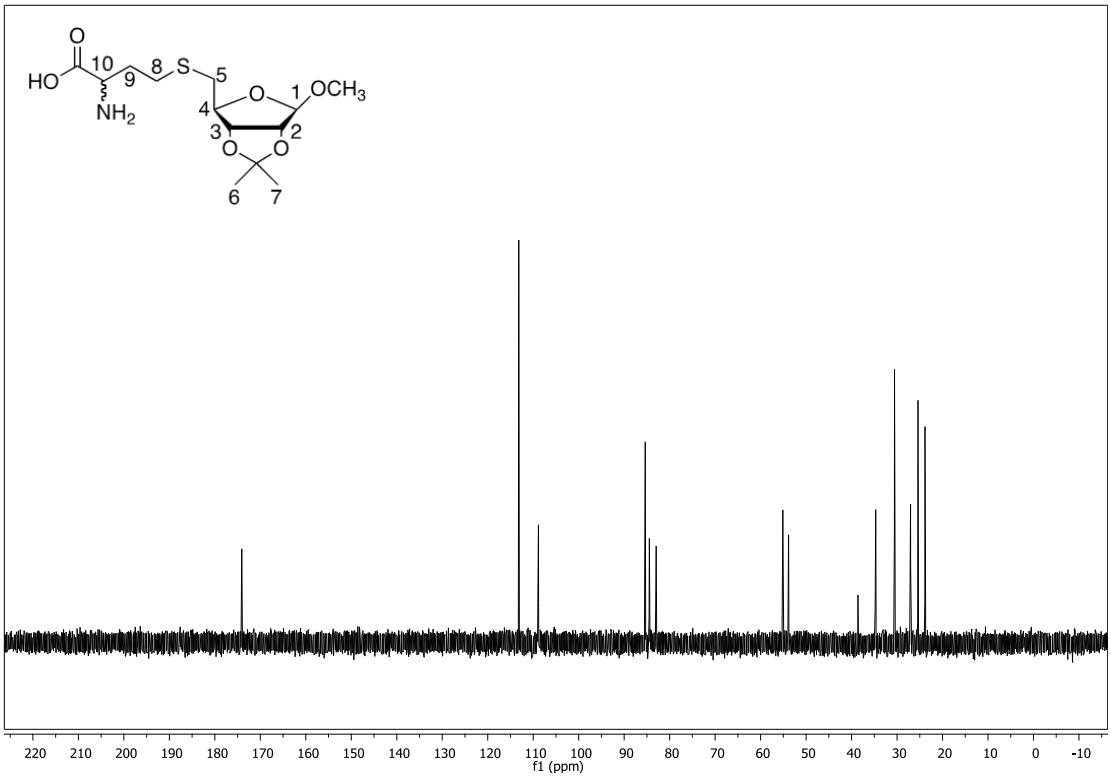
Compound (4a)


9 8
10
O
S
O
O
5
4
O
1
OCH3
3
2
O
O
6 7
f1 (ppm)

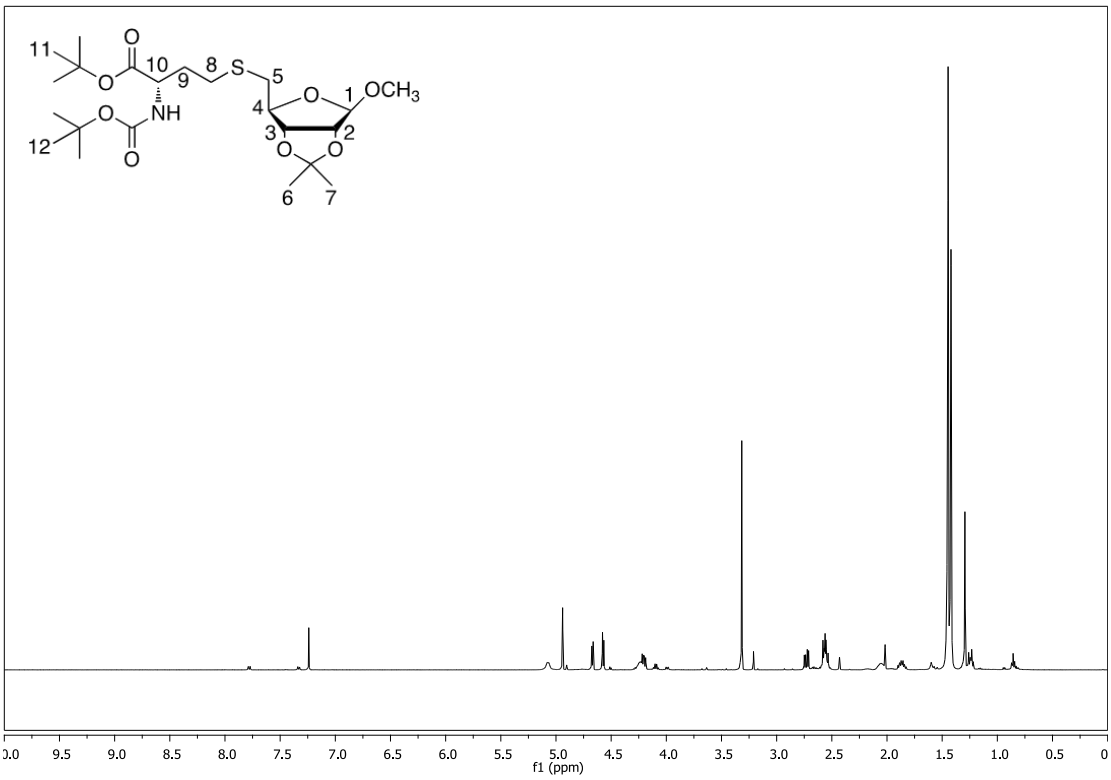

9 8
10
O
S
O
O
5
4
O
1
OCH3
3
2
O
O
6 7
f1 (ppm)

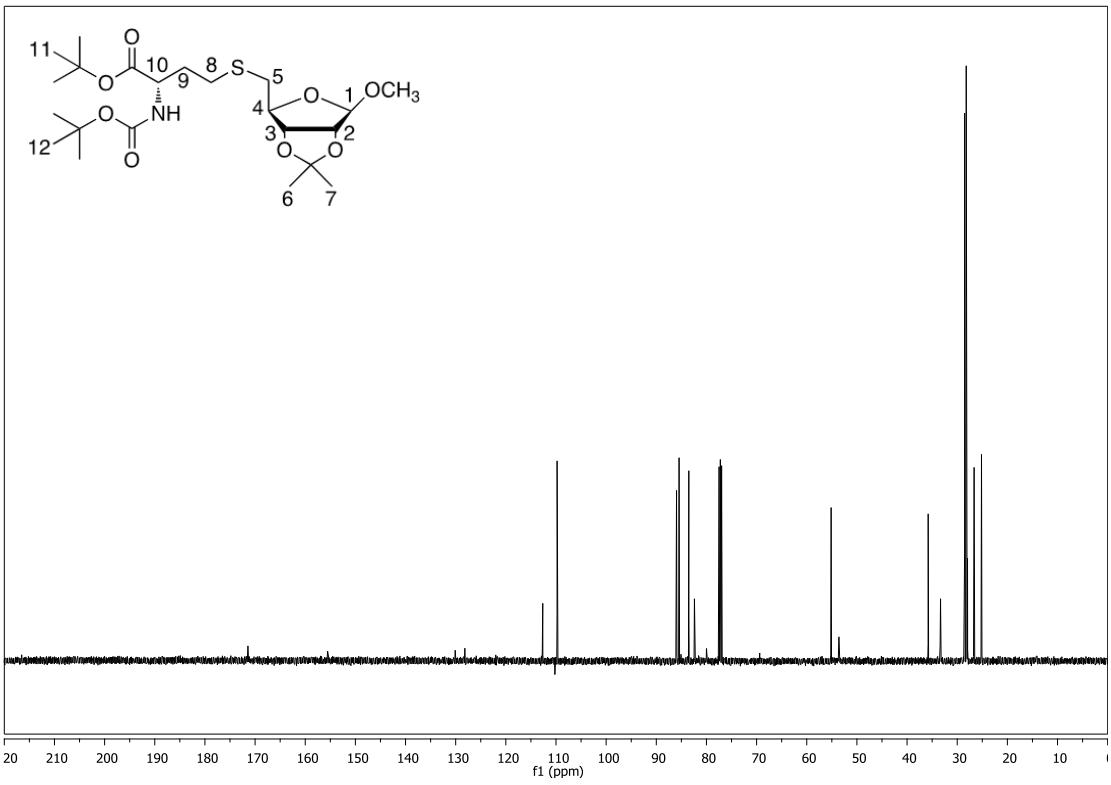
Compound (4b)


HO
O
10
9
8
S
5
4
3
O
1
OCH3
2
O
O
6
7
NH2
f1 (ppm)

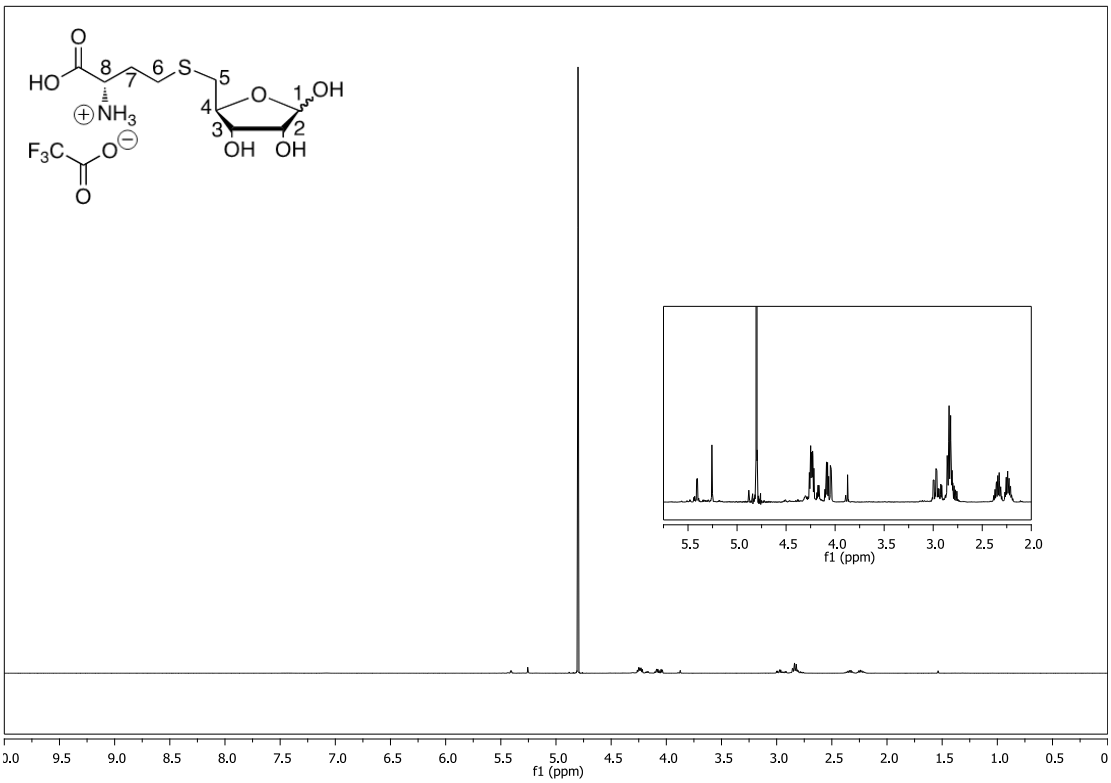

HO
O
10
9
8
S
5
4
3
O
1
OCH3
2
O
O
6
7
NH2
f1 (ppm)

Compound (5)

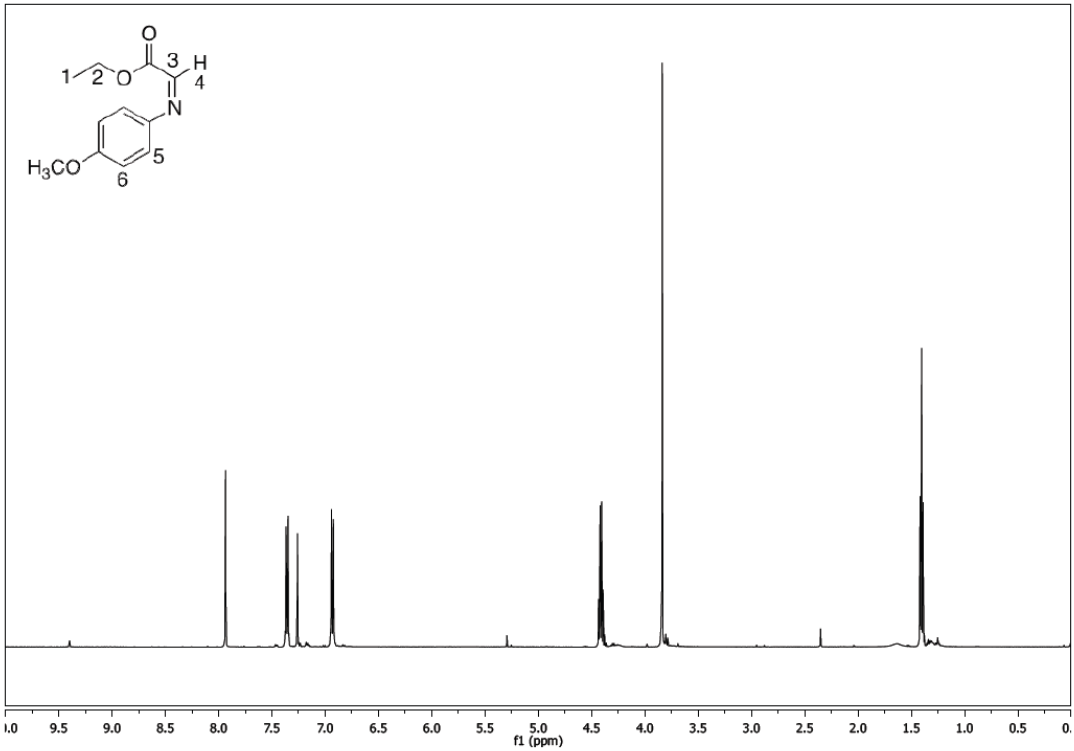

11
O
10
8
S
5
O
1
OCH3
9
4
NH
3
2
12
O
O
O
6
7
f1 (ppm)

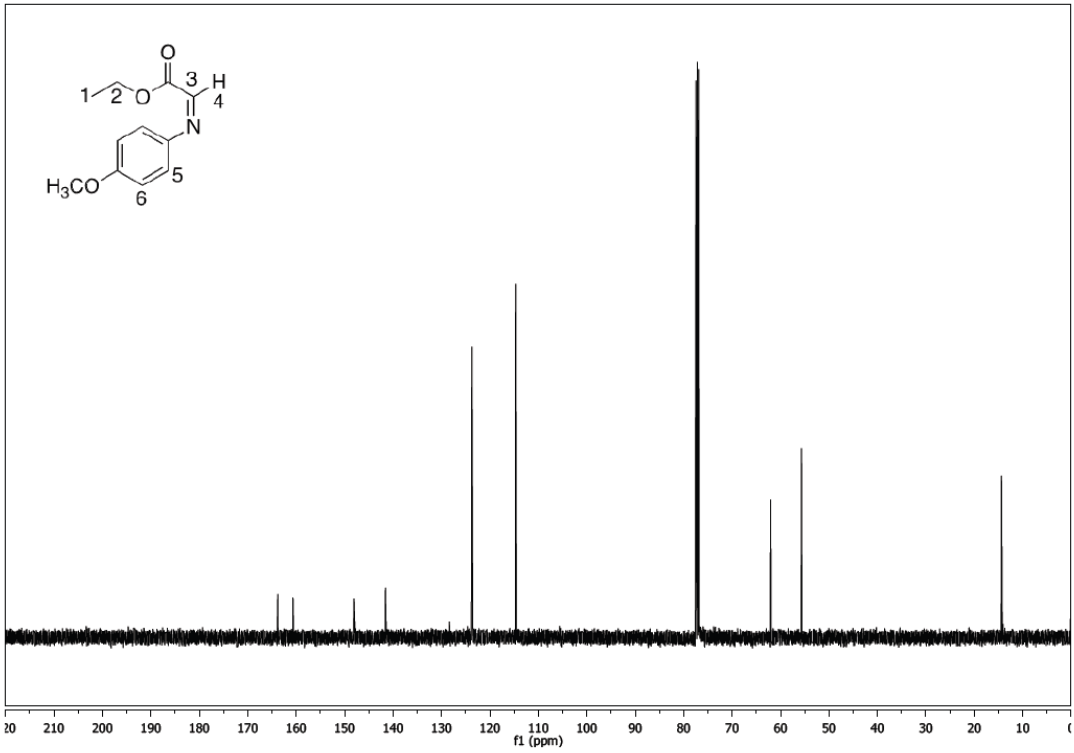

11
O
10
8
S
5
O
1
OCH3
9
4
NH
3
2
12
O
O
O
6
7
f1 (ppm)

Compound (7)

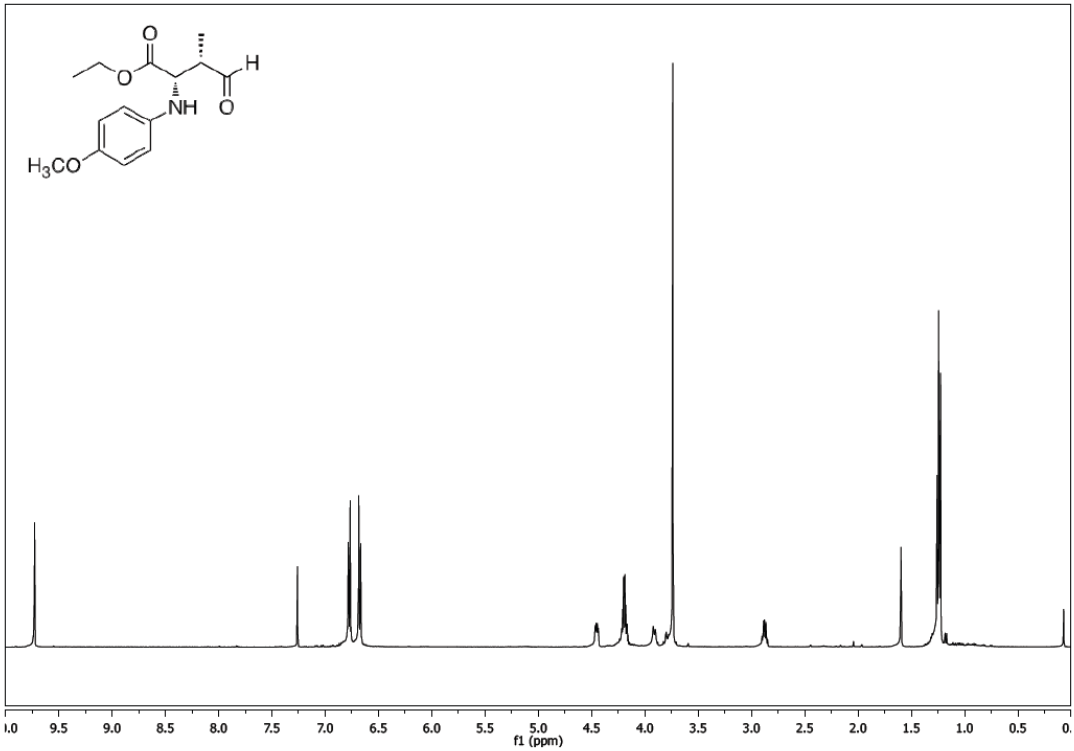

HO
O
8
7
6
S
5
4
O
1
OH
3
2
OH
OH
NH3
F3C
O
O
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
f1 (ppm)
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
f1 (ppm)

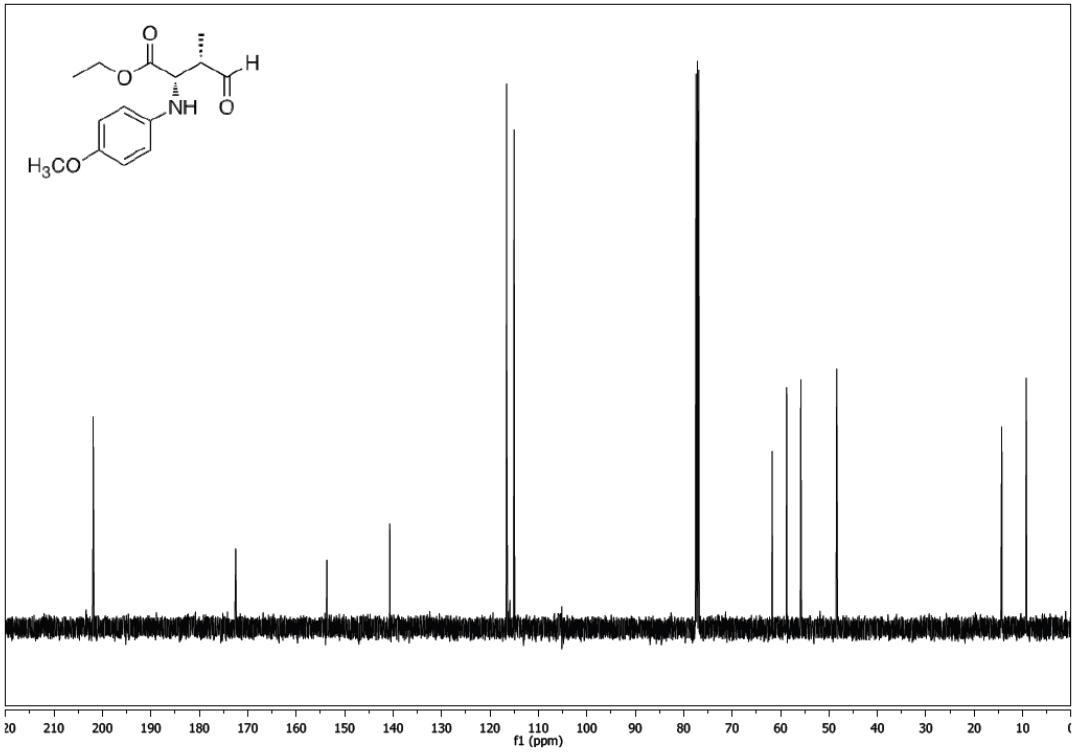
Compound (8)


O
3
H
4
1
2
O
N
5
H_3CO
6
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f1 (ppm)

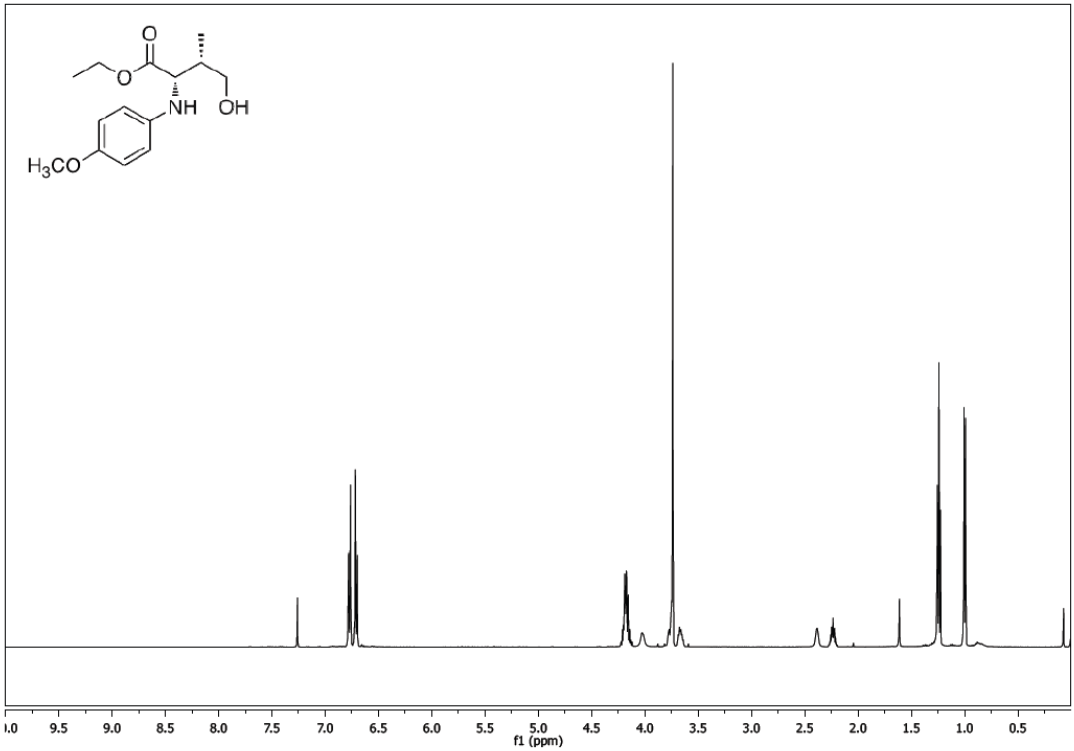

O
3
H
4
1
2
O
N
5
H_3CO
6
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

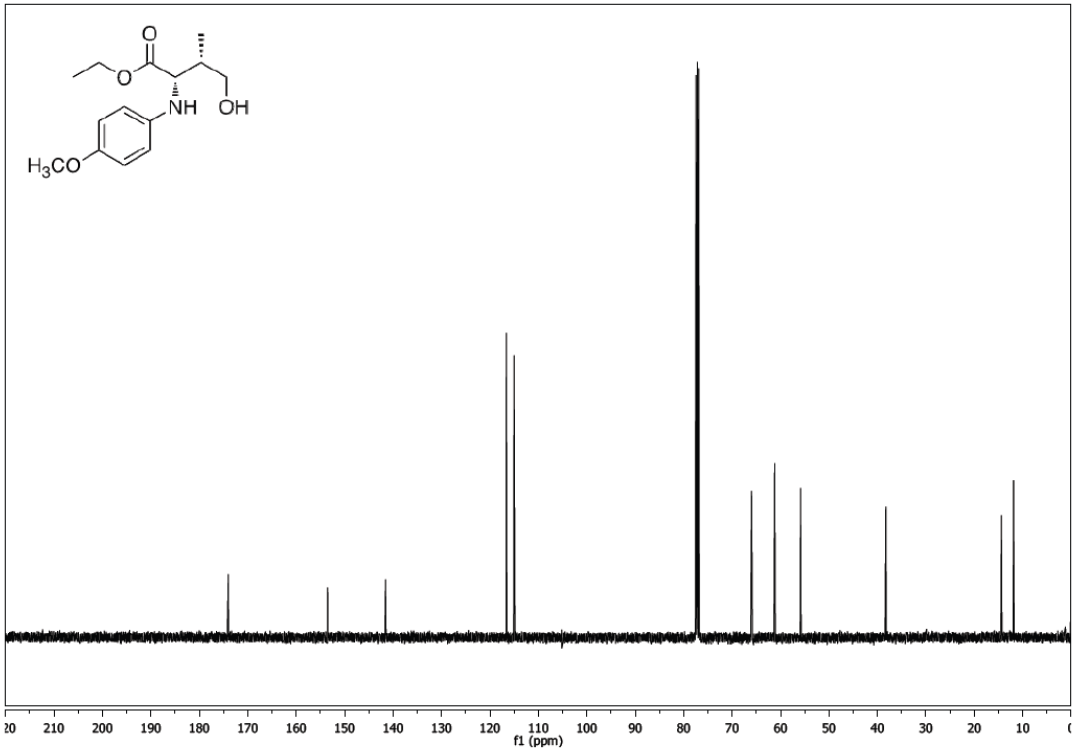
Compound (9)


O
O
H
NH
O
H3CO
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)

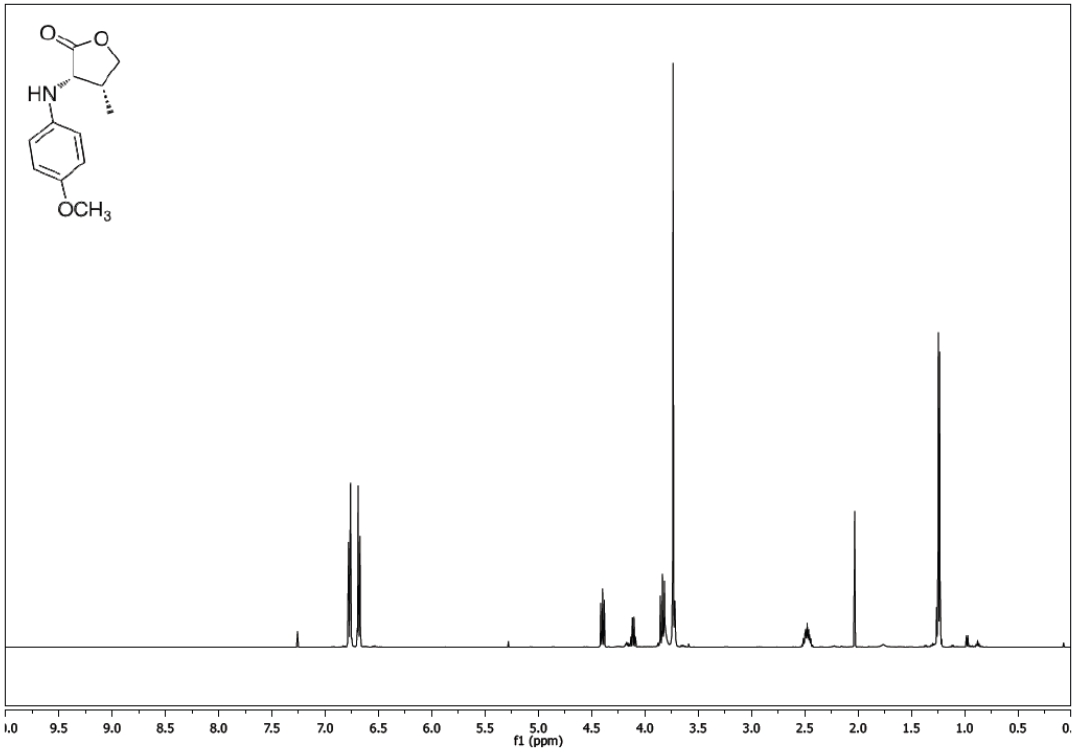

O
O
H
NH
O
H3CO
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
f1 (ppm)

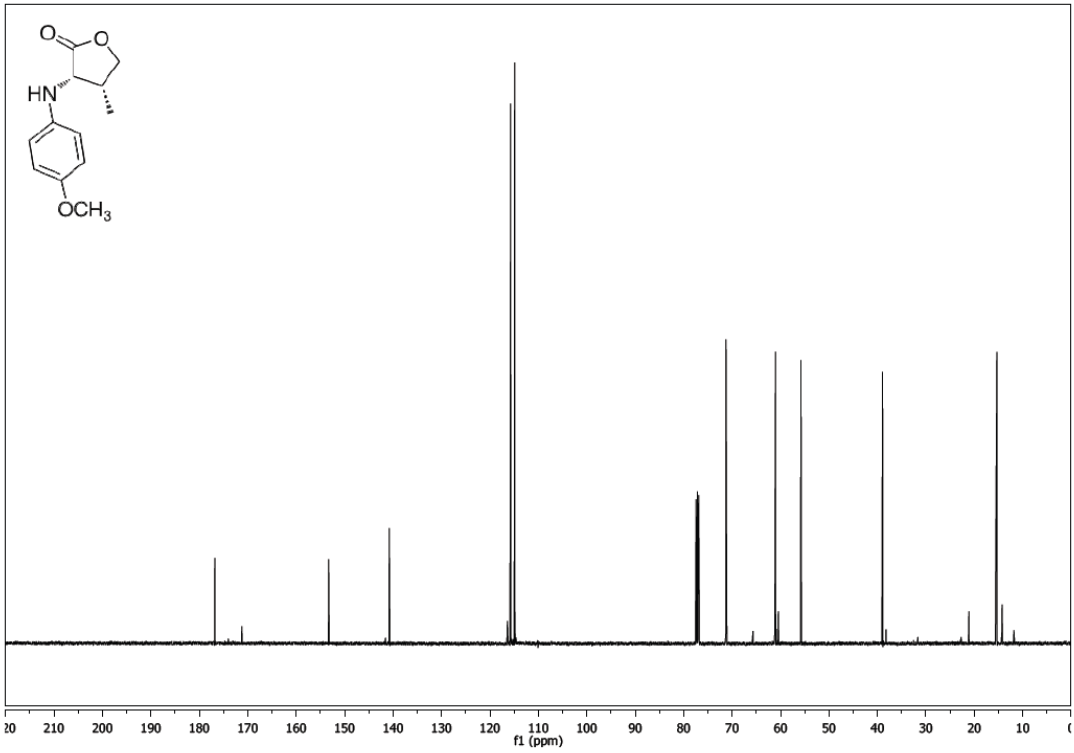
Compound (10)



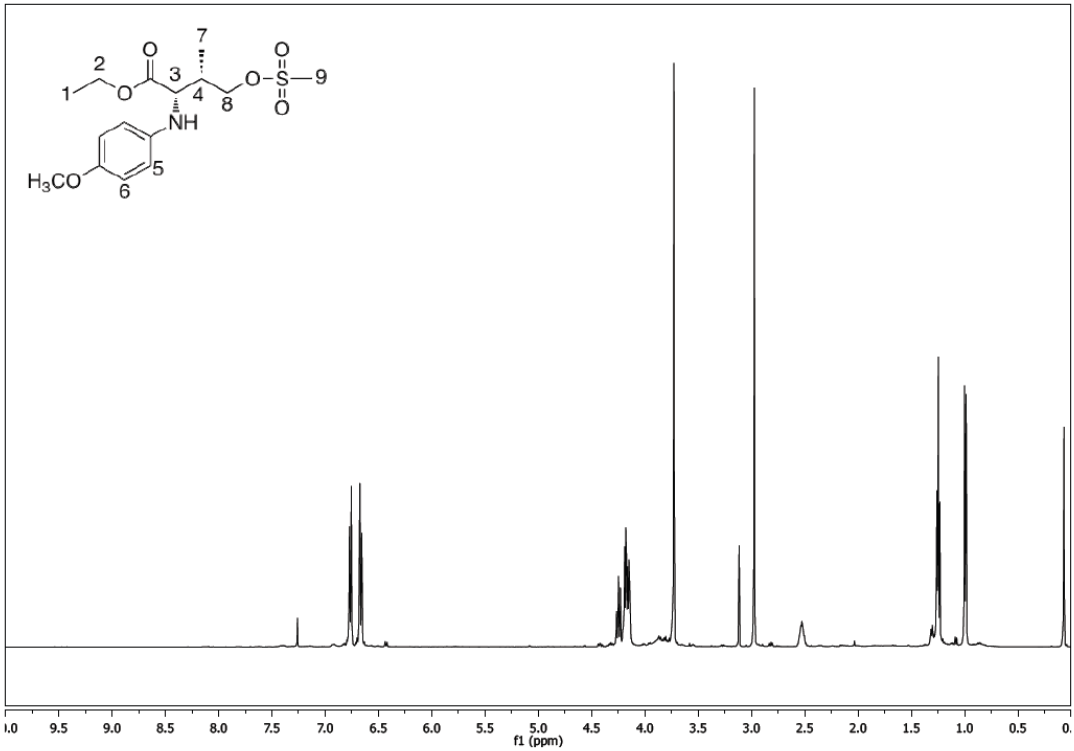


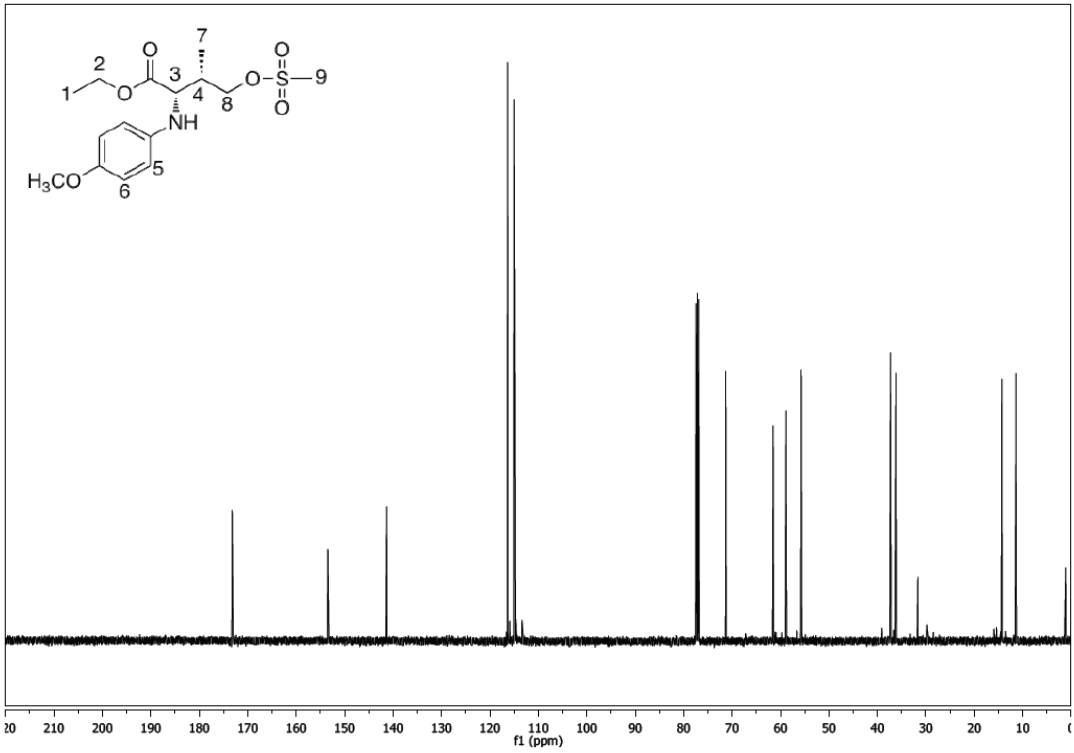
Compound (11a)





Compound (11b)





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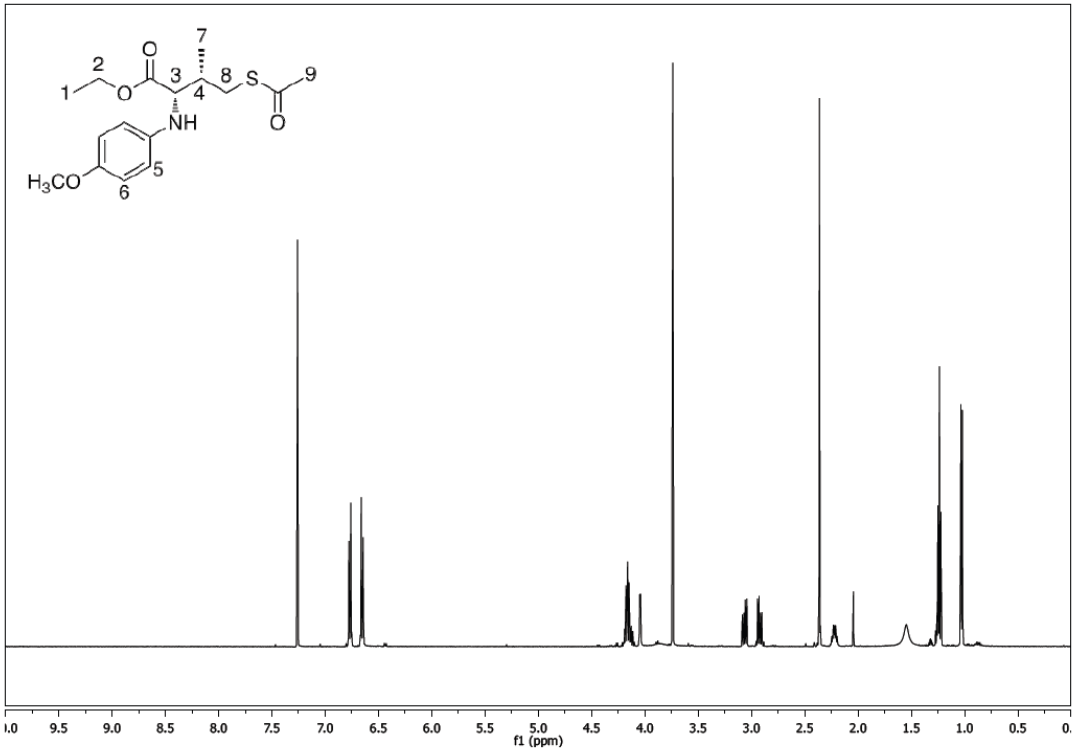

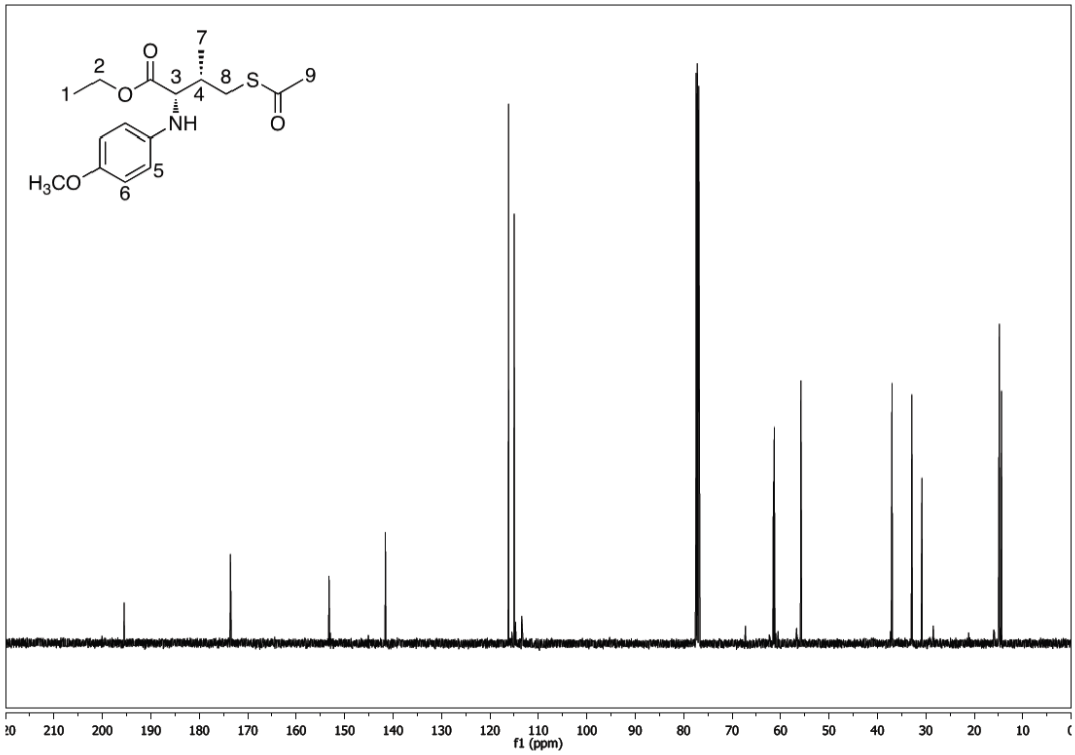

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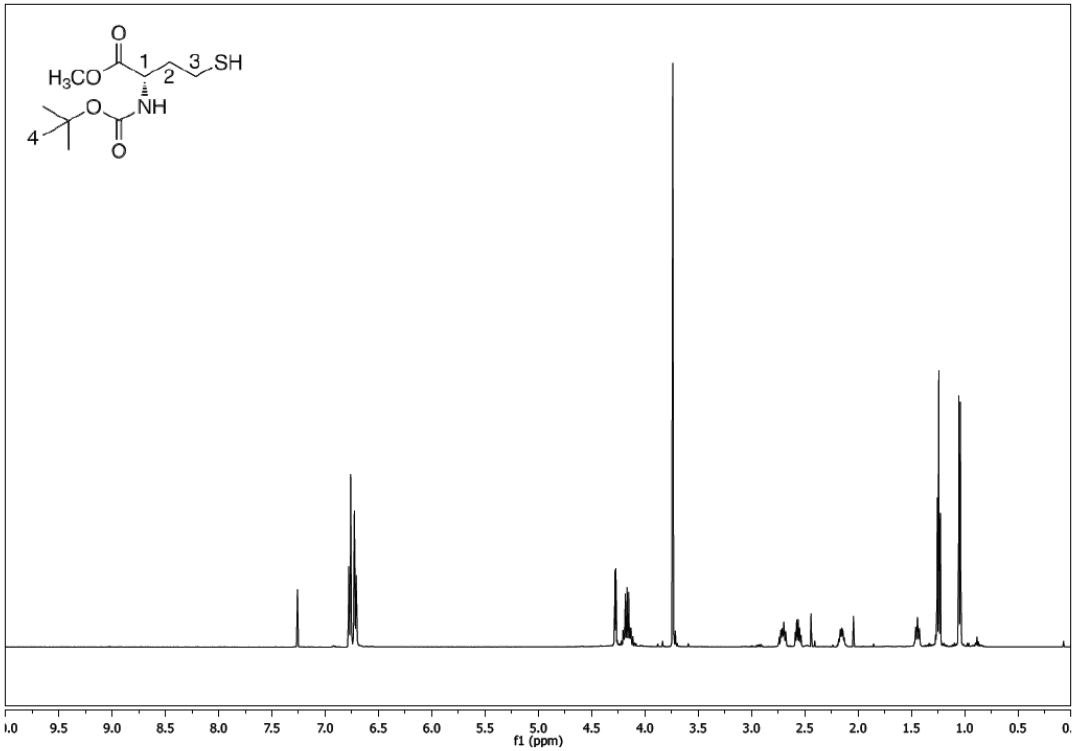
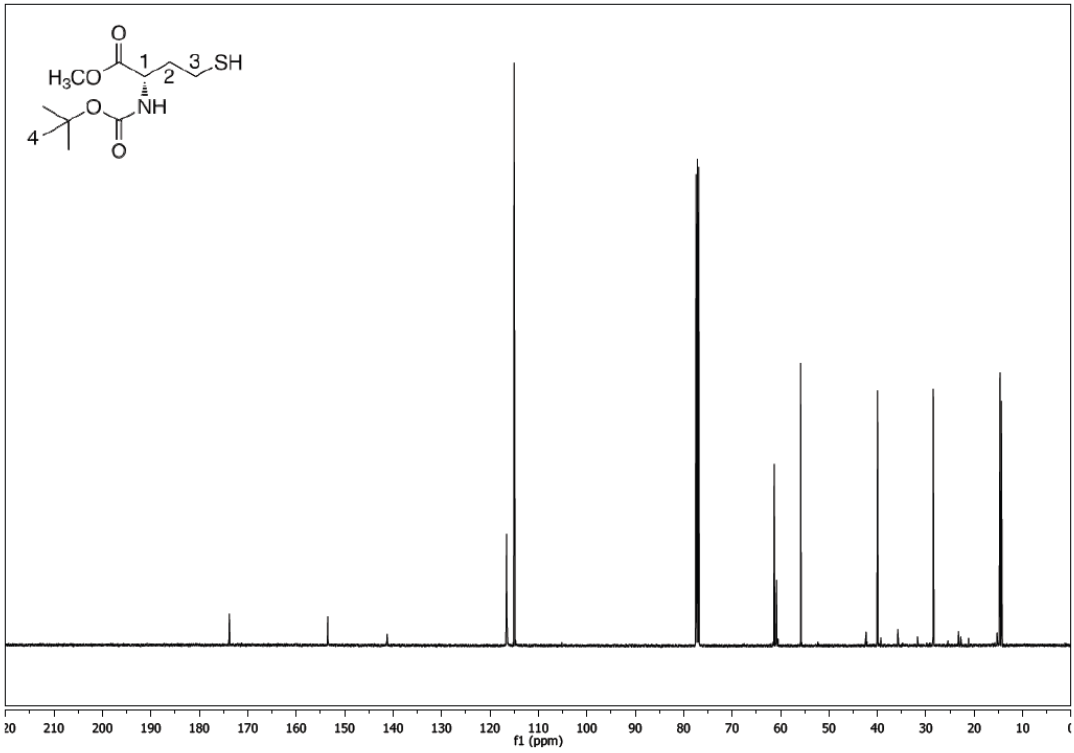
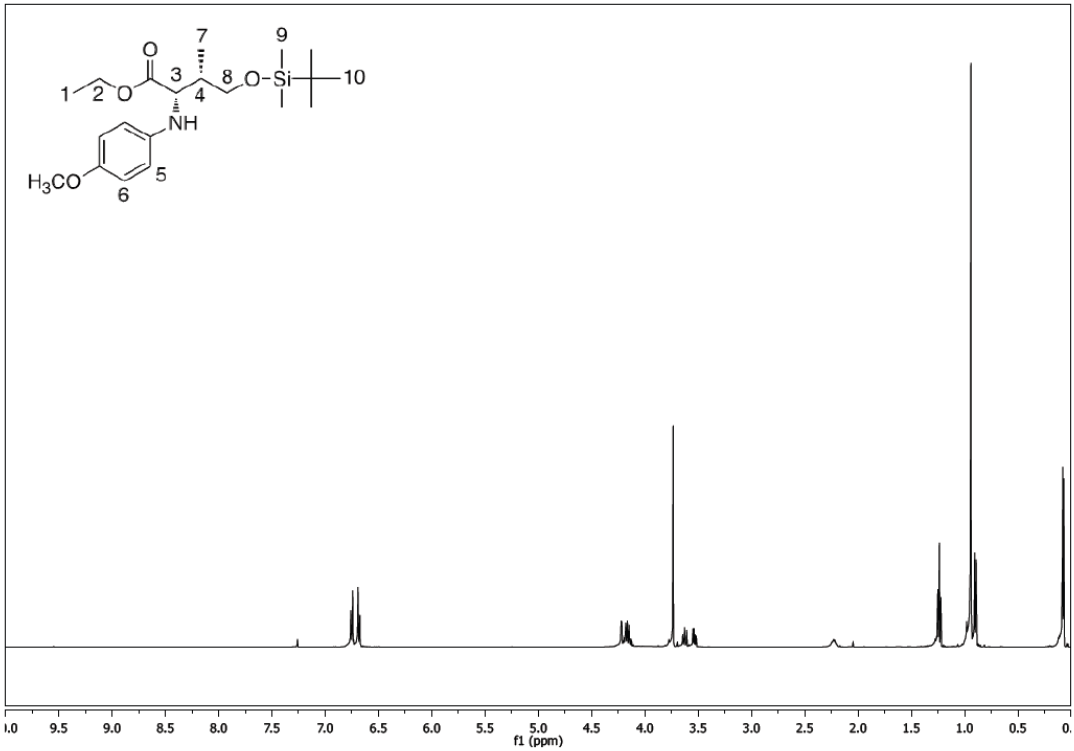
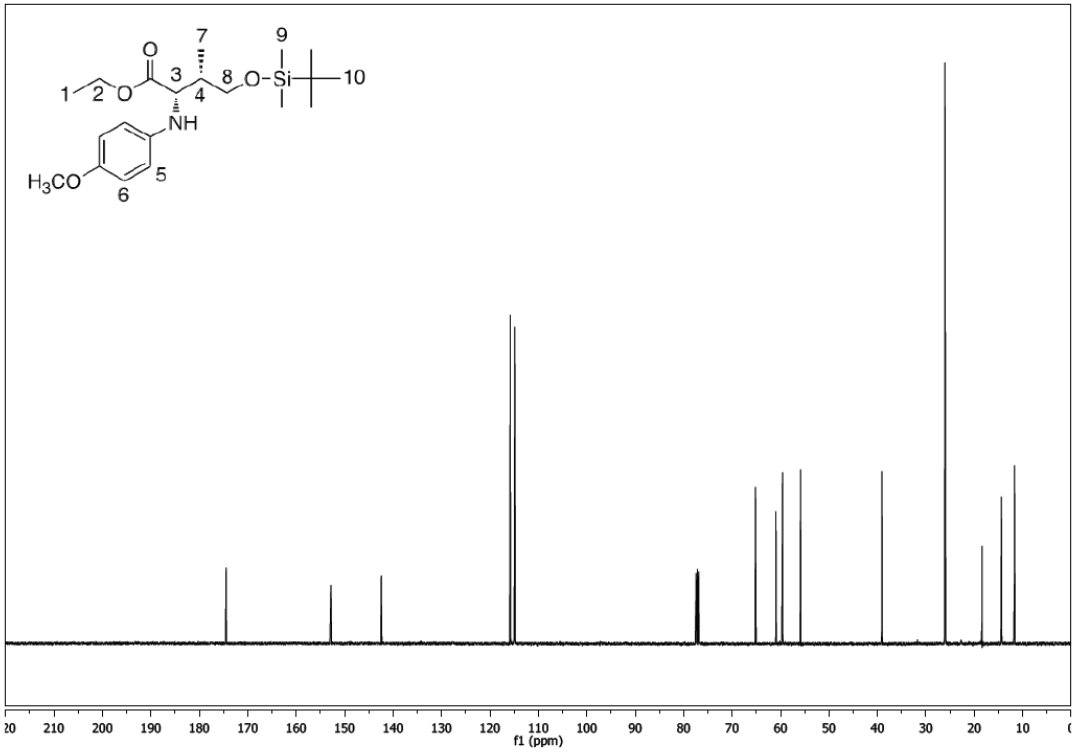


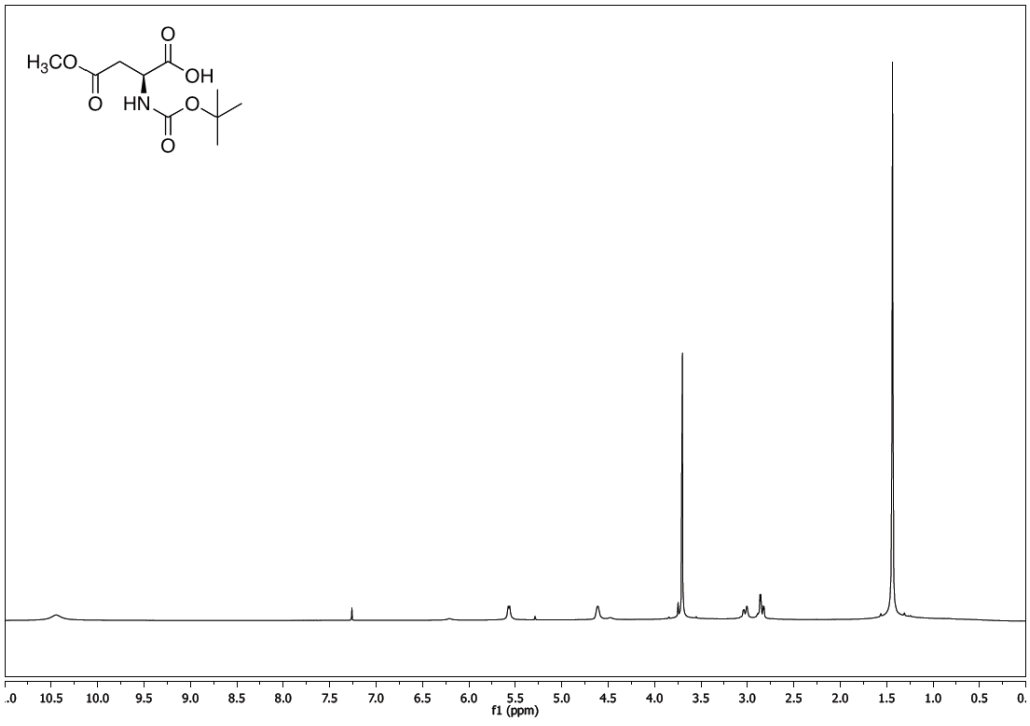


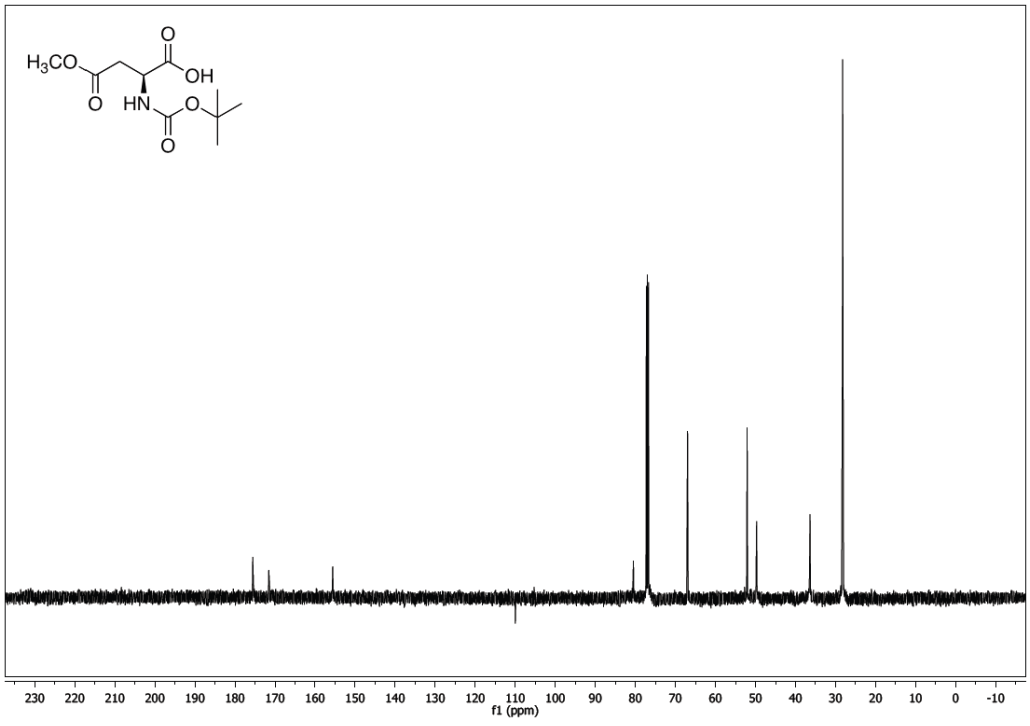
Compound (14b)


O
7
9
1
2
O
3
4
8
O-Si
10
NH
H3CO
5
6
f1 (ppm)


O
7
9
1
2
O
3
4
8
O-Si
10
NH
H3CO
5
6
f1 (ppm)

Compound (16)





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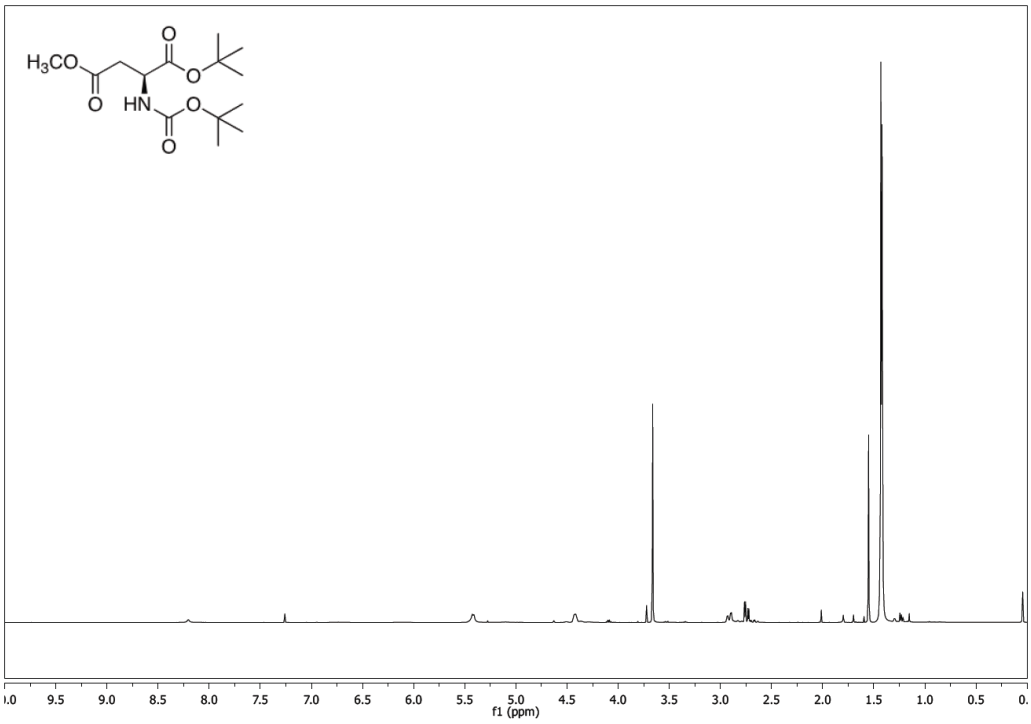

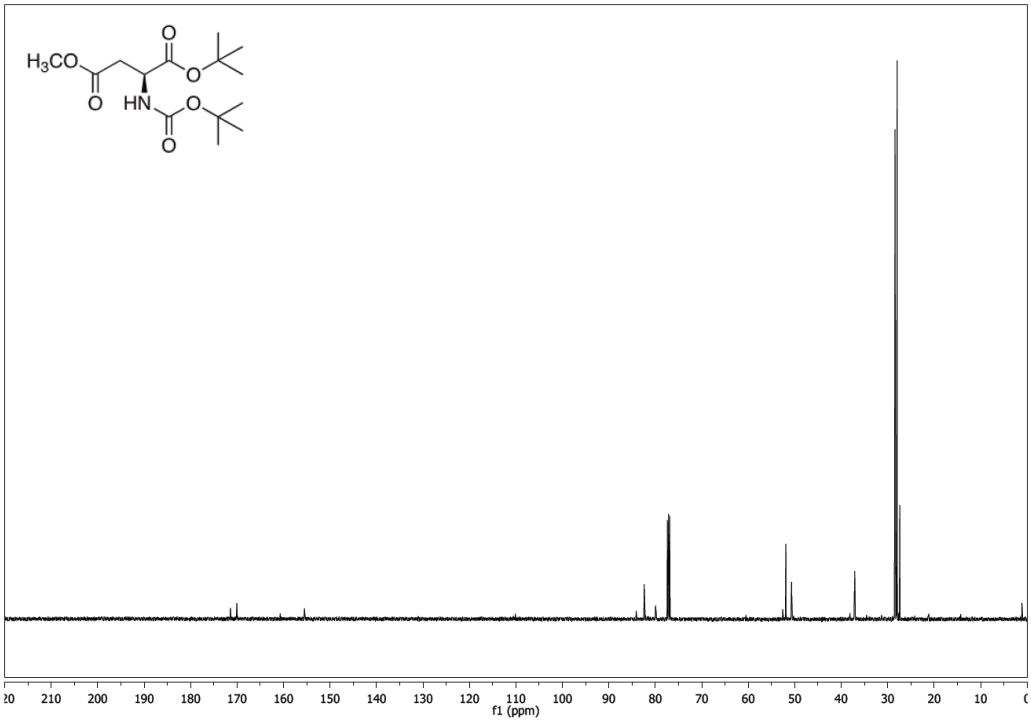

Compound (19)