STUDIES ON UPTAKE OF THIAMIN ANALOGS BY A THIAMIN DEFICIENT *E. coli* MUTANT STRAIN

A Thesis

By

SARAH ELIZABETH OLIVARD

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Approved by:

Chair of Committee,	Tadhg Begley
Committee Members,	Coran Watanabe
	Paul Straight
Head of Department,	David Russell

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ABSTRACT

Thiamin transport in *Escherichia coli* is a model system to establish the tolerance of derivatives for transport into the cell. Since little is known about what types of thiamin derivatives may be successfully taken into the cell through the transport system, a series of thiamin derivatives are synthesized. A thiamin amino analog is synthesized and tested to determine the use of the analog as an alternate source of thiamin for growth of an *E. coli* thiamin mutant. Formate, acetate, and benzoate thiamin esters are synthesized and tested as alternate sources for growth of an *E. coli* thiamin mutant.

Thiamin esters or amides may provide a scaffold for attaching other small molecules of interest to be imported into the cell by thiamin transport system. Thiamin containing formate, acetate, and benzoate esters were synthesized and tested as alternative growth source for thiamin using an *E. coli* mutant strain incapable of synthesizing thiamin. All three synthesized ester thiamin forms gave a zone of growth determined by disk-assay study. Also, an amino thiamin is synthesized to determine uptake through thiamin transport system by growth study using an *E. coli* mutant incapable of synthesizing thiamin. The growth curves resulting show concentration-dependent growth in the absence of natural thiamin, indicating amino thiamin is taken up by thiamin transport system as an alternate source of thiamin for growth. More characterization of the thiamin transport system is desired in order to develop thiamin conjugates of interest such as a photoaffinity probe for isolating thiamin-utilizing enzymes.

DEDICATION

For my mother, father, and sisters

ACKNOWLEDGMENTS

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NOMENCLATURE

ABC	ATP-binding cassette
AcOH	Acetic acid
ATP	Adenosine triphosphate
Calcd	Calculated
BtuCD	Transport proteins of vitamin B12
ESI-MS	Electrospray ionization mass spectrometry
HBr	Hydrobromic acid
HMP	Hydroxymethyl pyrimidine
HPLC	High pressure liquid chromatography
kDa	Kilodalton
MHz	Megahertz
NBD	Nucleotide binding domain
NMR	Nuclear magnetic resonance
TbpA	Thiamin binding protein
TDP	Thiamin diphosphate
ThiB	Periplasmic binding protein of *S. typhiurium*
ThiE	Thiamin phosphate synthase
TMD	Transmembrane domain
TMP	Thiamin monophosphate

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INTRODUCTION AND LITERATURE OVERVIEW

ABC Transporters and Relevant Systems

ABC transport in gram-negative bacteria consists of a periplasmic binding protein that binds the substrate, a transmembrane protein that receives the substrate from the binding protein, and a cytosolic ATPase (ABC) that supplies the energy for movement of the substrate into the cell by catalyzing hydrolysis of two ATP molecules.[1] ABC transporters represent the largest class of transporters.[2a,2b] The primary sequence of the nucleotide binding domain have a highly conserved set of motifs in archaea, eubacteria, and eukarya; the transmembrane domains, however, have a varied sequence and architecture, which allows for different substrates to be translocated by specific transmembrane domains.[2a] The ABC transport system architecture consists of two transmembrane domains (TMDs) that are embedded in the membrane and two nucleotide binding domains (NBDs, or ABCs) that are located in the cytoplasm.[2a] The periplasmic binding protein (for gram negative bacteria) serves as a high-affinity binding protein for particular substrates and delivers the substrate from the periplasm to the specific TMD for translocation in to the cell.

The first crystal structure of a whole ABC transporter was reported for *E. coli* Vitamin B12 BtuCD.[3] This crystal structure initially provided insight on proposed mechanisms of transport as well as determining architecture for ABC transporters. Since then, crystal structures of many ABC transporters have been solved, and at least 65

transporters have been identified in *E. coli* K-12.[1, 2b, 4, 5, 6] Nutrients that are transported through ABC transport are amino acids, phosphate, metals such as iron, carbohydrates, vitamins, and other essential nutrients.

Thiamin Transport in *E. coli* and Relevant Studies

Thiamin transport in *E. coli* has been studied using mutants that require thiamin monophosphate or diphosphate for growth.[7] Transport through a specific protein was identified through measurement of exit of radiolabeled [^{35}S]-thiamin after addition of thiamin to the medium.[7] The periplasmic thiamin binding protein was overexpressed, purified, and characterized in 2002, and an assay has been used to determine the dissociation constant of thiamine, thiamin monophosphate, and thiamin diphosphate.[8] All three substrates bind the periplasmic protein with high affinity (between 2.3 and 7.4 nM).[1,8] Structurally similar periplasmic binding proteins include thiamin binding protein ThiB from *S. typhimuirum* and also the Fe^{3+}- binding protein from *H. influenzae*.[4,9] Moreover, thiamin binding protein of *E.* coli (TbpA) is found to be structurally similar to thiaminase-I, a thiamin degrading enzyme, based on modeling studies that suggests the two proteins developed from a common ancestor.[1]

Thiamin transport system in *E. coli* has also been studied through inactivation of transport using chemical inhibitors. Pyrithiamine and oxythiamine are found to inhibit the rate of thiamin uptake through measurement of uptake of [^{35}S]-thiamin, and chloroethylthiamine is also found to inhibit thiamin uptake in *E. coli* through

measurement of ^{14}C thiamin uptake in the presence of the analog.[7,10] ABC transport is also inhibited by N-ethylmaleimide in *E coli*, although not directly inhibiting the thiamin-binding protein (TbpA) of the transport system.[7,11] Finally, inactivation of thiamin transport system has been studied more extensively with thiamin analogs in *Saccharomyces cerevisiae*, a eukaryotic system.[12,13]

Transport Systems for "Smuggling" Small Molecules

Transport systems have been exploited to import vitamin conjugates of interest into the cell. Folate transport has been studied extensively for uptake of folate conjugates such as chemotherapeutic agents, liposomes, imaging agents, immune stimulants, dendrimers, and nanoparticles.[14,15,16] A Vitamin B12-insulin conjugate has been developed for uptake of insulin at clinical doses through vitamin B12 transporters.[17] Furthermore, peptides larger than six amino acids cannot cross the membrane of gram negative bacteria; biotinylated peptides up to 31 amino acids in length have been taken up by *E. coli* and other gram negative bacteria through the biotin transporter.[18]

Ligand attachments occur at parts of both the molecule and vitamin/cofactor of interest where there is no substantial recognition for binding. Also, linker chains have been used to attach a molecule of interest at a distance from the vitamin to cause minimal effects on binding of the natural substrate.[17] Finally, some insight on the mechanism of translocation of substrates has been gained through study of maltose transport system. A mutant of maltose binding protein has been developed that has

preferable binding to sucrose.[19] The mutant is used to determine whether sucrose, an unnatural substrate for the maltose binding protein, will still be translocated into the cell. Sucrose is indeed imported through the maltose transport system. The transport of the unnatural substrate is due to change in conformation of the maltose binding protein upon binding the substrate followed by protein-protein interaction with the maltose-ATPase, which triggers ATP hydrolysis and allows translocation of sucrose. The results relay that the translocation event is dependent on protein interaction between the substrate binding protein conformation and the ATPase, not substrate recognition.[19]

Utilizing Thiamin Transport for Uptake of Analogs

Thiamin transport in *Escherichia coli* actively occurs through ATP-binding cassette (ABC) transporters.[8] The periplasmic protein, thiamin binding protein (TbpA), has been identified in *E. coli* and *Samonella typhiurium*, and TbpA in *E. coli* has been co-crystallized with one of the forms of thiamin.[1] As previously mentioned, the protein has the ability to bind all three forms of thiamin (thiamin-OH, thiamin monophosphate, and thiamin diphosphate) with similar dissociation constants on the nanomolar scale.[2] Because the side chain of thiamin has no evidence for significance in binding, thiamin analogues with conjugate attachment at the hydroxyl side chain may also bind to the protein and be successfully transported into the cell. The hypothesis that TbpA can bind other thiamin analogues with functionalized side chains has been proposed.

Synthesis of Thiamin Analogs for Biological Studies

Many studies on thiamin-dependent enzymes have been performed to trap a reaction intermediate through the use of synthesized thiamin analogs.[20,21] A ring opened form of thiamin, benfotiamin, is used as a more easily absorbed compound for treatment of thiamin deficiency.[22] Other ring-opened forms have been synthesized as possible inhibitors for treatment of cancer.[22] Finally, phosphate analogs have been synthesized as a probe for new phosphorylation pathways.[23] Development of new thiamin analogs with ligands attached at the hydroxyl side chain may be useful for delivering reagents of interest into the cell. By employing a ligand attachment that allows for hydrolysis of reagents upon entering the cell, thiamin transport may provide a useful function for "smuggling" small molecules into biological systems such as antibiotics or fluorescent reagents. Furthermore, exploration of the tolerance of thiamin transport to conjugates may lead to development of a photoaffinity probe for isolation of thiamin-utilizing enzymes in a new biological system.

Purpose of Study

The ability for thiamin analogs to go through the transport system may provide access for delivering reagents to the cell. A synthetic scheme for thiamin analogs and preliminary studies of analog uptake are outlined. Characterizing tolerance of the thiamin transport system in *E. coli* may lead to investigation of new thiamin chemistry.

SYNTHESIS OF AMINO THIAMIN 6 AND UPTAKE OF AMINO THIAMIN 6 IN *E. COLI* BW25113 MUTANT

Introduction

A synthetic scheme for thiamin amine has been revised and results in an improved 62% yield of final product (Figure 1).[24] The amino analog may provide a scaffold for attaching small molecules of interest to thiamin via amide bond formation with the free amine. Growth and toxicity studies are performed on an *E. coli* mutant strain that contains no thiamin pyrophosphate synthase (ThiE), an enzyme which assembles thiamin monophosphate from the thiazole and pyrimidine components that are synthesized separately along the pathway (Figure 2).[25]

A single-gene knockout mutant has been developed for a ThiE- *E. coli* strain.[26] Through recombination, a kanamycin cassette has replaced the reading frame coding for thiamin phosphate synthase (ThiE), giving the ThiE- mutant kanamycin resistance in the absence of ThiE gene. Thiamin must be provided for growth of the mutant cells because the mutant strain no longer has the ability to synthesize thiamin. The amino analog is an alternative to thiamin and therefore results in growth as well as a level of toxicity at higher millimolar concentrations.

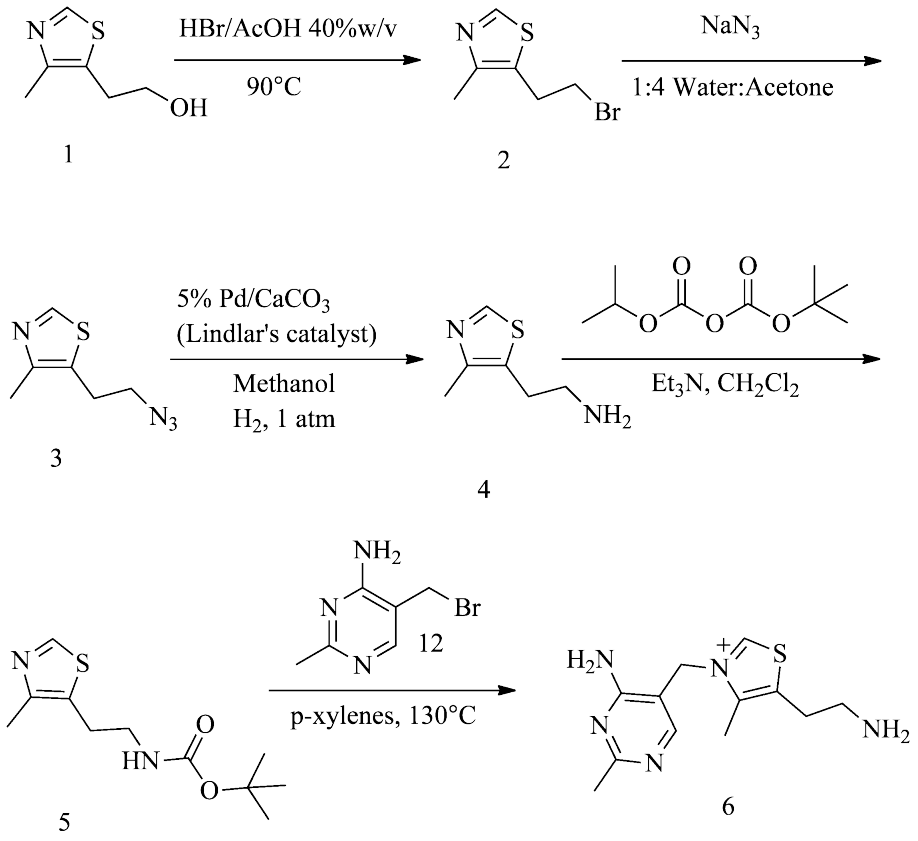

Figure 1. Synthesis of Amino Thiamin 6

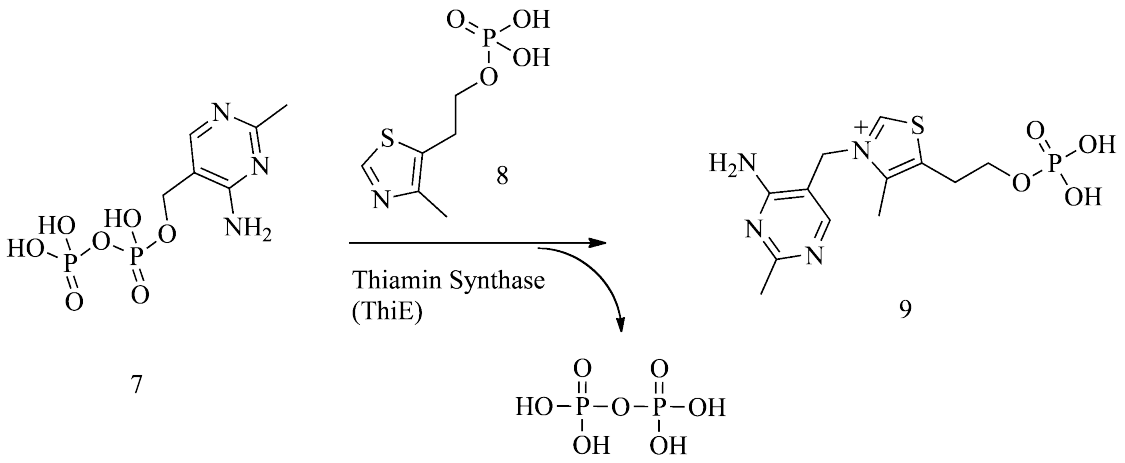

Figure 2. Catalytic Reaction of Thiamin Synthase to Form Thiamin Monophosphate

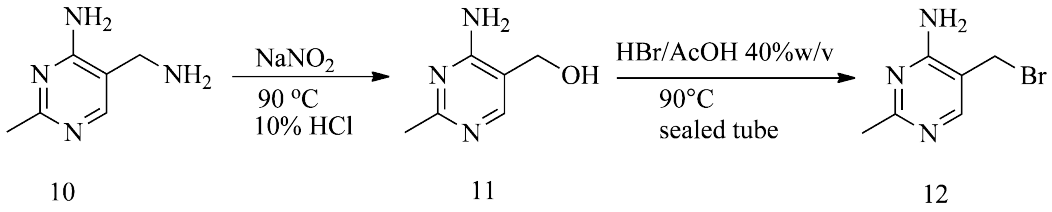

Figure 3. Synthesis of Pyrimidine Reagent 12

Materials and Methods

Grewediamine is supplied as a gift from Hoffman LaRoche. All reagent grade solvents and starting materials are supplied from Sigma Aldrich, unless otherwise noted. NMR is taken with a Mercury instrument (300 mHz). Mass spectrometry analysis is performed on (TOF+) ESI-MS.

Synthesis of Bromo Thiazole 2

Thiazole 1 (7 mmol, 1.0 g) is added to a (#15 Ace glass) pressure tube. Ten milliliters of 40% (w/v) solution of HBr and Acetic acid mixture is added to the tube, followed by tight closure of pressure screw cap. The mixture is stirred vigorously at reflux conditions (90°C) overnight. The remaining acetic acid is then distilled off, leaving an oily residue. The residue is precipitated with ether, followed by several washings with ether for purification. 1.1 g solid is recovered (76% yield). **Spectroscopic Data:** ^{1}H NMR (300 MHz, Methanol-d_4)– δ 2.56 (s, 3H, C**H$_3$**), 3.54 (t, 2H, -C**H$_2$**CH$_2$OH), 3.75 (t, 2H, -CH$_2$C**H$_2$**OH), 9.96 (s, 1H, **H-** thiazolium). ESI-MS (m/z): [M+H]+ calcd for C_6H_8BrNS 205.9561; found 205.9639

Synthesis of Azido Thiazole3

Bromo thiazole 2 (4.85 mmol, 1.0g) is dissolved into 50 mL of 1:4 water: acetone mixture in a 250 mL round bottom flask. Sodium azide (4 eq., 19.4 mmol, 1.26g) is added to the round bottom flask. The reaction is stirred vigorously for 48 hours. A rotary evaporator is used to remove acetone from the solvent mixture. The oil droplet suspension is diluted with water and extracted into dichloromethane. The dichloromethane is subsequently washed with water, 5% sodium bicarbonate, brine, and water respectively. 0.8 g material is recovered (98% yield). **Spectroscopic Data:** ^{1}H NMR (300 MHz, methanol-d_4) - δ 2.40 (s, 3H, C**H$_3$**), 3.30 (t, 2H, -C**H$_2$**CH$_2$N$_3$), 3.54 (t, 2H, -CH$_2$C**H$_2$**N$_3$), 8.80 (s, 1H, **H**-thiazolium). ESI-MS (m/z): [M+H]+ calcd for $C_6H_8N_4S$ 169.0545; found 164.0548

Synthesis of Amino Thiazole 4

The synthesis is as previously reported in literature.[27] A tan oil results (0.18g, 93% yield). **Spectroscopic data:** ^{1}H NMR (300 MHz, chloroform-*d*) - δ 1.43 (br s, 2H, N**H$_2$**), 2.40 (s, 3H, C**H$_3$**), 2.92 (m, 4H, -C**H$_2$**C**H$_2$**NH$_2$), 8.57 (s, 1H, **H-**thiazolium). ESI-MS (m/z): [M+H] + calcd for $C_6H_{10}N_2S$ 142.0643; found 143.0647.

Synthesis of Protected Amino Thiazole 5

Amino thiazole 7 (1.3 mmoles, 0.18g) is dissolved in dichloromethane followed by addition of triethylamine (1.5 equivalents). 1 equivalent of *tert*-butyl isopropyl dicarbonate is added to the vessel under argon, and the reaction is stirred overnight. The solution is diluted with dichloromethane and is washed with water, sodium bicarbonate, brine, and water respectively. No further purification is performed as determined by NMR analysis (0.22 g, 70% yield). **Spectroscopic Data:** ^{1}H NMR (300 MHz, chloroform-*d*) - δ 1.40 (s, 9H, -C(C**H$_3$**)$_3$), 2.36 (s, 3H, -C**H$_3$**), 2.94 (t, 2H, -C**H$_2$**CH$_2$NH), 2.96 (q, 2H, -CH$_2$C**H$_2$**NH-), 4.79 (br s, 1H, -N**H-**), 8.54 (s, 1H, **H**-thiazolium). ESI-MS (m/z): [M+H] + calcd for $C_{11}H_{18}N_2O_2S$ 243.1167; found 243.1167.

Synthesis of HMP Alcohol 11

Synthesis is performed as previously reported, and 0.4g is recovered resulting in 50% yield).[28] **Spectroscopic Data:** ^{1}H NMR (300 MHz, methanol-d_4) - δ 2.40 (s, 3H, C**H$_3$**-), 4.83 (s, 2H, -C**H$_2$**OH), 7.95 (s, 1H, aromatic **H**-C). ESI-MS (m/z): [M+H] + calcd for $C_6H_9N_3O$ 140.0824; found 140.0828.

Synthesis of Pyrimidine Reagent 12

The procedure is updated according to a previous method (Figure 3).[29] To a pressure tube containing HMP Alcohol 11 (2.3 mmol, 0.4 g) is added ten milliliters of HBr: Acetic acid mixture (40% w/v). A screw cap is secured to the pressure tube and the reaction mixture is refluxed at 90°C overnight. Excess acetic acid is removed by distillation, leaving an oily product residue. Product is precipitated with anhydrous ethyl ether and is washed several times with ether. No further purification is need as determined by NMR (0.44 g, 68% yield). **Spectroscopic Data:** ^{1}H NMR (300 MHz, methanol-d_4)- δ 2.56 (s, 3H, -C**H$_3$**), 4.55 (s, 2H, -C**H$_2$**Br), 8.29 (s, 1H, aromatic **H**-C). ESI-MS (m/z): [M+H] + calcd for $C_6H_8N_3Br$ 201.9980; found 201.9985.

Synthesis of Amino Thiamin 6

Amino thiazole 5 (1.6 mmol, 0.380g) and 1 equivalent of pyrimidine reagent 12 (1.6 mmol, 0.44g) are weighed into a 50 mL round bottom flask. Two milliliters of p-xylenes is added to suspend the mixture. The reaction mixture is refluxed with a condenser at 130°C for three hours. Rotary evaporator removes the solvent. The residue is washed with absolute ethanol, suspended in ether, and vacuum filtered. No further purification is needed as confirmed by NMR shown (Figure 4, 0.35G, 62% yield). Spectroscopic Data: 1H NMR (300 mHz, D2O)- δ 2.59 (s, 3H, CH3-pyrimidine), 2.63 (s, 3H, CH3-thiazolium), 3.38 (m, 4H, -CH2CH2NH2), 5.58 (s, 2H, Bridging –CH2-),

8.10 (s, 1H, H- pyrimidine), 9.67 (s, 1H, H-thiazolium). ESI-MS (m/z): [M+] calcd for C12H18N5S+ 264.1283; found 264.1281 (Figure 5).

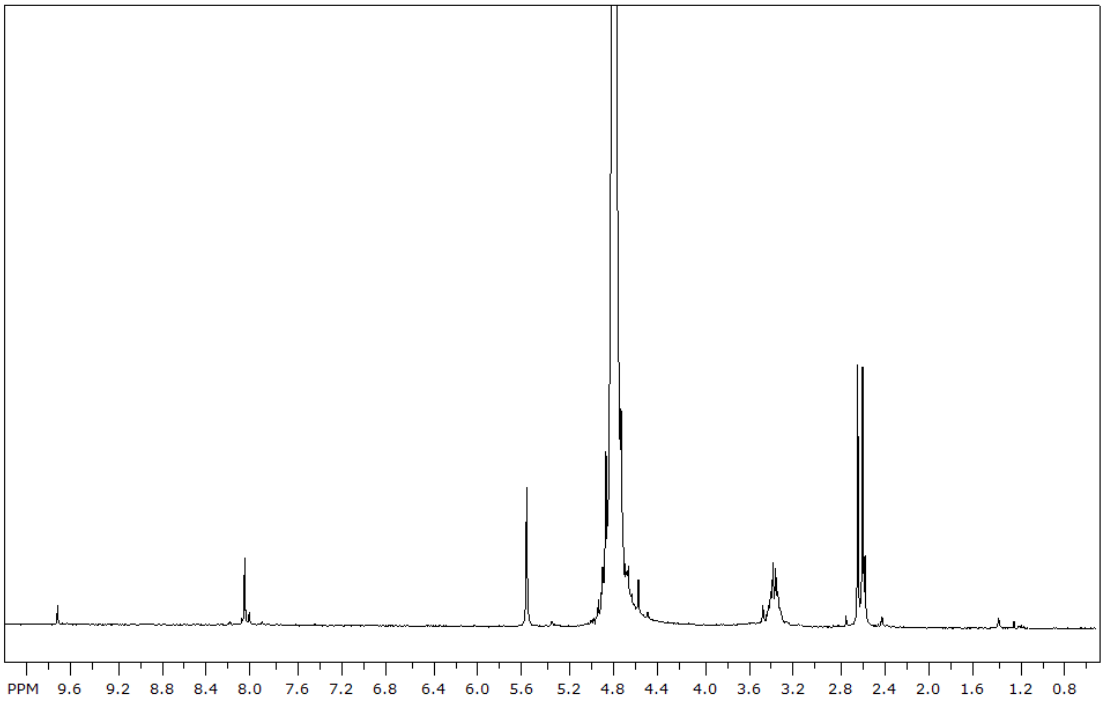

Figure 4. Proton NMR Spectrum of Amino Thiamin 6

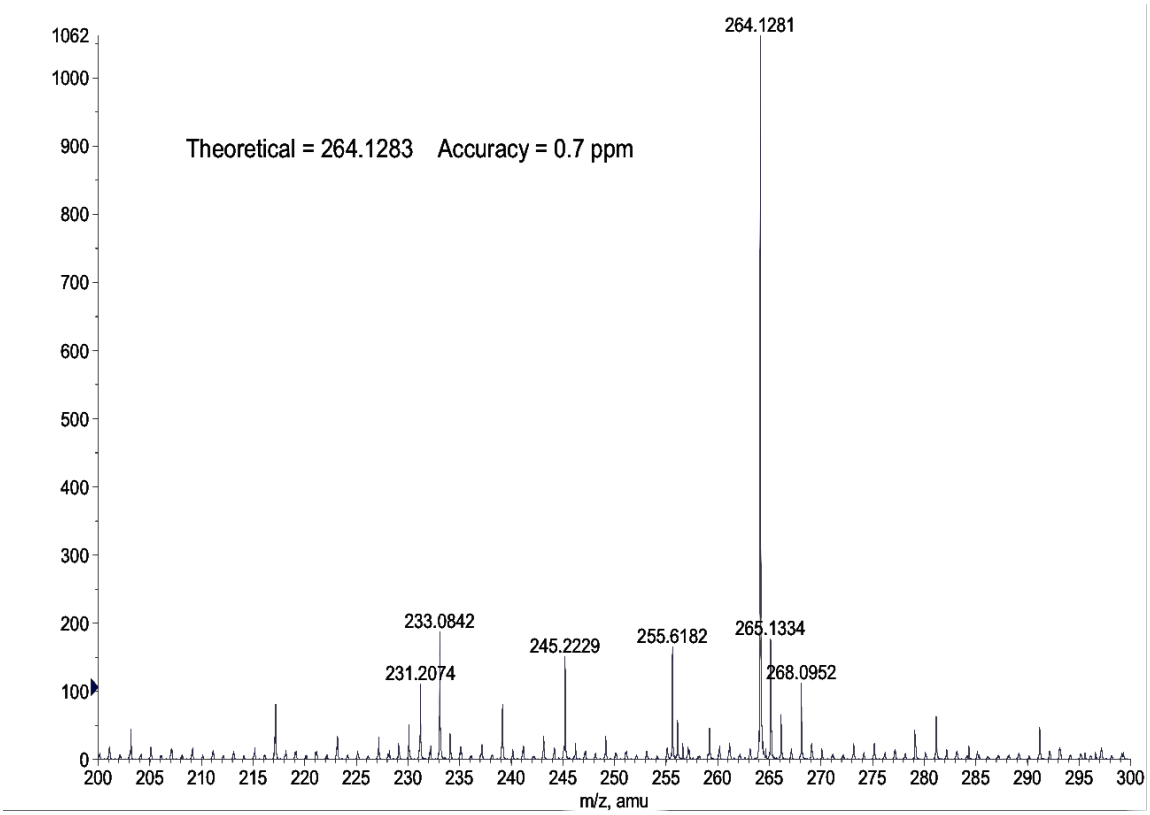

Figure 5. Mass Spectrum of Amino Thiamin 6

Growth Culture of Thiamin Depleted BW25113 Mutant

BW25113 ThiE- *E. coli* is streaked onto an LB plate and incubated at 37°C for 16 hours. A colony is selected and inoculated into a 5 mL LB starter culture containing 40 ug/mL of kanamycin. The culture is incubated in a shaker at 37°C and 200 rpm overnight. Since a kanamycin cassette replaces the reading frame coding for ThiE gene, the mutant strain exhibits kanamycin resistance. As a result, a final concentration of 40 ug/ml is used for all subsequent cultures to select for the ThiE- mutant strain.[26] The

starter LB culture is centrifuged at 5000 RPM for 8 minutes, and the supernatant is removed. The cells are washed with minimal M9 media. Centrifugation and washing is repeated four times to remove most excess thiamin from the media. The washed starter culture is used to inoculate 20 mL of M9 media to a 1% final concentration. Cell density is measured (OD_{600}) several times over 27 hours to monitor growth plateau, indicating the cells are depleted from a thiamin source. Amino thiamin 6 is added in increasing concentrations of 1 uM, 10 uM, 1 mM, 50 mM, 75 mM, and 100 mM to separate media samples containing the thiamin depleted *E. coli* mutant (at a 0.05% concentration of cells). M9 media containing only 0.05% of *E.* coli mutant is used as a control for no growth and comparison with toxic levels of analog. As another control, the same increasing concentrations of thiamin hydrochloride is added to separate samples of culture to compare with growth due to uptake of amino thiamin 6. Growth is monitored over a time of 45 hours (OD_{600}) using a UV-Vis spectrometer.

Results

The slowest growth of the ThiE- mutant is observed at 1 uM of amino thiamin 6, and most rapid growth is observed between 1 and 50 mM amino thiamin 6 (Figure 6). Concentration-dependent growth is observed.

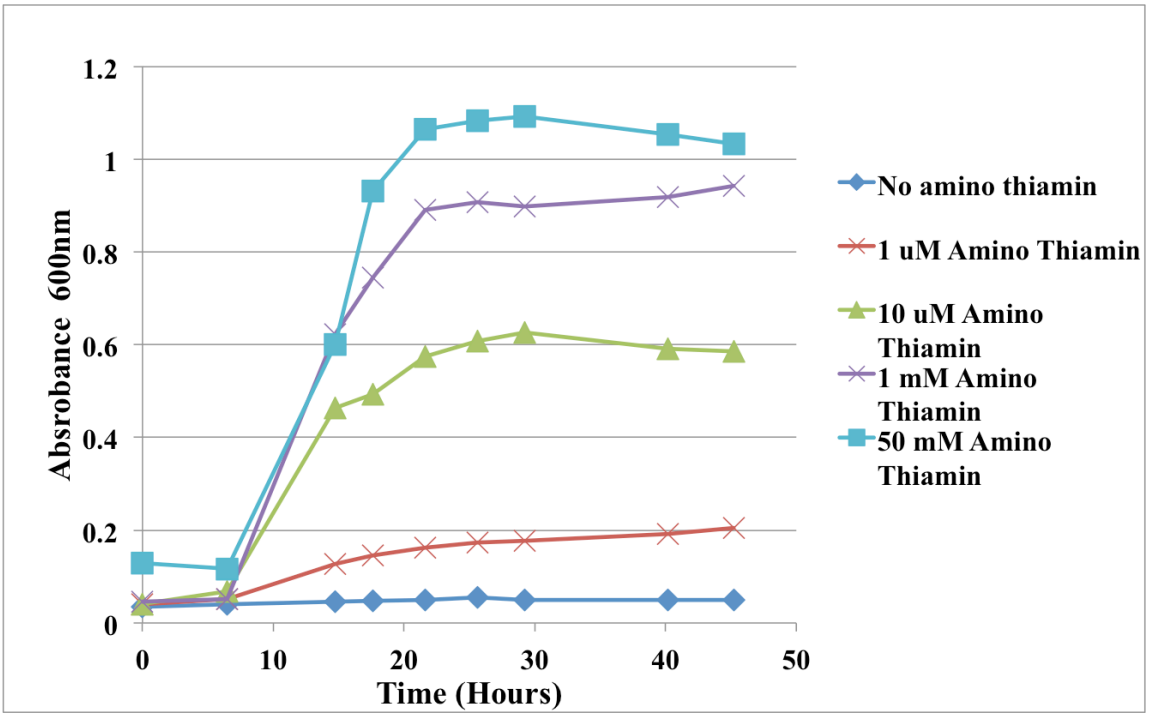

Figure 6. Growth Curve for Uptake of Amino Thiamin 6 by ThiE- E. coli Mutant Strain

Thiamin not synthesized by the prokaryote may enter the biosynthetic pathway followed by pyro phosphorylation by thiamin phosphate kinase and thiamin phosphate kinase. As a hypothesis, enzymes which remove amino groups in the cell may catalyze imine formation, and further hydrolysis forms thiamin, which may then be utilized as a metabolite (Figure 7). No further studies have been performed to confirm this hypothesis.

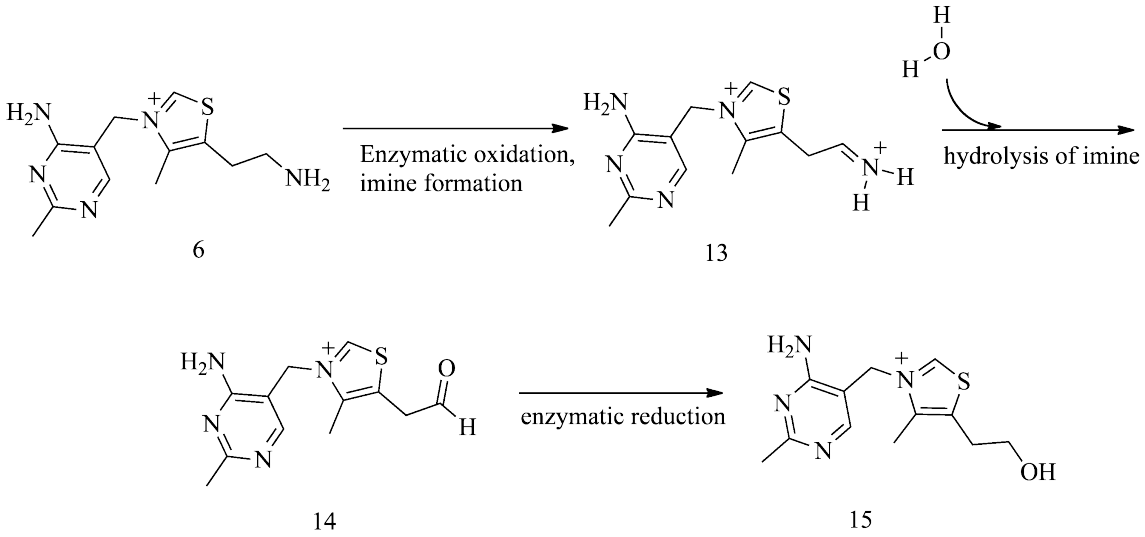

Figure 7. Imine Formation and Hydrolysis of Amino Thiamin 6 to Form Thiamin 15

Amino thiamin 6 provides an alternative source of thiamin for growth and has therefore successfully been imported into the cell. Thiamin amino analog may provide a scaffold for ligand attachment through amide bond formation.

SYNTHESIS OF THIAMIN ESTER ANALOGS AND INITIAL UPTAKE STUDY OF ESTERS USING A THIAMIN DEFICIENT E. COLI MUTANT

Introduction

After establishing a scaffold for thiamin conjugates, ester analogs containing a formyl, acetyl, and benzoyl group are synthesized to study uptake and tolerance of the thiamin transport system. Thiamin esters are easily afforded through refluxing thiamin hydrochloride in acid, which produces a pure product and gives decent yield. As an alternative method, thiazole ester is first formed followed by condensation with the pyrimidine component. Both methods rely on precipitation of final product for purification, given that thiamin contains a positive charge and acts as a salt. The latter method results in very low yield. Finally, thiamin esters are more readily synthesized initially to determine tolerance of the uptake system. Thiamin amide conjugates requires several more steps to yield a final product.

Materials and Methods

Chloromethyl pyrimidine is provided as a gift from Hoffeman-LaRoche. All reagents are from Sigma-Aldrich, unless otherwise noted.

Synthesis of Thiamin Formate 16

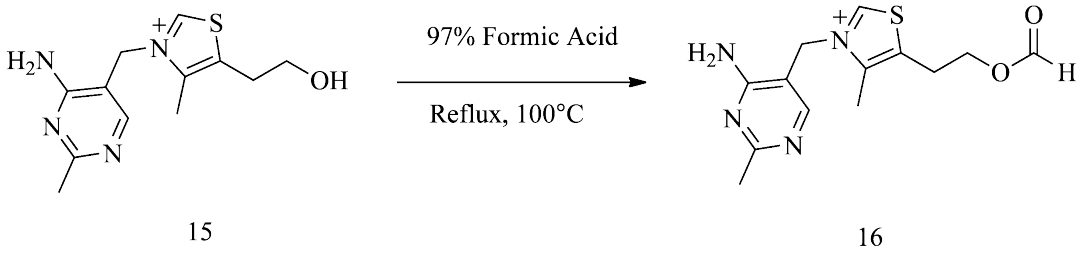

Figure 8. Synthetic Scheme for Thiamin Formate 16

To a pressure tube containing thiamin hydrochloride 15 (0.16 mmol, 0.055 g) is added 2 milliliters of 99% formic acid (Figure 8). A screw cap is secured to the tube, and the reaction mixture is refluxed at 100°C overnight. The excess formic acid is removed by distillation, leaving a solid residue. The residue is washed several times with anhydrous diethyl ether and is dried on house vacuum for two hours. No further purification is needed as determined by NMR analysis (Figure 9). 0.023g is recovered, leaving a 43% yield. **Spectroscopic Data:** ^{1}H NMR (300 MHz, methanol-d_4)- δ 2.59 (s, 3H, C**H$_3$**-pyrimidine), 2.65 (s, 3H, C**H$_3$**-thiazolium), 3.41 (t, 2H, -C**H$_2$**CH$_2$CHO), 4.44 (t, 2H, -C**H$_2$**CHO), 5.51 (s, 2H, Bridging –C**H$_2$**), 8.08 (s, 1H, **H**-formate), 8.13 (s, 1H, **H**-pyrimidine), 8.23 (s, 1H, **H**-thiazolium). ESI-MS (m/z): [M+] calcd for $C_{12}H_{17}N_4O_2S$+ 293.1072, found 293.1084 (Figure 10).

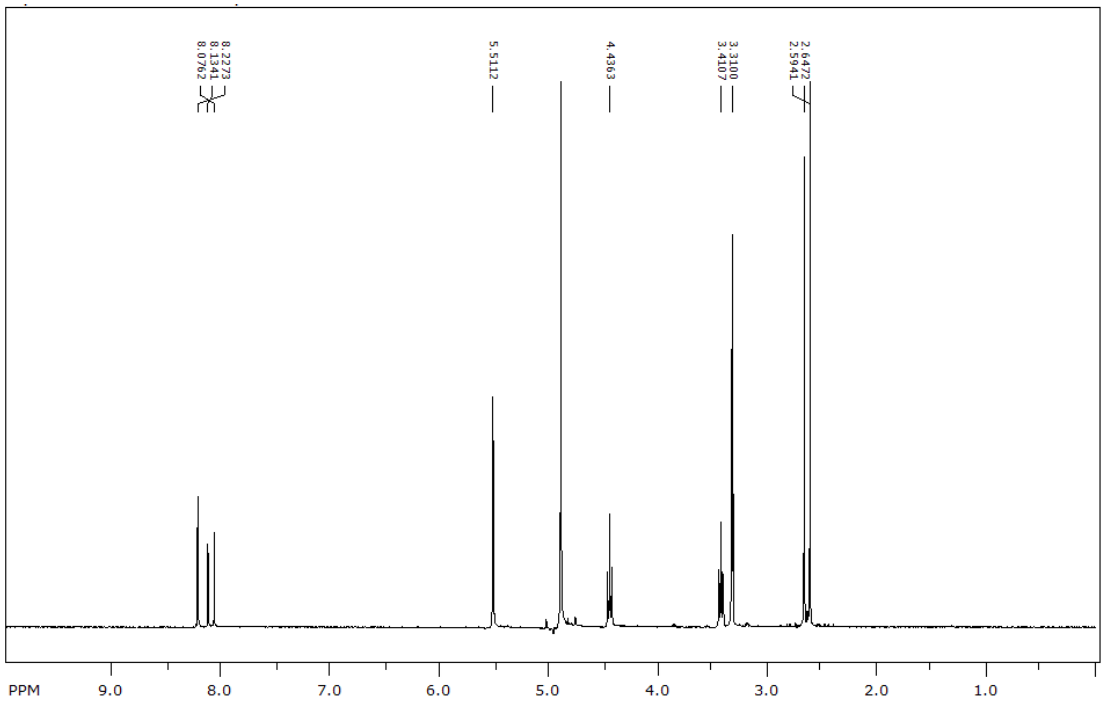

Figure 9. Proton NMR Spectrum of Thiamin Formate 16

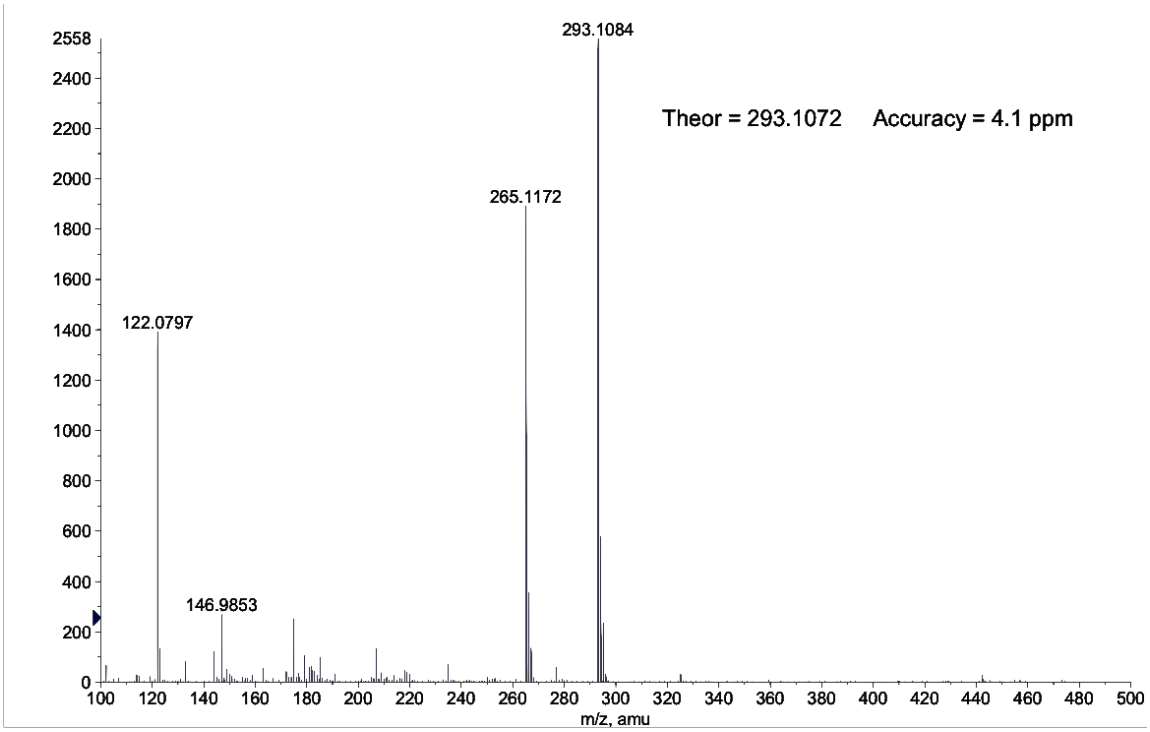

Figure 10. Mass Spectrum of Thiamin Formate 16

Synthesis of Thiamin Acetate 17

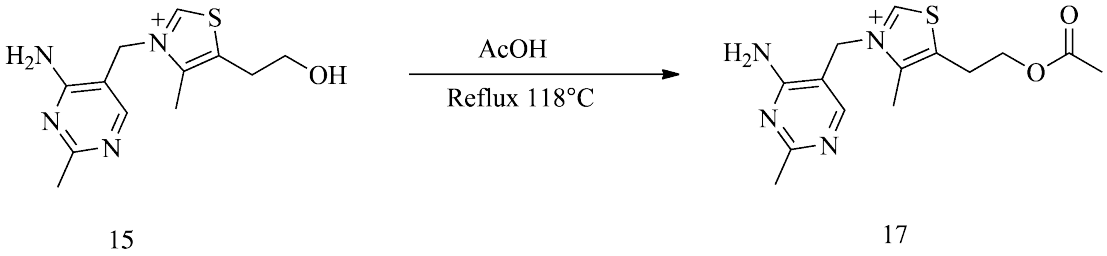

Figure 11. Synthetic Scheme for Thiamin Acetate 17

To a pressure tube containing thiamin hydrochloride (0.16 mmol, 0.055g) is added 2 milliliters of glacial acetic acid (Figure 11). A screw cap is secured to the tube, and the reaction mixture is refluxed at 100°C overnight. The excess acetic acid is removed by distillation, leaving a solid residue. The residue is washed several times with anhydrous diethyl ether and is dried on house vacuum for two hours. No further purification is needed as determined by NMR analysis (Figure 12). 0.037g is recovered, leaving a 67% yield. **Spectral Data:** ^{1}H NMR (300 MHz, methanol-d_4)- δ 2.08 (s, 3H, C**H$_3$**-acetyl), 2.64 (s, 3H, C**H$_3$**-pyrimidine), 2.64 (s, 3H, C**H$_3$**-thiazolium), 3.38 (t, 2H, -C**H$_2$**CH_2COCH_3), 4.34 (t, 2H, -CH_2C**H$_2$**$COCH_3$), 5.55 (s, 2H, Bridging –C**H$_2$**), 8.27 (s, 1H, **H**-pyrimidine), 9.84 (s, 1H, **H**-thiazolium). ESI-MS (m/z): [M+] calcd for $C_{14}H_{19}N_4O_2S$+ 307.1229; found 307.1214 (Figure 13).

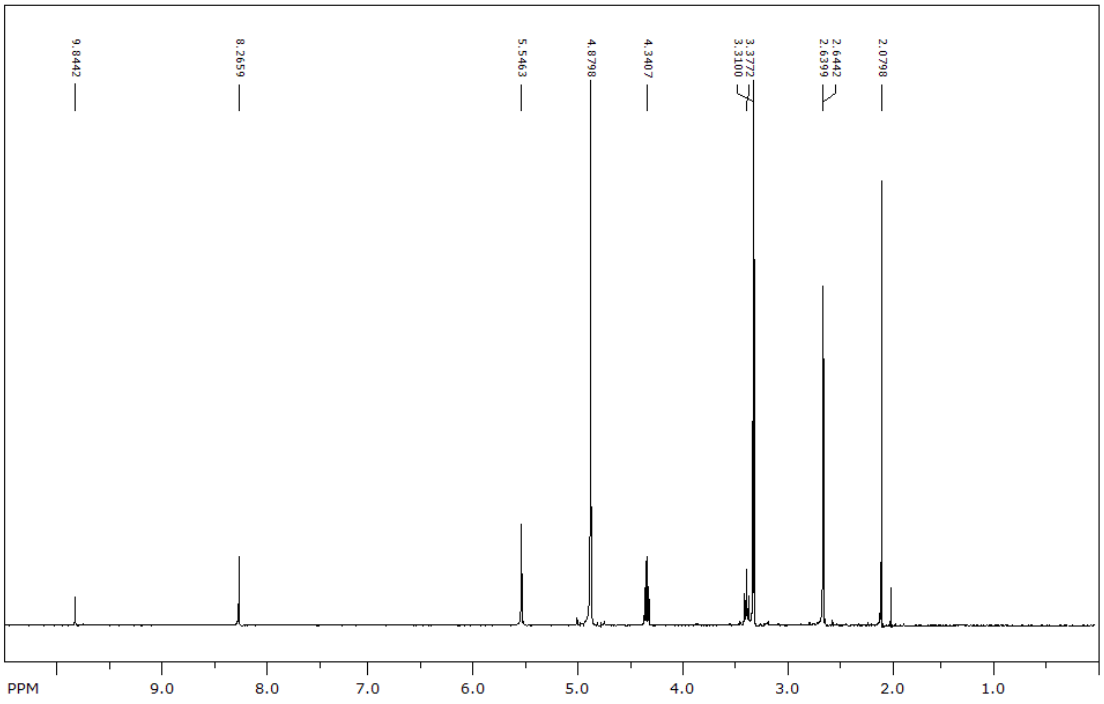

Figure 12 . Proton NMR Spectrum of Thiamin Acetate 17

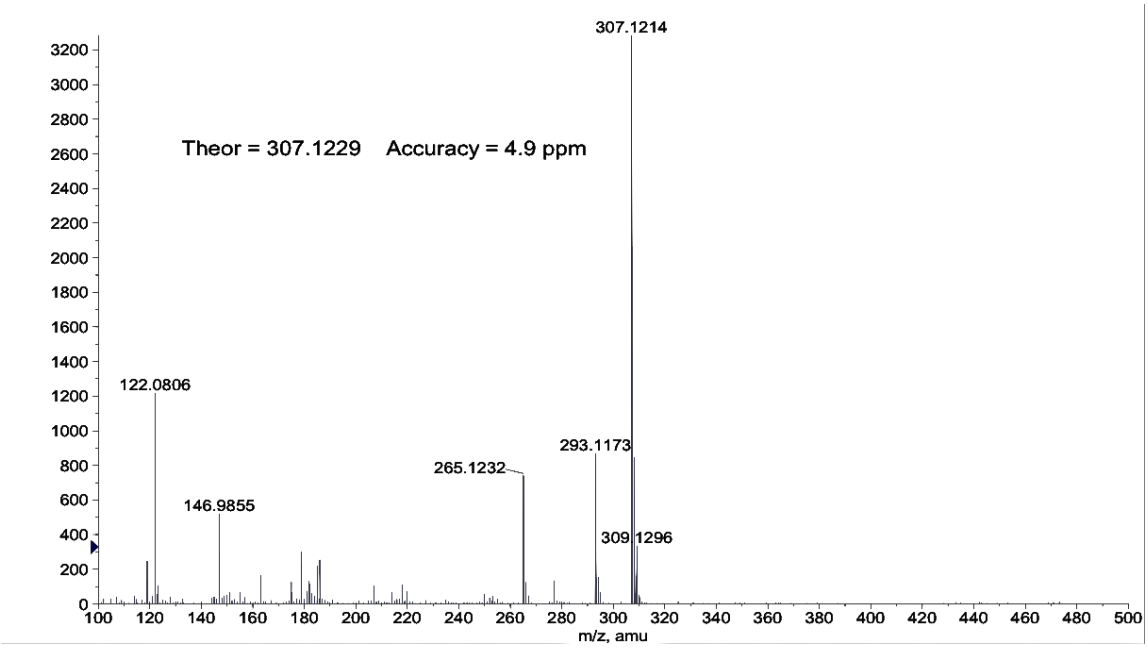

Figure 13. Mass Spectrum of Thiamin Acetate 17

Synthesis of Thiamin Benzoate 18

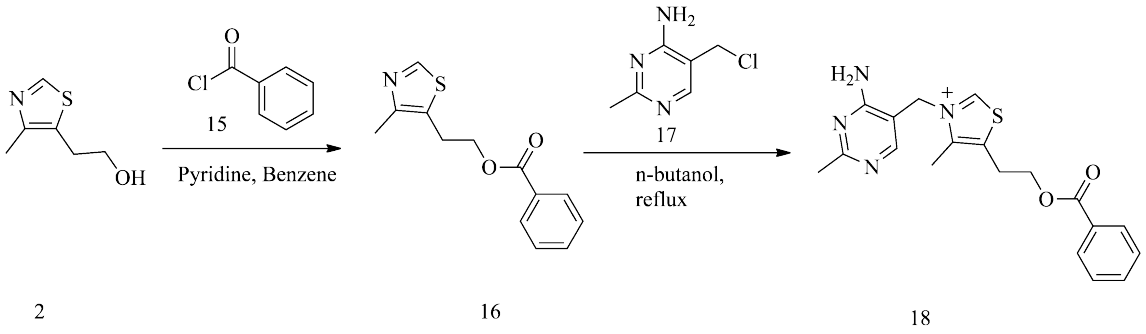

Figure 14. Synthetic Scheme for Thiamin Benzoate 18

The thiazole ester is synthesized first (Figure 14). The procedure is adapted from a similar synthesis of thiamin ester.[12] To a round bottom flask containing 0.65 G thiazole 2 dissolved in 2 milliliters of benzene and 0.5 milliliters of pyridine is added 1 eq. benzoyl chloride 13 dropwise. The reaction is stirred for one hour. Rotary evaporator removes the solvent, which leaves a crystalline product (0.9G, 99% yield). The crude product is used for the subsequent step without purification. Thiamin is then formed through reflux of thiazole ester **16** with chloromethyl pyrimidine **17** in n-butanol. After 75 minutes, the resulting precipitant is immediately filtered, followed by several washings with ethanol and acetone. No further purification is required as determined by NMR (figure 15, 0.037 G, 2.5% yield). **Spectral Data**: ^{1}H NMR (300 MHz, Methanol-d_4): δ 2.63 (1, 3H, C**H$_3$**-pyrimidine), 2.68 (1, 3H, C**H$_3$**-thiazolium), 3.54 (t, 2H, -C**H$_2$**CH$_2$-benzoate), 4.62 (t, 2H, -CH$_2$C**H$_2$**-benzoate), 5.55 (s, 1H, Bridging –C**H$_2$**), 7.51 (m, 2H, **H**-meta), 7.65 (m, 1H, **H**-para), 8.07 (dd, 2H, **H**-ortho), 8.27 (s, 1H, **H**-pyrimidine), 9.84 (s, 1H, **H**-thiazolium). ESI-MS (m/z): calcd for $C_{18}H_{21}N_4O_2S$+ 369.1385; found 369.1402 (Figure 16).

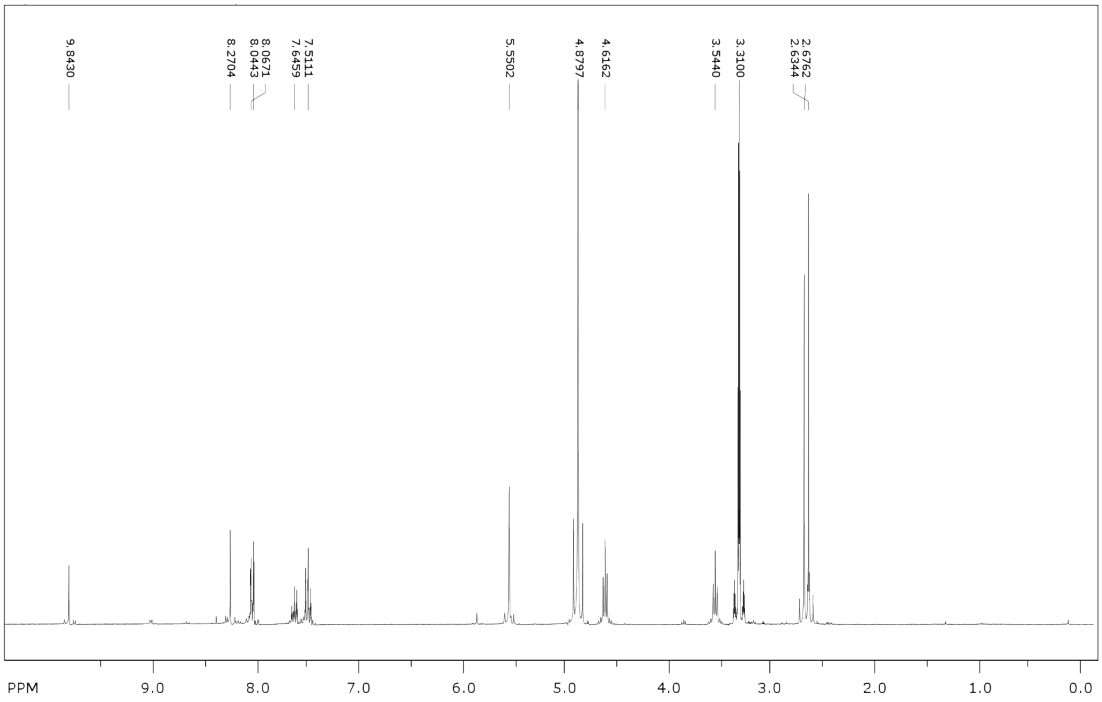

Figure 15. Proton NMR Spectrum of Thiamin Benzoate 18

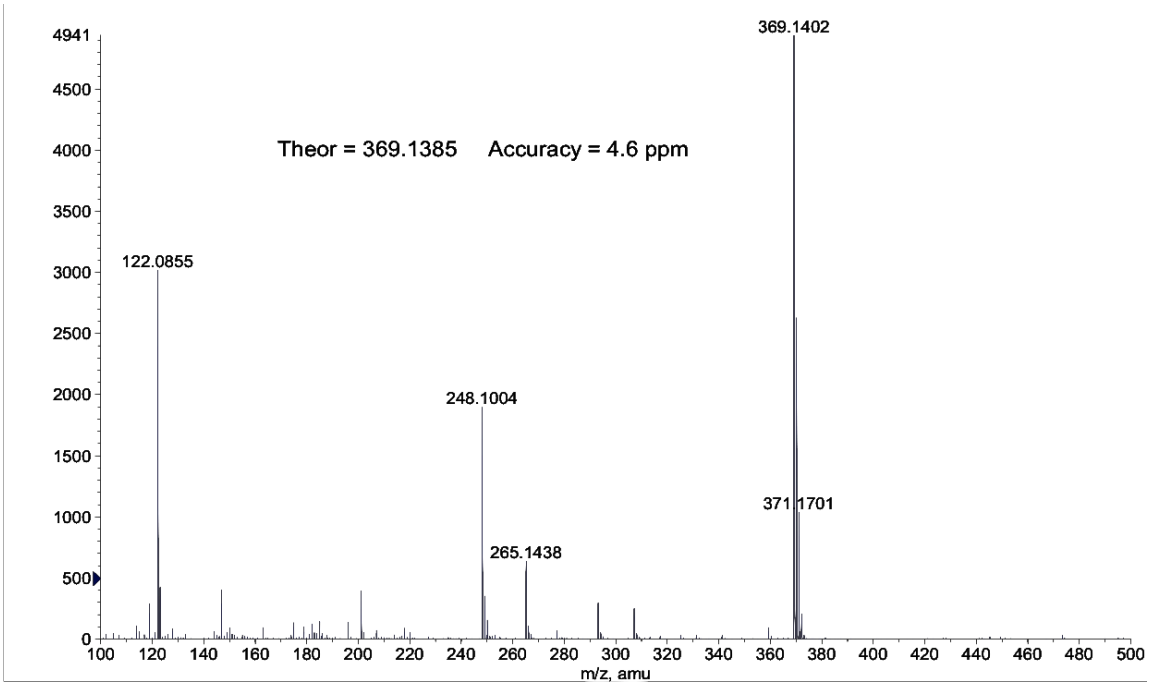

Figure 16. Mass Spectrum of Thiamin Benzoate 18

Testing Uptake of Thiamin Esters by E. coli Mutant Using A Disk-Assay Growth Method

To determine whether the thiamin esters are taken up and metabolized by the cell, a disk assay method is employed. The *E. coli* mutant is depleted of all exogenous forms of thiamin in minimal growth media in the same method as previously stated. The resulting culture is added to fresh liquid M9 minimal media and agarose to a final concentration of 0.05%. The liquid media is immediately poured onto plates containing four separated wells. After the seeded media has solidified, sterilized filter paper disks are placed on each separated agarose well using a sterilized toothpick. As a control, 3µl of sterile filtered water, (40 mg/ml) ampicillin, and 1mM thiamin hydrochloride is

pipetted onto separate disks in separate wells. 3 µl of 1 mM each analog is also placed onto a separate disk in separate wells. The plates are then incubated at 30°C.

Table 1. Positive or Negative Growth of E. coli Mutant on Thiamin Esters

Name	Growth (+/-)
Water (vehicle)	-
Ampicillin (negative control)	-
Thiamin hydrochloride (positive control)	++
Thiamin formate	++
Thiamin acetate	+
Thiamin benzoate	+

Results

As expected, there is no growth on sterile filtered water (Table 1). Since the mutant is not resistant to ampicillin, there is also no growth. Growth is observed on thiamin hydrochloride as another control. Finally, growth is observed for the formate, acetate, and benzoate analogs of thiamin. As a hypothesis, analogs are taken up by thiamin transport system, and esterases present inside the cell hydrolyze the small molecule component. Thiamin may then be used as a metabolite, allowing for growth.

An alternate hypothesis is that the amino thiamin may become phosphorylated by the kinase. The molecule may then be incorporated into the last step of the biosynthetic pathway and then be used as a cofactor.

CONCLUSION: PROSPECT FOR USE OF THIAMIN TRANSPORT SYSTEM AS SOURCE OF UPTAKE OF THIAMIN CONJUGATES

To further exploit the uptake properties of thiamin transport system in *E. coli*, effect of esterase activity outside the cell on thiamin esters must be explored. An experimental procedure is proposed as well as prospect for future studies.

Effect of Secreted Esterase on Thiamin Esters

Esterases may be secreted from the cell and cause hydrolysis of thiamin esters before entering the cell. Media from cell culture may be used as a source of secreted esterase to test hydrolysis of esters over time. A one-liter culture may be grown into log phase, and the cells may then be spun down by centrifuge. The separated supernatant will be collected and concentrated with a speed vacuum down to the smallest volume possible without causing precipitation. Any precipitant is filtered, and as an experimental proposal, 10 μM of thiamin ester may be added to the concentrated supernatant. After about one hour, an aliquot of supernatant may be filtered through a ten kDa cutoff membrane to separate small molecules from proteins secreted in the supernatant. The small molecule pool may be concentrated, and an aliquot may be injected on the HPLC to determine the amount of hydrolyzed ester. A standard of each ester and thiamin hydrochloride may be used to identify the presence of each compound in the media.

Synthesis of Thiamin Amide Analogs for Future Studies

Upon determining whether esterases secreted from the cell are causing hydrolysis of thiamin conjugates, thiamin amides may be synthesized and tested as a more stable form for thiamin conjugate studies. As an additional study, a fluorescent thiamin analog may be synthesized to observe uptake of the analog through the thiamin transport system. Fluorescence confocal microscopy may be used to observe uptake of a fluorescent molecule. If the analog has gone inside the cell, the cytoplasm will show fluorescence due to the analog itself.

Future Scope of Study

After uptake has been confirmed via disk assay method, growth studies, and visual determination using microscopy, the use of thiamin analogs to exploit thiamin transport becomes a more accessible tool to explore the cell biology of *E. coli* and other microorganisms. Reagents of interest that are not currently compatible for uptake may become of use upon forming a thiamin analog. Reagents of interest may include a thiamin analog containing an antibiotic that may be smuggled inside the cell as well as a thiamin derivative photo affinity label or activity-based probe for isolating and characterizing thiamin-utilizing enzymes from a proteome.

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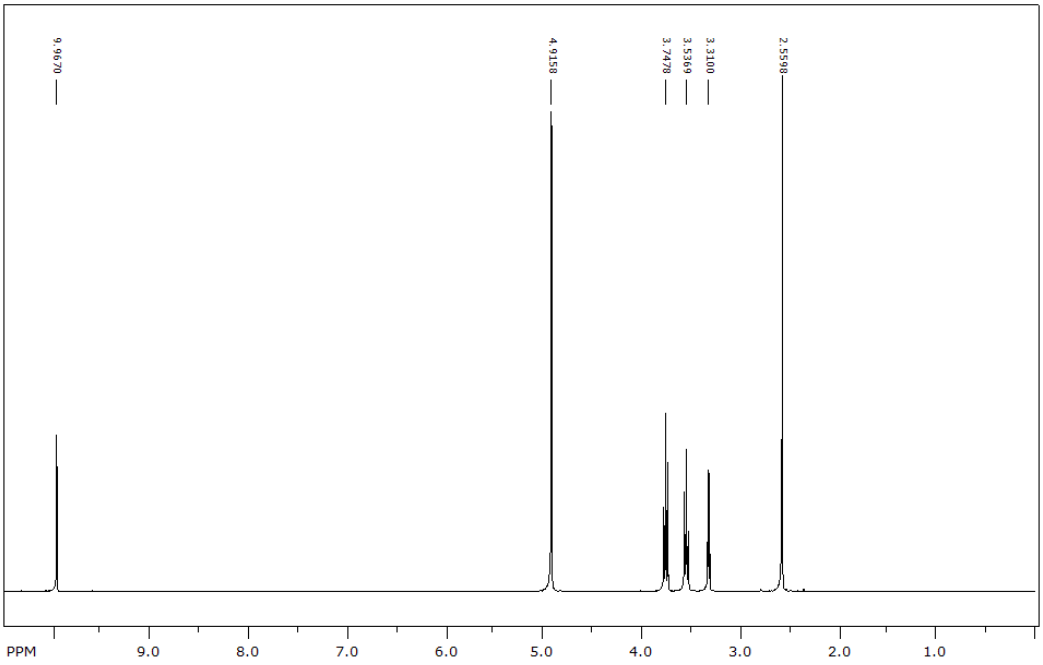
APPENDIX

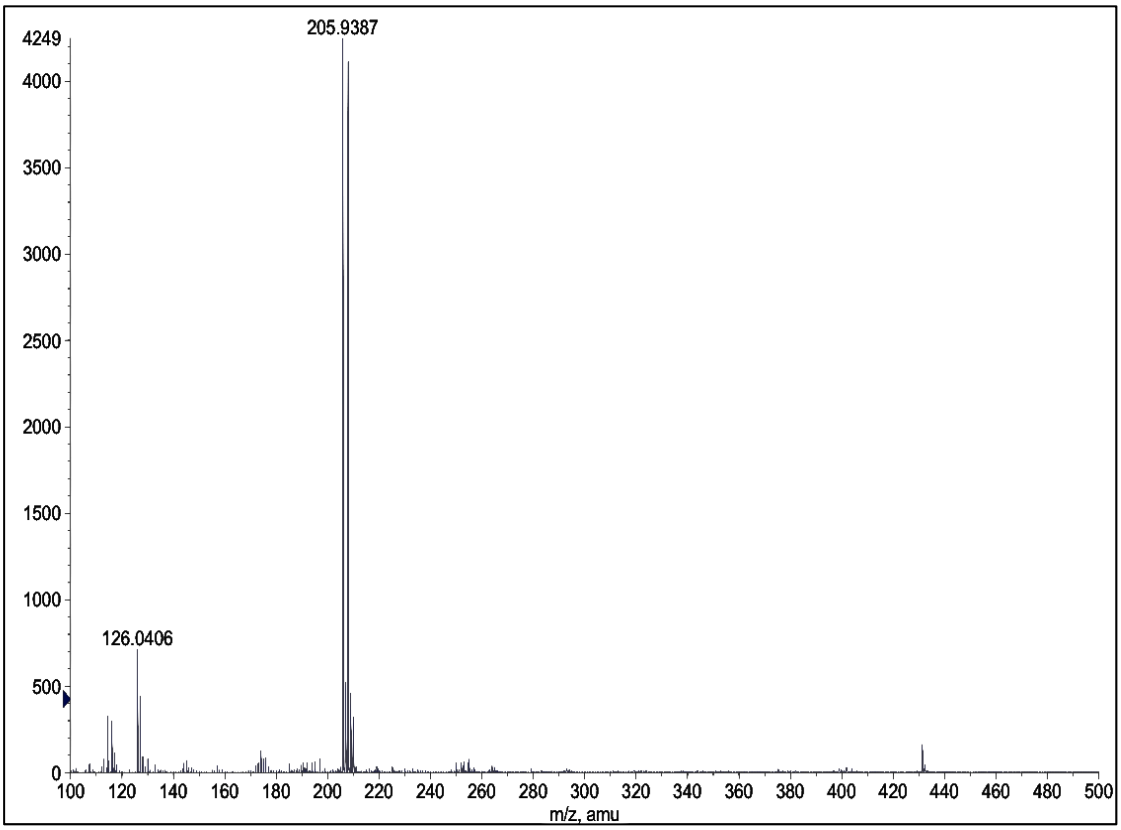
Figures

Bromo Thiazole 5. Spectroscopic Data



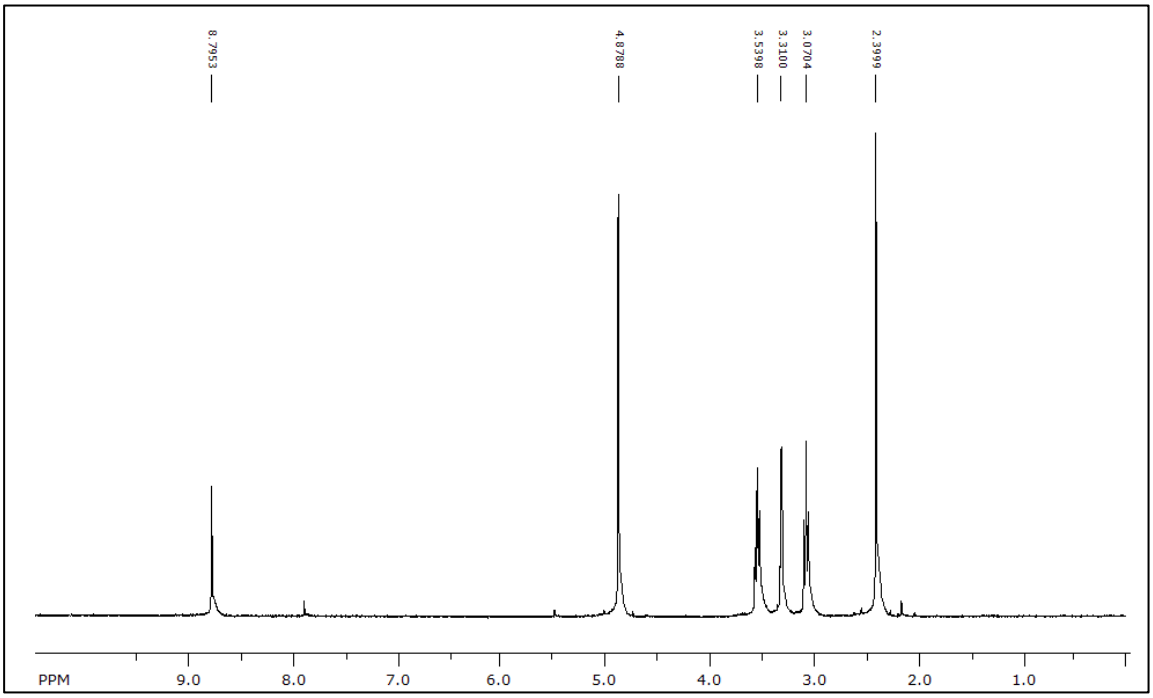
Proton NMR Spectrum of Bromo Thiazole 5

Assignment of Protons: **^{1}H NMR:** (300 MHz, Methanol-d_4)– δ 2.56 (s, 3H, C**H$_3$**), 3.54 (t, 2H, -C**H$_2$**CH$_2$OH), 3.75 (t, 2H, -CH$_2$C**H$_2$**OH), 9.96 (s, 1H, **H-** thiazolium)



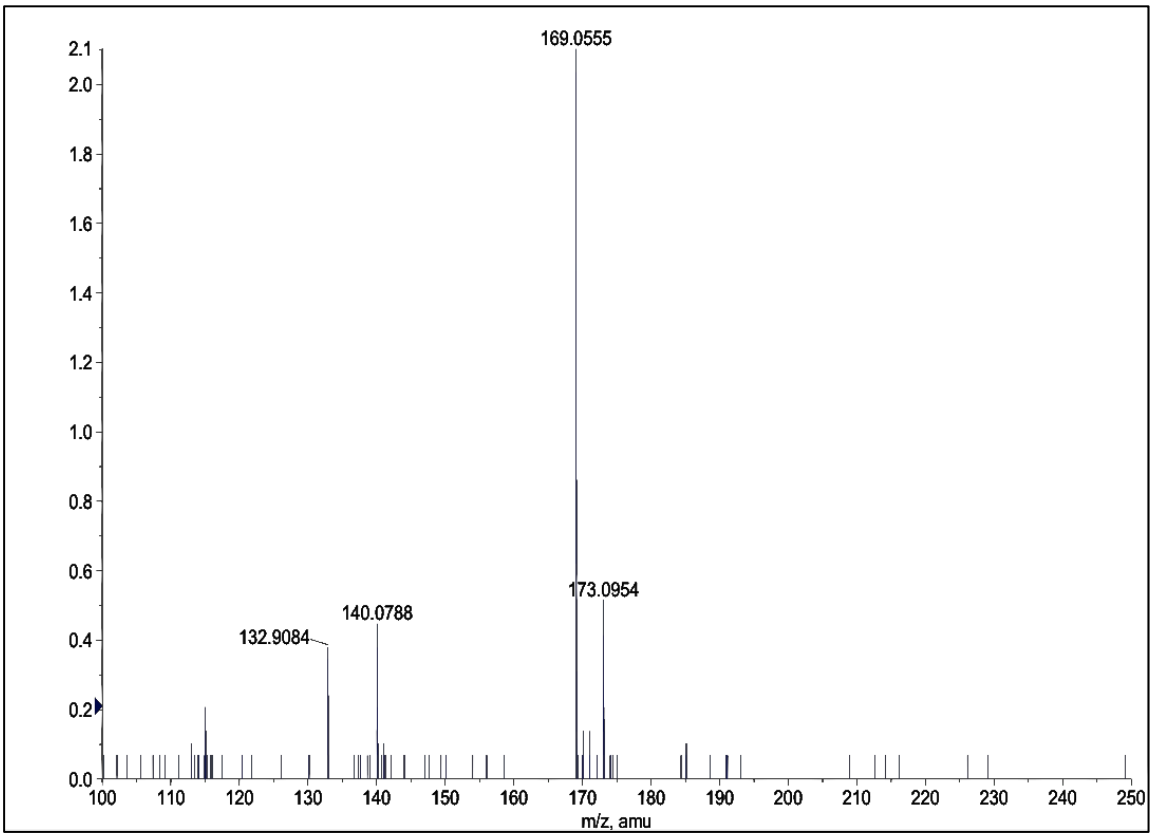
Mass Spectrum of Bromo Thiazole 5: ESI-MS (m/z): [M+H]+ calcd for C_6H_8BrNS 205.9561; found 205.9639

Azido Thiazole 6 Spectroscopic Data



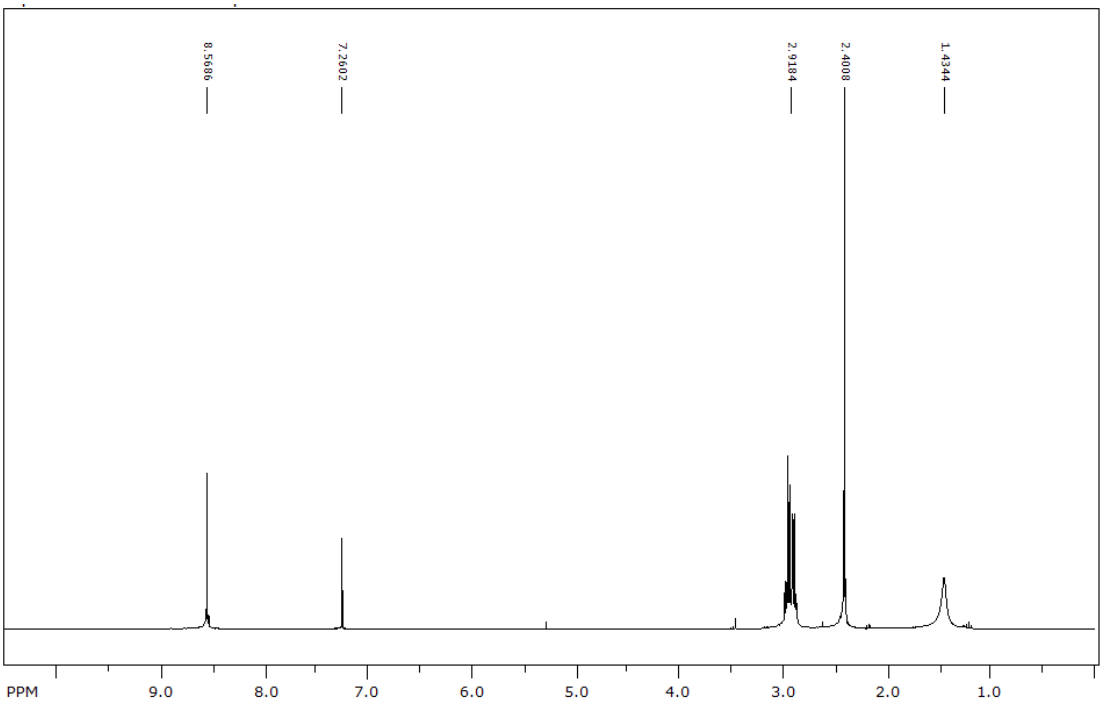
Proton NMR Spectrum of Azido Thiazole 6

Assignment of Protons: **^{1}H NMR** (300 MHz, methanol-d_4)- δ 2.40 (s, 3H, C**H$_3$**), 3.30 (t, 2H, -C**H$_2$**CH$_2$N$_3$), 3.54 (t, 2H, -CH$_2$C**H$_2$**N$_3$), 8.80 (s, 1H, **H**-thiazolium)



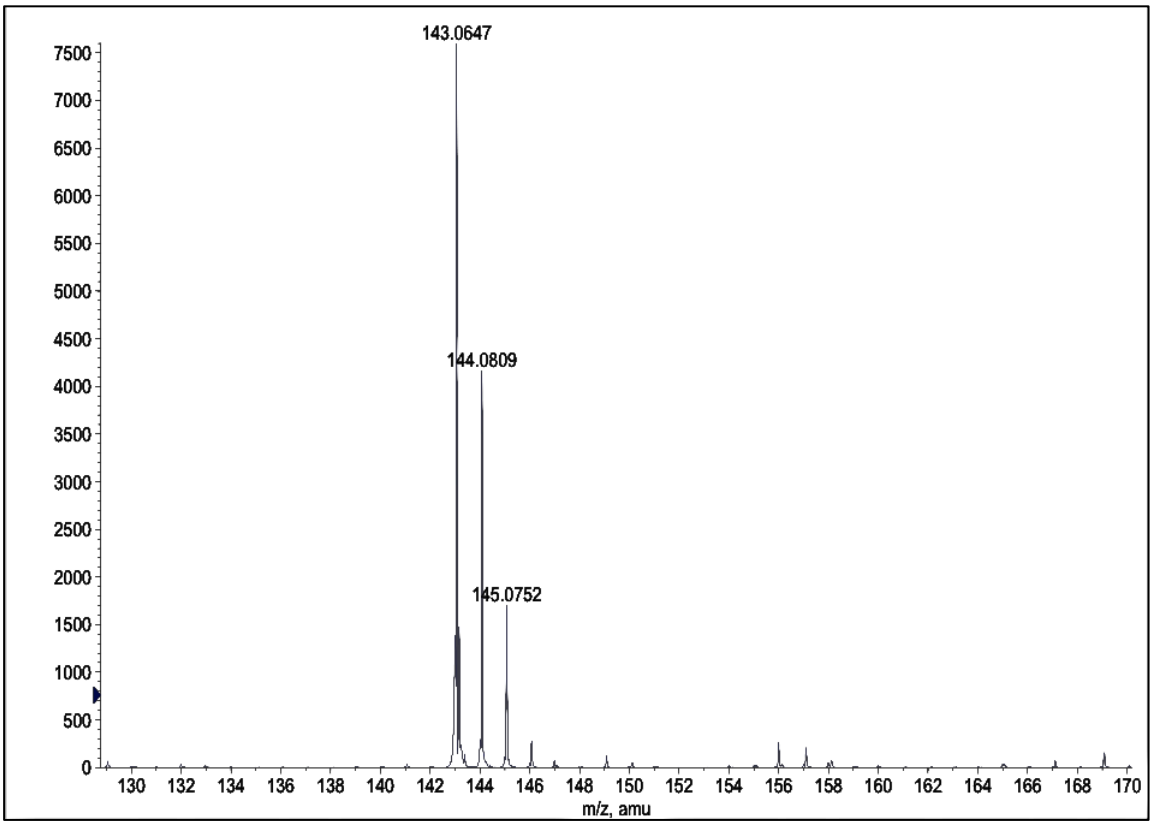
Mass Spectrum of Azido Thiazole 6: ESI-MS (m/z): [M+H]+ calcd for $C_6H_8N_4S$ 169.0545; found 164.0548

Amino Thiazole 7 Spectroscopic Data



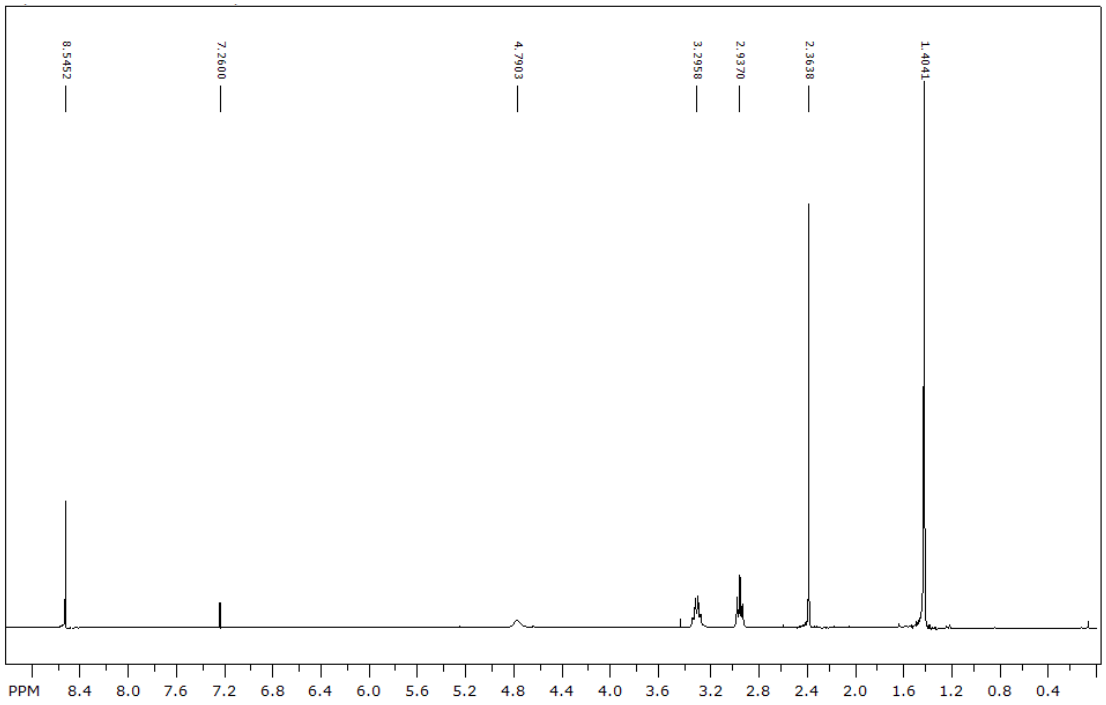
Proton NMR Spectrum of Amino Thiazole 7

Assignment of Protons: **^{1}H NMR** (300 MHz, chloroform-*d*)- δ 1.43 (br s, 2H, N**H$_2$**), 2.40 (s, 3H, C**H$_3$**), 2.92 (m, 4H, -C**H$_2$**C**H$_2$**NH_2), 8.57 (s, 1H, **H-**thiazolium).



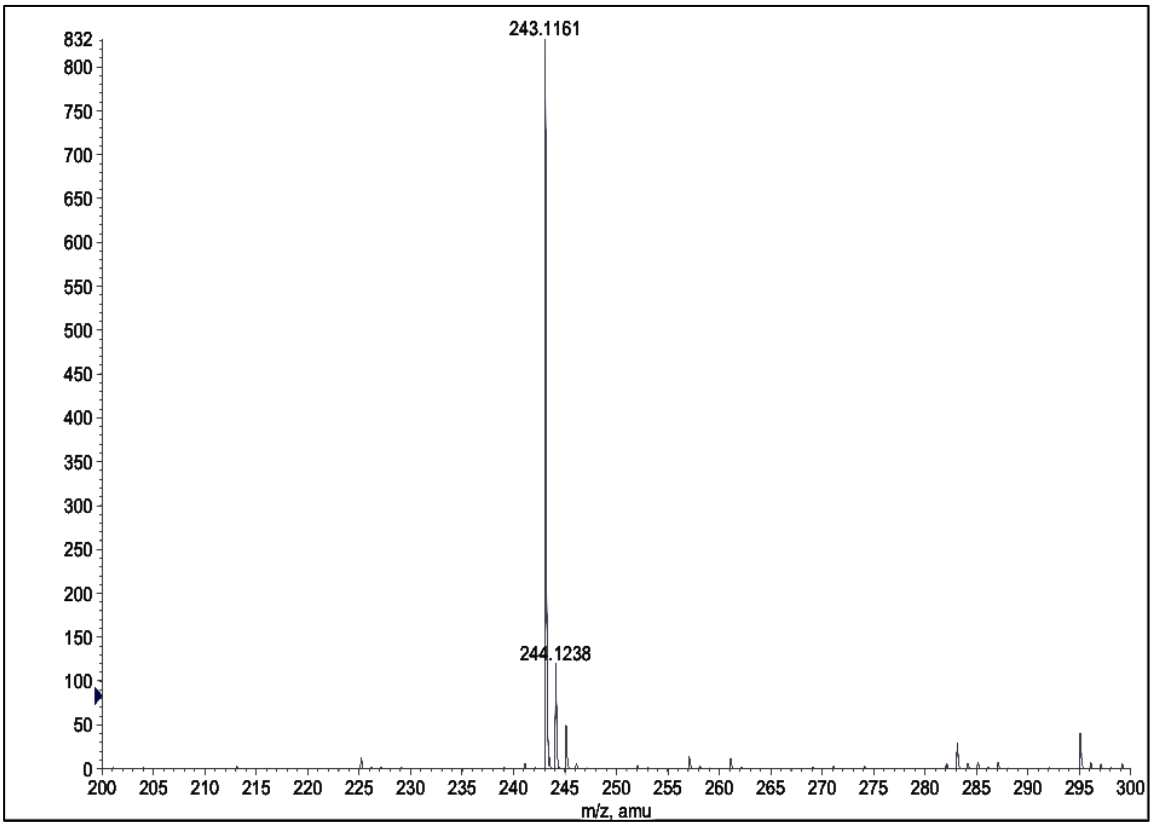
Mass Spectrum of Amino Thiazole 7: ESI-MS (m/z): [M+H]+ calcd for $C_6H_{10}N_2S$ 142.0643; found 143.0647

Protected Amino Thiazole 8 Spectroscopic Data



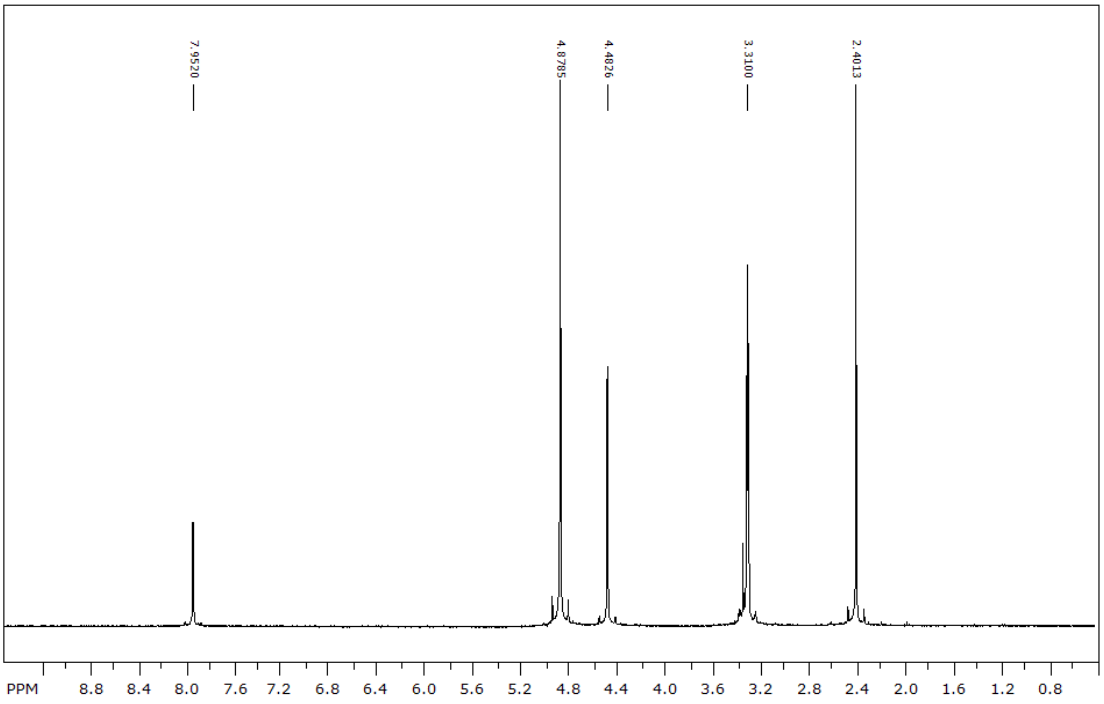
Proton NMR Spectrum of Protected Amino Thiazole 8

Assignment of Protons: **^{1}H NMR** (300 MHz, chloroform-*d*) - δ 1.40 (s, 9H, -C (C**H$_3$**)$_3$), 2.36 (s, 3H, -C**H$_3$**), 2.94 (t, 2H, -C**H$_2$**CH$_2$NH-), 2.96 (q, 2H, -CH$_2$C**H$_2$**NH-), 4.79 (br s, 1H, -N**H-**), 8.54 (s, 1H, **H**-thiazolium)



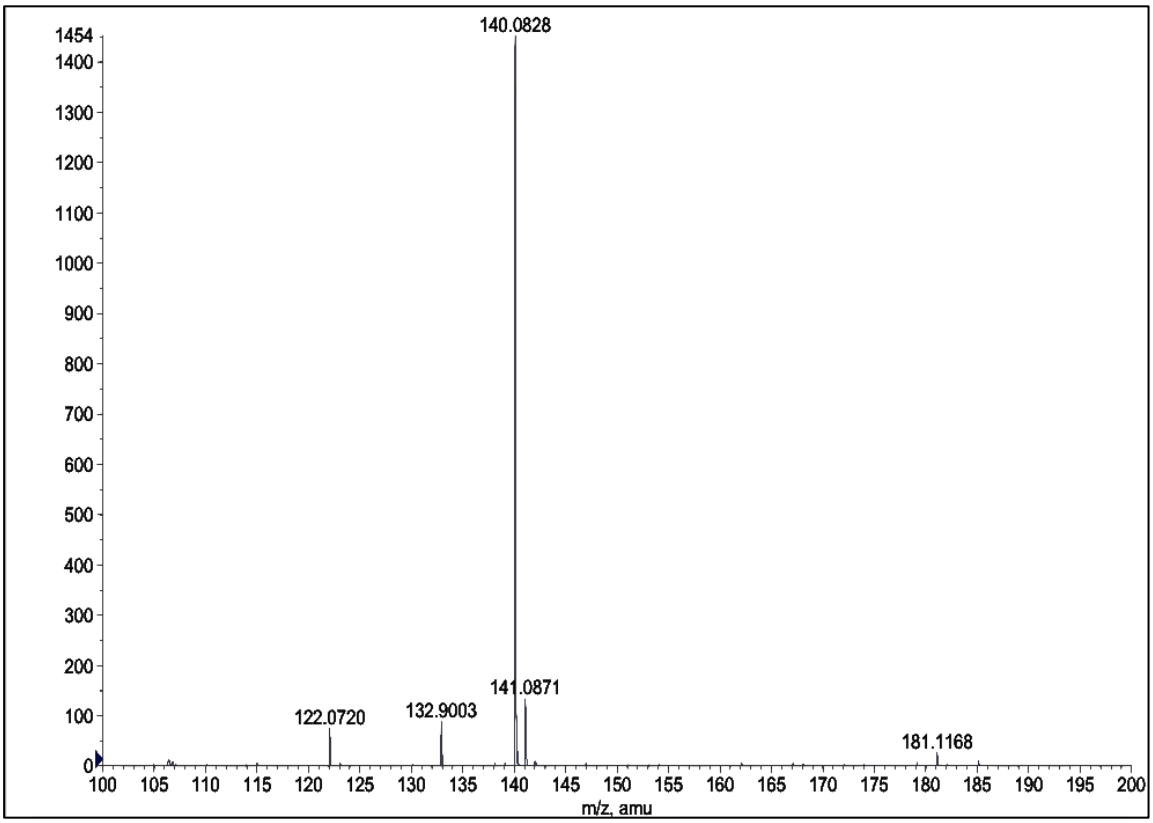
Mass Spectrum of Protected Amino Thiazole 8: ESI-MS (m/z): [M+H]+ calcd for $C_{11}H_{18}N_2O_2S$ 243.1167; found 243.1167

HMP Alcohol 11 Spectroscopic Data



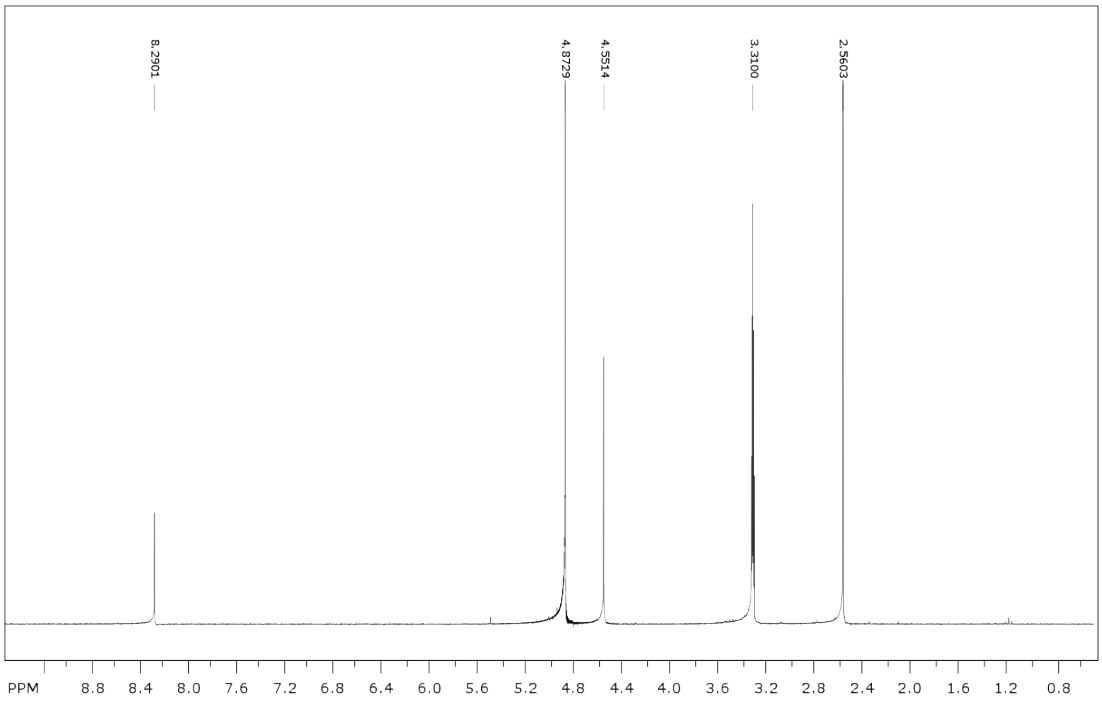
Proton NMR Spectrum for HMP Alcohol 11

Assignment of Protons: **^{1}H NMR** (300 MHz, methanol-d_4)- δ 2.56 (s, 3H, -C**H$_3$**), 4.55 (s, 2H, -C**H$_2$**Br), 8.29 (s, 1H, aromatic **H**-C).



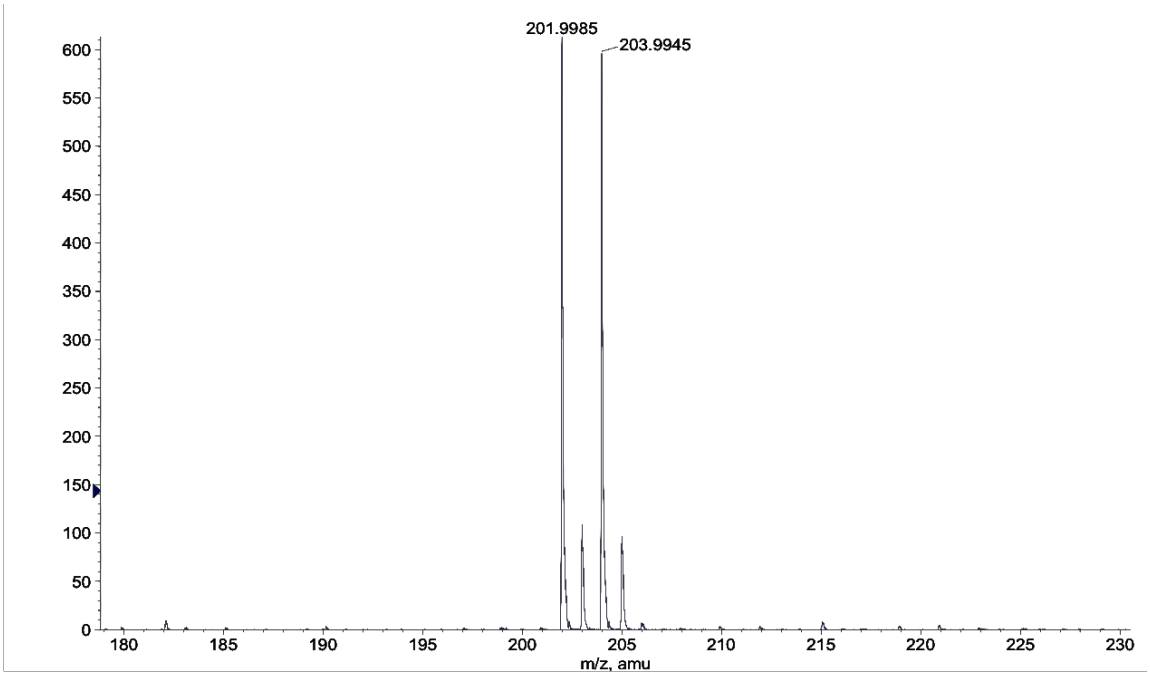
Mass Spectrum of HMP Alcohol 11: ESI-MS (m/z): [M+H]+ calcd for $C_6H_9N_3O$ 140.0824; found 140.0828

Pyrimidine Reagent 12 Spectroscopic Data



Proton NMR Spectrum of Pyrimidine Reagent 12

Assignment of Protons: **^{1}H NMR** (300 MHz, methanol-d_4) - δ 2.56 (s, 3H, -C**H$_3$**), 4.55 (s, 2H, -C**H$_2$**Br), 8.29 (s, 1H, aromatic **H**-C).



Mass Spectrum of Pyrimidine Reagent 12: ESI-MS (m/z): [M+H] + calcd for $C_6H_8N_3Br$ 201.9980; found 201.9985.