Synthesis of bisubstrate analog inhibitors against aspartate semialdehyde dehydrogenase

Harinath G. Muvvala

The University of Toledo

Follow this and additional works at: http://utdr.utoledo.edu/theses-dissertations

Recommended Citation
http://utdr.utoledo.edu/theses-dissertations/387

This Thesis is brought to you for free and open access by The University of Toledo Digital Repository. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of The University of Toledo Digital Repository. For more information, please see the repository's About page.
A Thesis

entitled

Synthesis of Bisubstrate Analog Inhibitors against Aspartate Semialdehyde Dehydrogenase

by

Harinath G. Muvvala

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in Chemistry

Dr. Ronald Viola, Committee Chair

Dr. Max O. Funk, Committee Member

Dr. Steven J. Sucheck, Committee Member

Dr. Patricia R. Komuniecki, Dean

College of Graduate Studies

The University of Toledo

December 2012
Aspartate semialdehyde dehydrogenase (ASADH) is an essential enzyme found in bacteria, fungi and plants. This enzyme is responsible for the production of several essential amino acids, and its absence in humans makes ASADH an attractive target for the development of antibiotics. The goal of the project is to design, synthesize and build species-selectivity into bisubstrate analogue inhibitors against ASADH. Analogues were choosen to synthesize based on the docking model predictions for binding affinities and selectivities against ASADHs that have been purified and characterized from different microbial species. The synthetic approach involves the parallel synthesis of secondary amine derivatives and phenyl alkyl halides. The two synthons are being coupled through nucleophilic substitution followed by hydrolysis to form the desired bisubstrate analogues. Four compounds were identified based on docking score and selectivity analyses. These analogs were synthesized and tested against ASADH. These studies have led to identification of sub-millimolar inhibitors of ASADH. This work is supported by a grant from the NIH (AI077720).
Acknowledgements

I owe my deepest gratitude for the guidance and support of Dr. Ronald Viola throughout my research. He has provided me with technical and theoretical insights that have expanded my knowledge and understanding in the field of Chemistry. I would like to thank my graduate research committee, Dr. Max O. Funk and Dr. Steven J. Sucheck for their knowledge and assistance. I would like to take this opportunity to thank my research group, colleagues and friends for their support. I am grateful to one and all who helped me in many ways throughout my project.

Special Thanks to:

Department of Chemistry, University of Toledo

Graduate School, University of Toledo

Dr. Amarjit Luniwal

Dr. Alexander G. Pavlovsky
Table of Contents

Abstract........................................................................................................... iii

Acknowledgements....................................................................................... iv

Table of Contents.......................................................................................... v

List of Tables.................................................................................................. viii

List of Figures................................................................................................ ix

List of Schemes.............................................................................................. x

List of Abbreviations .................................................................................... xii

1 Introduction.................................................................................................. 1

1.1 Background of inhibitor development ................................................. 4

1.2 Role of active site functional groups in ASADH ............................... 6

1.3 Chemical mechanism of ASADH......................................................... 8

1.4 Molecular modeling and docking studies........................................... 9

2 Results and Discussion ............................................................................. 17

2.1 Synthesis of N-benzylglycine methyl ester ....................................... 18

2.2 Synthesis of benzyl protected tertiary amine ................................... 19
2.3 Debenzylation

2.4 Alkylation

2.5 Parallel synthetic approach

2.5.1 Synthesis of Phenyl alkyl halides (synthon B)

2.5.2 Coupling of two synths

2.5.3 Hydrolysis

3 Summary

4 Experimental

4.1 General procedures

4.2 Synthesis of secondary amine derivative 6 or synthon A

4.2.1 N-benzylglycine methyl ester

4.2.2 Tertiary amine derivative 5

4.2.3 Secondary amine derivative 6 or synthon A

4.3 Synthesis of GS-2

4.3.1 Phenyl alkyl tertiary amine 24 or coupling of two synths A and B

4.3.2 Target compound GS-2

4.4 Synthesis of GS-8

4.4.1 Phenyl alkyl tertiary amine 25 or coupling of two synths A and B

4.4.2 Target compound GS-8

4.5 Synthesis of GS-9

vi
4.5.1 Phenyl alkyl tertiary amine 26 or coupling of two synthons A and B........47

4.5.2 Target compound GS-9.................................................................................48

4.6 Synthesis of GS-10 ..........................................................................................49

4.6.1 Phenyl alkyl tertiary amine 27 or coupling of two synthons A and B........49

4.6.2 Target compound GS-11...............................................................................51

4.7 Synthesis of S-1 ...............................................................................................51

4.8 Enzymatic assay...............................................................................................52

References.............................................................................................................55

Appendix A

Supporting information for chapter 4.................................................................60
List of Tables

1.1 Some inhibitors of ASADH identified by fragment library screening.¹ ............................................. 5
1.2 Proposed series of new bisubstrate analogs .................................................................................. 14
1.3 Surflex-dock score for bisubstrate analogues ............................................................................. 16
2.1 Optimizing the alkylation of secondary amine derivatives ..................................................... 23
2.2 Optimizing the synthesis of tertiary amine alkyl phenyl derivative ...................................... 29
2.3 Kᵢ values for Bisubstrate analogs .............................................................................................. 33
List of Figures

1-1 Reaction catalyzed by aspartate β-semialdehyde dehydrogenase (ASADH) ..................2
1-2 Aspartate pathway of amino acid biosynthesis.........................................................3
1-3 Optimal inhibitory pharmacophore core structure ......................................................6
1-4 Ribbon drawing of the ASADH from *Escherichia coli* ..............................................7
1-5 The active site structure of *Vibrio cholerae* ASADH with bound NADP and the covalent inactivator SMCS ...........................................................................................................8
1-6 Chemical mechanism of aspartyl semialdehyde dehydrogenase. ..............................9
1-7 Binding of D-2-aminoadipic acid (2-aaa) in the active site of *S.pneumoniae* ASADH. 11
1-8 Binding of 2-aminoadipate (cyan) in the active site of ASADH .................................12
1-9 Initial set of synthetic inhibitors.......................................................................................13
1-10 Different binding modes of new bisubstrate analog GS-14 (brown color) in *V.cholerae* ASADH (left) and *S.pneumoniae* ASADH (right). .........................................................15
4-1 Assay procedure for ASADH. ..........................................................................................53
4-2 Enzyme assay by various concentrations of bisubstrate analogue GS-8 ......................53
4-3 A Dixon plot of 1/[Vmax] versus inhibitor (GS-8) concentration ....................................53
List of Schemes

Scheme 1 : Synthesis of bisubstrate analogues .................................................................18
Scheme 2 : Synthesis of N-benzylglycine methyl ester .......................................................19
Scheme 3 : Synthesis of N-benzyl tertiary amine derivative ............................................19
Scheme 4 : Synthesis of secondary amine derivative or synthon A ....................................20
Scheme 5 : Synthesis of N-propanol derivative ..................................................................20
Scheme 6 : Synthesis of N-propanol derivative ..................................................................21
Scheme 7 : Synthesis of N-benzyl propanol derivative .....................................................21
Scheme 8 : Synthesis of N-benzyl propanol ......................................................................22
Scheme 9 : Synthesis of N-benzyl alky halide derivative ..................................................22
Scheme 10 : Synthesis of N-alkyl halide derivative ..........................................................23
Scheme 11 : Synthesis of target bisubstrate analogues ......................................................25
Scheme 12 : Synthesis of phenyl propyl bromide 17 ......................................................26
Scheme 13 : Synthesis of phenyl propyl bromide 19 ......................................................27
Scheme 14 : Synthesis of phenyl propyl bromide 21 ......................................................27
Scheme 15 : Synthesis of phenyl propyl bromide 23 ......................................................27
Scheme 16 : Synthesis of tertiary amine alkyl phenyl derivative 24 ..................................28
Scheme 17 : Synthesis of tertiary amine alkyl phenyl derivative 25, 26 and 27 ...............29
Scheme 18 : Synthesis of target compound S-1 ..................................................................30
Scheme 19 : Synthesis of target compound GS-2 ...............................................................31
Scheme 20 : Synthesis of target compounds GS-8, GS-9 and GS-10 .............................31
Scheme 21: Synthesis of GS-2, GS-8, GS-9, GS-10 and S-1 .................................................35
List of Abbreviations

ADP………………..Adenosine diphosphate
AMP……………….Adenosine monophosphate
ASA……………….Aspartate-β-semialdehyde
ASADH……………Aspartate-β-semialdehyde dehydrogenase
br……………………Broadened

CHES………………Cyclohexylamino ethanesulfonic acid
d…………………Doublet
dd…………………Doublet doublet
DTT………………..Dithiothreitol

EDTA……………..Ethylenediaminetetraacetic acid

HEPES……………Hydroxyethyl piperazine ethanesulfonic acid
Hz………………….Hertz

J………………….Coupling constant

m……………………Multiplet
MHz………………..Megahertz
m/z………………..Mass to charge ratio

NADP……………….Nicotinamide adenine dinucleotide phosphate

q…………………Quartet

s……………………Singlet

SMCS……………….S-methyl cysteine sulfoxide

sp……………………Streptococcus pneumonia

t……………………Triplet

THF……………….Tetrahydrofuran

TMS………………Tetramethylsilane

vc……………………Vibrio cholera

2-aaa……………….2-aminoadipic acid
Chapter 1

Introduction

Infectious microorganisms showing resistance to the most recently developed antibiotics that target cell wall assembly and protein biosynthesis are an increasing public health problem.\(^1\) *Enterococcus faecalis* showing plasmid-encoded resistance to erythromycin and tetracycline was discovered in the early 1970s. Now antibiotic resistant *Enterococci* are the leading cause of hospital-acquired infections.\(^2\) *Mycobacterium tuberculosis* strains are resistant to isoniazid, rifampin, and other common treatments have become an increasing clinical challenge.\(^3\) Many different bacterial genuses like *Staphylococcus, Enterococcus, Salmonella, Gonococcus, Streptococcus* and *Mycobacterium* that were previously susceptible to antibiotics now exhibit multidrug resistance.\(^1\) Identifying novel targets of key enzymes that play a crucial role in the biosynthesis of essential amino acids is a potentially effective way to combat antimicrobial drug resistance. The aspartate pathway for amino acid biosynthesis is essential for the viability of all microorganisms\(^4\) but the absence of this entire pathway in humans makes enzymes of this pathway an attractive target for the development of selective antibiotics. Aspartate-\(\beta\)-semialdehyde dehydrogenase (ASADH) catalyzes the second step in the aspartate pathway, formation of aspartate \(\beta\)-semialdehyde (ASA) by
the reductive dephosphorylation of β-aspartyl phosphate (Figure 1-1). The product of this reaction occupies the first branch point metabolite and is a precise bottleneck in the flow through the pathway.\textsuperscript{5} Inhibition of this enzyme will stop the production of four essential amino acids namely, lysine, methionine, threonine and isoleucine (Figure 1-2) each of which is required for protein synthesis.\textsuperscript{6} In addition, this pathway produces essential metabolites that play crucial roles in several cellular functions.

Figure 1-1. Reaction catalyzed by aspartate β-semialdehyde dehydrogenase (ASADH)

These include diaminopimelate precursor for cross-linking in bacterial cell wall biosynthesis,\textsuperscript{7} dipicolinic acid which is crucial for sporulation in Gram positive bacteria,\textsuperscript{8} homoserine lactone a key component required for quorum sensing signaling,\textsuperscript{9,9b} S-adenosyl methionine required for methylation reactions,\textsuperscript{9a} protective dormancy and virulence factor production\textsuperscript{10} (Figure 1-2). Thus, identifying new inhibitors against this key enzyme, ASADH, can produce lead compounds that could be developed into potential new antibiotics to combat the growing threat from antibiotic-resistant microorganisms.
**Figure 1-2.** Aspartate pathway of amino acid biosynthesis. The targeted metabolic pathway produces four essential amino acids (green color) and several metabolite precursors (orange color) involved in sporulation, bacterial cell wall cross-linking and quorum sensing (red color).
1.1 Background of inhibitor development

Inhibitor synthesis can be guided by two quite different approaches. Using known structures of substrates and products to guide the synthesis of inhibitor is one method. The other method is using kinetic studies to screen fragment molecule libraries to identify new compounds that bind to ASADH with high ligand efficiency. Earlier studies had identified substrate analogue inhibitors against ASADH such as S-methyl-L-cysteine sulfoxide, S-carbomyl-L-cysteine and S-allyl-L-cysteine,\textsuperscript{12} an active site directed inactivators, aspartyl β-difluorophosphonate, a reversible competitive inhibitor,\textsuperscript{1,13,14} chloroketone, an irreversible inhibitor, and phosphoramidate (N-phosphoryl asparagine), a good competitive inhibitor.\textsuperscript{15} Coenzyme analogue inhibitors of ASADH include 2'-phosphoribose AMP, an analog of NADP missing the nicotinamide portion of the dinucleotide which has been shown to be a good competitive inhibitor\textsuperscript{16} of the archael \textit{mj}ASADH ($K_i = 50$ µM), and the Gram-negative bacteria \textit{vc}ASADH ($K_i = 2.5$ mM) while another coenzyme analogue, 2',5'-ADP, that contains only the adenosine portion of NADP have also been observed having a similar pattern of inhibition against the Gram-positive \textit{sp}ASADH.\textsuperscript{17} These studies suggest that in addition to targeting the substrate binding pocket, targeting the co-enzyme binding pocket is another viable strategy to inhibit ASADH.

The other method to identify new inhibitors uses kinetic studies to screen fragment molecule libraries to identify new compounds that bind to ASADH with high ligand efficiency. Previously in our lab several interesting compounds such as 2,3-diaminopropionate, a 5-carbon aminocarboxylate (5-aminocaproate), a 5-carbon
aminodicarboxylate (glutamate), a nitro analogue of succinate (3-nitropropionate), 2-
amino adipate and 2,4-diaminobutyrate have each been identified as enzyme inhibitors ($K_i$ values in lower millimolar range, Table 1.1) of ASADH by fragment based library screening. Based upon structural knowledge of the above identified analogues and our understanding of enzyme active site structure, it is desirable to incorporate a 4 to 6 carbon
dicarboxylate (one carboxyl group at each end) that helps in binding to this two essential
active site arginines (R245 & R99 for ASADH). There is also a preference for an amino
group in the pharmacophore core structure of these new analogs (Figure 1-3).

Table 1.1. Some inhibitors of ASADH identified by fragment library screening.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Chemical Formula</th>
<th>$K_i$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3-diaminopropionate</td>
<td>CH$_2$(NH$_2$)CH(NH$_2$)COO$^-$</td>
<td>0.47±0.08</td>
</tr>
<tr>
<td>2,4-diaminobutyrate</td>
<td>CH$_2$(NH$_2$)CH$_2$CH(NH$_2$)COO$^-$</td>
<td>0.14 ± 0.04</td>
</tr>
<tr>
<td>5-aminocaproate</td>
<td>H$_2$N(CH$_2$)$_4$COO$^-$</td>
<td>0.56 ± 0.08</td>
</tr>
<tr>
<td>glutamate</td>
<td>$^{\prime}$OOC(CH$_2$)$_2$CH(NH$_3^+$)COO$^-$</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>3-nitropropionate</td>
<td>$^{\prime}$O$_2$N(CH$_2$)$_2$COO$^-$</td>
<td>0.24 ± 0.06</td>
</tr>
<tr>
<td>2-amino adipate</td>
<td>$^{\prime}$OOC-(CH$_2$)$_3$CH(NH$_3^+$)COO$^-$</td>
<td>0.23 ± 0.05</td>
</tr>
</tbody>
</table>
**Figure 1-3.** Optimal inhibitory pharmacophore core structure \((n = 1-3, \, m = 1 \text{ and } n = 0-2, \, m = 2)\). Figure was adapted from reference.\(^1\)

### 1.2 Role of active site functional groups in ASADH

ASADH has been mechanistically characterized\(^1^8\) and representative structures have been determined from Gram-negative,\(^1^9\) Gram-positive,\(^1^7\) archael species\(^1^7\) and fungal species.\(^5\) ASADH is a homo dimer and each monomer has an active site formed by the N-terminal co-enzyme binding domain and a substrate binding domain as shown in **Figure 1-4.** Even though overall sequence diversity exists between different branches of the ASADH family, it has preserved the identity of the core active site functional groups throughout evolution (**Figure 1-5**). Viola *et al.*, have successfully identified the role of conserved active site residues of ASADH in substrate recognition and binding through studies of the kinetic and structural consequences of active site mutants of ASADH.\(^1^1\)

The presence of the essential cysteine 134 (**Figure 1-5**) locates the active site and it had been previously identified as the active site nucleophile.\(^6\) The essential histidine residue 274 (**Figure 1-5**) was identified as the critical acid-base catalyst.\(^1^1\) Arg 267, making an electrostatic interaction with the carboxyl group of the active site directed inactivator S-methyl cysteine sulfoxide (SMCS)\(^1^9\) and glutamine 240 making interactions with amino
group of SMCS, identified these residues as substrate binding groups (Figure 1-5).\textsuperscript{20,11} Arg 101 and Lys 243 making interactions with W 2 and W 1, respectively (Figure 1-5) are identified as the phosphate binding groups.\textsuperscript{11,19}

**Figure 1-4.** Ribbon drawing of the ASADH from *Escherichia coli*. The overall structure is shown in complex with bound coenzyme NADP (shown in orange) and the active site amino acids (blue sticks) are shown in one subunit (figure was taken from reference [12]).
Figure 1-5. The active site structure of *Vibrio cholerae* ASADH with bound NADP and the covalent inactivator SMCS (green color). *H. influenza* numbering is in parentheses (figure was taken from reference [12]).

1.3 Chemical mechanism of ASADH

The proposed chemical mechanism of ASADH involves the initial attack of the active site nucleophile Cys 136 on the carbonyl carbon of the aspartyl phosphate substrate. The active site nucleophile is generated by the deprotonation of Cys 136 by the adjacent His 273. His 273 is positioned for this role by a hydrogen bond to Gln-161. The binding of the aspartyl phosphate is supported by an electrostatic interaction of the guanido group of Arg-267 with the carboxyl group of this substrate. Attack of the active site thiolate on the carbonyl carbon of aspartyl phosphate generates a tetrahedral intermediate, followed by collapse of this tetrahedral intermediate with expulsion of the
phosphate group leads to hydride transfer to this intermediate by the adjacent bound NADPH. Collapse of the tetrahedral intermediate generated after hydride transfer leads to expulsion of the enzyme thiolate group (assisted by protonation from adjacent His 273) and yields aspartate semialdehyde (ASA, (Figure 1-6)).

![Chemical mechanism of aspartyl semialdehyde dehydrogenase](image)

**Figure 1-6.** Chemical mechanism of aspartyl semialdehyde dehydrogenase (figure was adapted from reference [7]).

### 1.4 Molecular modeling and docking studies

Our previous group member Dr. Luniwal conducted molecular modeling and docking studies utilizing SYBYL 8.0 installed on Linux operating system. Three dimensional structures for each compound were generated using the default settings of SKETCH option in SYBYL. Gasteiger–Huckel charges were applied to the molecules after adding all hydrogen atoms before energy calculations were performed using the Tripos force-fields. For energy minimizations, the powell conjugate- gradient algorithm with a termination criterion of 0.05 K.cal/mol was used. Docking studies were conducted using the docking package Surflex-dock™ on SYBYL which internally uses
Hammerhead docking system based empirical scoring function and its search engine relies on surface-based molecular similarity method.\textsuperscript{21} For these docking studies the structure of ASADH with the bound inhibitor 2-aminoadipate was used. Several docking protocols were generated by altering the bloat and threshold values with/without changing the mode for protomol generation such as automatic or ligand predicted methods.

Based upon the structural knowledge of the previously identified analogs\textsuperscript{1} and our understanding of enzyme active site structure, it is desirable to incorporate carboxyl groups into pharmacophore core structure that can potentially interact with the two essential active site arginines R 245 and R 99 for ASADH (\textbf{Figure 1-5}). To examine this possibility, docking models were generated using D-2-aminoadipic acid (2-aaa) bound to ASADH. 2-aaa was chosen because high resolution x-ray structural data are available for this complex\textsuperscript{22} and also our previous studies\textsuperscript{1} determined 2-aaa to have a low sub-millimolar activity against ASADH. The structural insight of 2-aaa bound to the enzyme active site shows that 2-aminoadipic acid selectively binds through two sets of bidentate interactions between the carboxyl groups of 2-aaa and the guanidium groups of Arginine 99 and Arginine 245 (\textbf{Figure 1-7}).\textsuperscript{22} In addition, the amino group on the alpha carbon of the inhibitor 2-aaa is stabilized by interactions with the carboxylate oxygens of Glu 220 and side chain carbonyl oxygen of Asn 127 (\textbf{Figure 1-7}).\textsuperscript{22} Docking studies were carried out to improve inhibitor design, to build additional binding interactions and to prioritize the analogs for synthesis.
Our previous group member Dr. Luniwal conducted molecular modeling studies for comparative analysis between the two active sites of *S. pneumoniae* (Gram-positive) ASADH and *V. cholerae* (Gram-negative) ASADH using high resolution x-ray data. The structural components of the two active sites (*S. pneumoniae* and *V. cholerae* ASADH) are almost identical. However, his studies revealed that there are subtle differences between these two active sites include the identification of an additional pocket in *V. cholerae* ASADH (Figure 1-8A) that is absent in *S. pneumoniae* ASADH (Figure 1-8B). This is because the additional loop made up of P191, S192, G 193, G 194, D 192 and Q 208 in *S. pneumoniae* ASADH is absent in *V. cholerae* ASADH, thus making
extra space available in *V.cholerae* ASAD. We have attempted to use this difference to gain selectivity in inhibitor design between these two homologues.

**Figure 1-8.** Binding of 2-amino adipate (cyan) in the active site of ASADH. (A) Gram-positive (*S.pneumoniae*) with an additional loop present only in *sp* ASADH; (B) Gram-negative (*V.cholerae*) with an extra pocket (shaded in green) relative to the active site of *S.pneumoniae* ASADH. Atom labeling: blue color represents nitrogen and red color represents oxygen)

Dr. Luniwal designed and synthesized an initial set of new inhibitors as structural analogs of 2-aaa (**Figure 1-9**). This compound was chosen as starting point geometry because previous studies in our lab had demonstrated that 2-aaa has a low millimolar Kᵢ value against ASADH. The following structural analogs S 1 to S 6 were synthesized by Dr. Luniwal and tested for inhibition by our previous group member Lin Wang. We initially tested the synthetic compounds S 1 to S 6 at 20 mM concentration using standard assay procedure (see section 4.8, experimental). Surprisingly, none of these targets showed any inhibition of the reaction.
**Figure 1-9.** Initial set of synthetic inhibitors.

Based on an analysis of molecular modeling studies and the subtleties of the active site architecture, a series of new bisubstrate inhibitors (Table 1.2) were designed aiming to fill two regions within the active site. The compounds in this new series consist of a parent chain and a side chain. The parent chain contains two carboxylates (one carboxyl group at each end) simulating the structure of 2-aminoadipic acid (Table 1.2). One of the carbons in the carbon skeleton of the parent chain is replaced with nitrogen so that the further derivatization process can be synthetically manageable. The derivatization process includes adding a desired side chain (based on target compound) onto the nitrogen. These additional functionalities should bring some new interactions with the additional cavities surrounding the active site. These additional pockets could be the NADP (co-enzyme) binding space or the additional pocket visualized near the *V.cholerae* ASADH active site. If these additional functionalities introduced through side chains could preferably occupy the additional pocket present in *V.cholerae* ASADH (but absent in *S.pneumoniae* ASADH) this would lead to enhanced selectivity between the homologous enzymes of this family (*Sp* and *Vc* forms of ASADH). If this new side chain occupies the coenzyme (NADP) binding pocket this would lead to increased binding affinity.
Table 1.2. Proposed series of new bisubstrate analogs

![Chemical structure](image)

| Compound | n | n1 | X | R | O
|----------|---|----|---|---|---
| GS-1     | 1 | 0  | O | H |
| GS-2     | 1 | 1  | O | H |
| GS-3     | 1 | 2  | O | H |
| GS-4     | 2 | 1  | O | H |
| GS-5     | 1 | 1  | N | H |
| GS-6     | 1 | 1  | S | H |
| GS-7     | 1 | 1  | O | 3-OMe |
| GS-8     | 1 | 1  | O | 3-F |
| GS-9     | 1 | 1  | O | 3-Cl |
| GS-10    | 1 | 1  | O | 3,5-F |
| GS-11    | 1 | 1  | O | 3-Me |
| GS-12    | 1 | 1  | O | 2-Me |
| GS-13    | 1 | 1  | O | 2-F |
| GS-14    | 1 | 1  | O | 4-OMe |
| GS-15    | 1 | 1  | O | 4-F |

The docking models were provided by our group member Dr. Luniwal. The docking models generated using *V. cholerae* ASADH further supported our assumption that the additional space visualized in *V. cholerae* ASADH could provide selectivity. In these docking models most of the compounds oriented in such a way that they could reach the additional pocket (Figure 1-10). Conversely, in docking models generated using *S. pneumoniae* ASADH most of the compounds oriented to reach the coenzyme NADPH binding pocket (Figure 1-10).
Figure 1-10. Different binding modes of new bisubstrate analog GS-14 (brown color) in *V.cholerae* ASADH (left) and *S.pneumoniae* ASADH (right). The extra pocket shaded in green in *vc* ASADH and NADPH shown in yellow in *sp*ASADH. (Figures were provided by Dr. Luniwal, only GS-14 was shown for the sake of clarity).

These results were utilized to prioritize the synthesis of the following analogs: GS-9, GS-8 and GS-11, which were selected because they have substantially different predicted scores between the two enzyme forms, Gram-positive *S.pneumoniae* and Gram-negative *V.cholerae* (Table 1.3). In addition, GS-2 was selected for synthesis, because we would like to see the activity of bisubstrate analogue without any substituent on third position of the side chain phenyl ring (Table 1.3).
Our previous group members had identified novel inhibitors against different forms of ASADHs through small molecule fragment library screening, which support our goal of designing lead compounds for the development of new antibiotics. However, until now we have not identified any potent and selective inhibitors. To achieve this aim our plan is to design and synthesize bisubstrate inhibitors which can interact with multiple functional groups in the active site. This will allow us to utilize the subtle differences in the enzyme structures of different forms of ASADH (which catalyze the same reaction and share identical active site functional groups) that will lead to selectivity and increased binding affinity of target compounds against ASADHs. My goal is to develop a novel scheme, optimize the reaction conditions to synthesize the desired bisubstrate analog inhibitors, and eventual biological testing against different forms of ASADHs to identify potent inhibitors.
Chapter 2

Results and discussion

The goal of my project is to synthesize bisubstrate analogue inhibitors against ASADH. Analogues were chosen to synthesis based on docking model predictions for binding affinities and selectivities against ASADHs that have been purified and characterized from different microbial species. The synthetic approach involves the parallel synthesis of secondary amine derivatives (Synthon A) and phenyl alkyl halides (Synthon B). The two synthons are then coupled through nucleophilic substitution followed by hydrolysis to form the desired bisubstrate analogues (Scheme 1).
2.1 Synthesis of $N$-benzylglycine methyl ester

Following a known procedure,²⁴ $N$-benzylglycine methyl ester 3 precursor for the parent chain of bisubstrate analog was synthesized by treating methyl bromoacetate 1 and benzyl amine 2. Following a known procedure, (ref) one mmol each of methyl bromoacetate 1 and benzyl amine 2 in THF was treated with two equivalents of triethyl amine for 18 h at room temperature and resulted in 77% yield of $N$-benzylglycine methyl ester 3 after column chromatography (Scheme 2).
2.2 Synthesis of benzyl protected tertiary amine

To a solution of N-benzylglycine methyl ester 3 in methanol, NaHCO₃ was added under argon. To this suspension bromo methylpropionate 4 dissolved in methanol was added at 50 °C. The mixture was refluxed under argon at 60 °C for 18 h and resulted in compound 5 in 67% yield after column chromatography (Scheme 3).

2.3 Debenzylation

After synthesizing the N-benzylated tertiary amine 5 (Scheme 3), the next key step involved the formation of intermediate compound 8 (Scheme 5). In order to form the key intermediate compound 8, we needed to debenzylate the compound 5. To form debenzylated secondary amine 6, compound 5 in ethyl acetate with 1 drop of acetic acid was treated with Pd/C (10% wt, 0.06 g/mmol 5) in the presence of H₂ at 35 psi for 18 h. This resulted in debenzylated compound 6 in 92% yield (Scheme 4).
Scheme 4: Synthesis of secondary amine derivative or Synthon A

\[
\text{O} \quad \text{O} \\
\text{O} \quad \text{O} \\
\text{O} \quad \text{Pd/C} \\
1 \text{ drop of acetic acid} \\
\text{H}_2, 35 \text{ psi}, 18 \text{ hr} \\
92\%
\]

2.4 Alkylation

After synthesizing the debenzylated secondary amine 6, we looked to attach the requisite carbons to form the key intermediate tertiary amino alcohol 8 (Scheme 5), which was later converted into desired bisubstrate analogs by modifying the alcohol group of compound 8. Unfortunately alkylation of compound 6 with bromopropanol and K\textsubscript{2}CO\textsubscript{3} as a base in acetonitrile failed (shown by a dashed arrow) to produce tertiary amino alcohol 8 (Scheme 5).

Scheme 5: Synthesis of N-propanol derivative

\[
\text{O} \quad \text{O} \\
\text{O} \quad \text{O} \\
\text{O} \quad \text{CH}_3\text{CN, K}_2\text{CO}_3 \\
70 \degree \text{C}, \\
70\%
\]

In the next attempt, we tried the same alkylation reaction using in situ Finkelstein reaction conditions, alkylation of compound 6 with bromopropanol, KI and K\textsubscript{2}CO\textsubscript{3} as a base in acetone also failed (shown by a dashed arrow) to produce tertiary amino alcohol 8 (Scheme 6).
Scheme 6: Synthesis of N-propanol derivative

\[
\begin{align*}
6 & \quad \text{+} \quad \text{Br-CH\textsubscript{3}COCH\textsubscript{3}, K\textsubscript{2}CO\textsubscript{3}} \\
7 & \quad \text{KI} \\
\text{70 \textdegree C} & \quad \text{8}
\end{align*}
\]

To avoid the depletion of compound 6 that required a three step synthesis, we ran the above alkylation reaction with bromopropanol as a model compound using starting materials (compound 3 and compound 1) of the previous steps. In this process, N-benzylglycine methyl ester 3 in acetonitrile was added K\textsubscript{2}CO\textsubscript{3}, KI and 3 equivalents of bromopropanol, refluxed for 15 h at 70 \textdegree C still failed to produce any tertiary amino alcohol 10 (Scheme 7).

Scheme 7: Synthesis of N-benzyl propanol derivative

\[
\begin{align*}
3 & \quad \text{+} \quad \text{Br-CH\textsubscript{3}COCH\textsubscript{3}, K\textsubscript{2}CO\textsubscript{3}} \\
7 & \quad \text{KI} \\
\text{70 \textdegree C} & \quad \text{10}
\end{align*}
\]

In the next attempt, benzyl amine 1 was used as the starting material for alkylation. Unfortunately, alkylation of benzyl amine 1 with 1.3 equivalents of bromopropanol and K\textsubscript{2}CO\textsubscript{3} as a base also failed to produce any amino alcohol 11 (Scheme 8).
As the above alkylation reactions of amines to bromopropanol had failed, we next tried the alkylation reactions using 1, 3-dibromopropane as the carbon source. In the first attempt, N-benzylglycine methyl ester dissolved in acetonitrile was treated with 2 equivalents of 1, 3-dibromopropane and K$_2$CO$_3$, refluxed for 24 hours at 60$^\circ$C to form amino propyl bromide 13. However this reaction resulted in only 36% yield of amino propyl bromide 13 (Scheme 9) after column chromatographic purification along with a byproduct tentatively identified as dimer 14 (according to $^1$H NMR analysis) in 30% yield.

Since the alkylation reactions with dibromopropane 12 were working, similar conditions were employed to synthesize the desired tertiary amino alkyl bromide 15 (Scheme 10). The secondary amine derivative 6 was treated with 4 equivalents of 1, 3-dibromopropane and K$_2$CO$_3$, refluxed for 6 hr at 60 $^\circ$C afforded amino propyl bromide 15.
along with some unidentified impurities. Column chromatography yielded pure product but only in 10% yield (entry 1, Table 2.1). In an attempt to increase the yield of tertiary amino propyl bromide 15, the reaction was performed using 10 equivalents of

**Scheme 10: Synthesis of N-alkyl halide derivative**

![Scheme 10](image)

**Table 2.1. Optimizing the alkylation of secondary amine derivatives**

<table>
<thead>
<tr>
<th>Entry</th>
<th>1,3-dibromopropane</th>
<th>Temp</th>
<th>Time (hr)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4 eq</td>
<td>60</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>10 eq</td>
<td>rt</td>
<td>24</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>10 eq</td>
<td>rt</td>
<td>40</td>
<td>10</td>
</tr>
</tbody>
</table>

dibromopropane at room temperature for 24 hours (entry 2, Table 2.1). This again resulted in only 9% yield after column chromatographic purification. We then increased the reaction time to 40 hours (entry 3, Table 2.1). These conditions also afforded amino propyl bromide 15 along with some unidentified impurities. Column chromatography once again yielded pure product in 10% yield (entry 3, Table 2.1). Unfortunately, these alkylation reactions using dibromopropane as carbon source resulted in a low yield of the desired amino propyl bromide 15 (Scheme 10) along with some unidentified byproducts.
In order to synthesize the desired bisubstrate analogues, we required the synthesis of alkylated amines by adding the requisite number of carbons to secondary amines in much higher yields. Synthesis of phenyl alkyl bromide 17 (Scheme 12), that would serve as carbon source for alkylation reaction was a viable option (Scheme 11). The synthetic approach involves the parallel synthesis of secondary amine derivatives (Synthon A) and phenyl alkyl halides (Synthon B). The two synthons are then coupled through nucleophilic substitution, followed by hydrolysis to form the desired bisubstrate analogs (Scheme 11).
Scheme 11: Synthesis of bisubstrate analogue inhibitors

**Scheme Details:**

1. **Synthesis of bisubstrate analogue inhibitors**
2. **Synthons A and B**
   - **Synthon A:**
     - Reaction: O\(\text{H}_2\text{N}\) \(\text{O}\) \(\text{N}\) \(\text{O}\) \(\text{Et}_3\text{N} (2\text{eq}), \text{THF}\) \(\text{rt, 18 hr}\) \(77\%\)
     - Result: O\(\text{H}_2\text{N}\) \(\text{O}\) \(\text{N}\) \(\text{O}\)
   - **Synthon B:**
     - Reaction: O\(\text{H}_2\text{N}\) \(\text{O}\) \(\text{N}\) \(\text{O}\) \(\text{Br} \text{-CH}_2\text{Br}\) \(\text{CH}_3\text{CN}, \text{K}_2\text{CO}_3\) \(60 \degree\text{C, 18 hr}\) \(67\%\)
     - Result: O\(\text{H}_2\text{N}\) \(\text{O}\) \(\text{N}\) \(\text{O}\) \(\text{RX}\), \(\text{ROH}\)

**Hydrolysis:**

- **Bisubstrate Analogue**
  - Reaction: Hydrolysis
  - Result: O\(\text{H}_2\text{N}\) \(\text{O}\) \(\text{N}\) \(\text{O}\)
2.5 Parallel synthetic approach

2.5.1 Synthesis of Phenyl alkyl halides (Synthon B)

First we looked to attach the requisite carbons to phenol to form the side chain (Synthon B) of glycine substrate 2 (GS - 2) analog. Alkylation of phenol 16, with excess dibromopropane and K₂CO₃ as a base in acetonitrile, refluxed at 60 °C for 18 hours afforded phenyl propyl bromide 17 along with unreacted dibromopropane. However the extractive workup was unable to remove the excess dibromopropane. The product was separated from dibromopropane by column chromatography to give phenyl propyl bromide 17 in 65% yield (Scheme 12).

![Scheme 12: Synthesis of Phenyl propyl bromide 17](image)

We then applied these results to the alkylation required for forming the side chain of glycine substrate 8 (GS-8) analog. Alkylation of 3-fluorophenol 18, with excess dibromopropane and K₂CO₃ as a base in acetonitrile, refluxed at 60 °C for 18 hours afforded phenyl propyl bromide 19 along with excess dibromopropane again the extractive workup was unable to remove the excess dibromopropane. The product was separated from dibromopropane by column chromatography to give phenyl propyl bromide 20 in 63% yield (Scheme 13).
Scheme 13: Synthesis of Phenyl propyl bromide 19

\[
\begin{align*}
\text{HO-} & + & \text{Br-} & \text{CH}_3\text{CN, K}_2\text{CO}_3 \\
18 & & 12 & 60^\circ \text{C, 18 hr} \\
\end{align*}
\]

Similar conditions were employed to synthesize the side chain of glycine substrate 9 (GS-9) analog. 3-chlorophenol 20 was treated with excess dibromopropane, acetonitrile and K\textsubscript{2}CO\textsubscript{3}, refluxed at 60 °C for 18 hours afforded phenyl propyl bromide 21 along with dibromopropane. The product was separated from the excess dibromopropane by column chromatography to give phenyl propyl bromide 21 in 65% yield (Scheme 14).

Scheme 14: Synthesis of Phenyl propyl bromide 21

\[
\begin{align*}
\text{HO-} & + & \text{Br-} & \text{CH}_3\text{CN, K}_2\text{CO}_3 \\
20 & & 12 & 60^\circ \text{C, 18 hr} \\
\end{align*}
\]

Finally, we were interested in forming the side chain of glycine substrate 10 (GS-10) analog. To that end, the alkylation reaction was performed with m-cresol 22 and K\textsubscript{2}CO\textsubscript{3} to yield pure phenyl propyl bromide 23 in 66% yield after column chromatography (Scheme 15).

Scheme 15: Synthesis of phenyl propyl bromide 23

\[
\begin{align*}
\text{HO-} & + & \text{Br-} & \text{CH}_3\text{CN, K}_2\text{CO}_3 \\
22 & & 12 & 60^\circ \text{C, 18 hr} \\
\end{align*}
\]
2.5.2 Coupling of two synthons

The next step in the parallel synthetic approach is coupling of the secondary amine derivatives (Synthon A) and the phenoxy alkyl halides (Synthon B). The two synthons are coupled via a C-N bond formed by nucleophilic displacement of bromide with the secondary amine to yield the penultimate compounds of the desired bisubstrate target molecules. Under acidic hydrolytic reaction conditions deblocking of the penultimate compounds led to the synthesis of bisubstrate target molecules. First coupling of two synthons was performed using secondary amine derivative 6, 1.5 equivalents of phenoxy alkyl bromide 16 in acetone at 50 °C for 24 hours (entry 1, Table 2.2) which resulted in penultimate compound 24 in 35% yield after column chromatographic purification. In an attempt to increase the yield we then tried the coupling reaction using the same conditions as entry 1 but increasing the reaction time to 48 hours (entry 2, Table 2.2). This resulted in penultimate compound 24 in 72% yield after column chromatographic purification (Scheme 16).

Scheme 16: Synthesis of tertiary amine alkyl phenyl derivative 24
Table 2.2. Optimizing the synthesis of tertiary amine alkyl phenyl derivative

<table>
<thead>
<tr>
<th>Entry</th>
<th>Time (h)</th>
<th>Solvent</th>
<th>Yield % (after column purification)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>Acetone</td>
<td>35</td>
</tr>
<tr>
<td>2</td>
<td>48</td>
<td>Acetone</td>
<td>72</td>
</tr>
</tbody>
</table>

Applying the above optimized conditions, the requisite phenyl alkyl halides for GS-2, GS-8, GS-9 and GS-10 target compounds were then coupled with secondary amine derivative 6 to form the corresponding penultimate compounds (Scheme 17). Phenyl alkyl halides 19, 21 and 23 were treated with 1.5 equivalents of secondary amine derivative 6 in dry acetone at 55 °C for 48 hours to yield penultimate intermediates 25, 26 and 27 in 70%, 75% and 70% respectively after column chromatographic purification.

Scheme 17: Synthesis of tertiary amine alkyl phenyl derivative 25, 26 and 27

19, \( R = F \)  
21, \( R = Cl \)  
23, \( R = Me \)  
25, \( R = F, 70\% \)  
26, \( R = Cl, 75\% \)  
27, \( R = Me, 70\% \)
2.5.3 Hydrolysis

The next step in the synthesis is to produce the final target compounds GS-2, GS-8, GS-9 and GS-10. An acidic hydrolytic reaction conditions were employed to convert each penultimate compounds to the bisubstrate target molecules. Secondary amine derivative 6 was dissolved in THF, H\text sub{2}O and excess amount of 1N HCl was added, refluxed at 80 °C for 18 hours and resulted in 100% conversion of starting material to compound 28. After completion, the solvents were evaporated using lyophilizer to obtain the hydrochloride salt of compound 28 as a white solid in 85% yield (Scheme 18).

**Scheme 18: Synthesis of target compound S-1**

Similar conditions were employed to synthesize the target compound GS-2. Penultimate intermediate 24 was dissolved in THF, H\text sub{2}O and excess amount of 1N HCl was added, refluxed at 80 °C for 18 hours, resulting in 100% conversion of starting material to target compound GS-2. After completion, the solvents were evaporated using lyophilizer to obtain hydrochloride salt of compound GS-2 as white solid in 83% yield (Scheme 19).
The optimized acid hydrolytic conditions for forming the target compound GS-2 were then successfully used to synthesize target compounds GS-8, GS-9 and GS-10. The penultimate intermediate compounds (methyl esters) 25, 26 and 27 were treated under the same conditions (excess HCl, THF, water and stirring at room temperature for 18 hours) to form target compounds GS-8, GS-9 and GS-10 in 85%, 80% and 83% yields, respectively (Scheme 20).

Based on the analysis of active site subtleties and molecular modeling studies a series of compounds were designed aiming to fill two regions within the active site. The
Surflex-Dock results were provided by our group member Dr. Amarjit Luniwal (Table 1.2). We utilized these results to prioritize the synthesis of the following analogs: GS-2, GS-8, GS-9 and GS-10, which were selected for synthesis and eventual biological testing. GS-2, GS-8 and GS-9 were selected because they have substantially different predicted scores between the two enzyme forms, Gram-positive *S.pneumoniae* and Gram-negative *V.cholerae*. Additionally, all four compounds have the same number of carbon atoms, the chain length between the two carboxyls are the same and also the alkyl chain length of the side chains are identical in the four analogs. The substituent at position 3 of the phenyl ring was varied in the four selected analogs. The biological results showed that each of these analogs have good inhibition (lower millimolar K<sub>i</sub> values, Table 2.3). Based on these results we have been able to improve from no activity (analog S1) to a low millimolar inhibitor activity. GS-8 (fluoro phenyl alkyl derivative) was identified as the best inhibitor and GS-8 and GS-11 also showed some selectivity between *Sp* and *Vc* forms of ASADH (Table 6). Enzymatic assay conditions were provided in experimental part.
Table 2.3. $K_i$ values for Bisubstrate analogs

<table>
<thead>
<tr>
<th>Structure</th>
<th>Name</th>
<th>Experimental $K_i$ (mM)</th>
<th>sp ASADH</th>
<th>vc ASADH</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Structure GS-9" /></td>
<td>GS-9</td>
<td>0.42 ± 0.04</td>
<td>0.54 ± 0.07</td>
<td></td>
</tr>
<tr>
<td><img src="image2.png" alt="Structure GS-11" /></td>
<td>GS-11</td>
<td>0.40 ± 0.07</td>
<td>0.60 ± 0.08</td>
<td></td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure GS-8" /></td>
<td>GS-8</td>
<td>0.18 ± 0.03</td>
<td>0.29 ± 0.05</td>
<td></td>
</tr>
<tr>
<td><img src="image4.png" alt="Structure GS-2" /></td>
<td>GS-2</td>
<td>0.66 ± 0.12</td>
<td>0.56 ± 0.10</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 3

Summary

For the synthesis of bisubstrate analog inhibitors of ASADH we employed a direct synthetic approach. First, the N-benzylglycine methyl ester 3 was synthesized from commercially available starting materials with 77% yield. Next it was converted to tertiary amine by coupling with bromo propionate 4 in 67% yield. In order to attach the side chain of bisubstrate analogs tertiary amine 5 needed to be debenzylated. The benzyl protecting group was removed using palladium as catalyst, resulting in secondary amine derivative 6 (Synthon A) in 92% yield. Next the requisite carbons were attached to secondary amine derivative 6 using bromopropanol as alkylating reagent. However, even with numerous attempts, the secondary amine derivative was unable to be alkylated using bromopropanol as alkylating agent. Alkylation was next attempted using dibromopropane. Unfortunately, alkylation of secondary amine derivative using dibromopropane resulted in only 10% yield of tertiary amine alkyl bromide 15 (Scheme 10). Because of these low yield issues, a parallel synthesis of phenyl alkyl halides (Synthon B) and secondary amine derivative (Synthon A) was used. First phenyl alkyl bromide was synthesized by phenol 16 and dibromopropane 12 in 65% yield. In the second step, secondary amine derivative 6 (synthon A) was coupled with phenyl alkyl bromide (synthon B) to form tertiary amine alkyl bromide 24 in 72% yield. Finally acid hydrolytic conditions were employed to convert penultimate intermediate, tertiary amine alky bromide to hydrochloride salt of the target compound GS-2 in 83% yield. GS-2 was synthesized in
six steps with an excellent overall yield of 81%. By applying similar conditions, target bisubstrate analogs GS-8, GS-9, GS-10 and S-1 analogue were also synthesized in good yields (Scheme 21)

Unfortunately, the experimental $K_i$ values measured for the synthesized compounds did not achieve the expected level of affinity that was predicted through docking studies. Also, significant selectivity has not yet been achieved between $Sp$ and $Vc$ forms of ASADH. The predicted set of scores generated through Surflex dock was based on maximizing the number of electrostatic interactions between the ligand and the enzyme active site. However it appears that some of these predicted interactions did not form, perhaps because of the ligand conformational flexibility. In support of this hypothesis, studies conducted by Sotriffer et al. revealed that the success rate in identification of a correct pose of a ligand can drop to below half as the number of rotatable bonds increases. Their findings illustrate the difficulty in finding correct poses for highly flexible ligands.

Also we did not observe expected level of selectivity between the two enzyme forms for the synthesized bisubstrate analogues. The reason for this lack of selectivity could be because of different orientations of ligand binding in the active site than the predicted pose. In those cases ligand binding could not take advantage of possible interactions in the extra binding pocket in $Vc$ ASADH relative to $Sp$ ASADH. In addition, the study conducted by Govern et al. demonstrated that even subtle protein conformational changes that result from ligand binding were sufficient to significantly influence the quality of docking results, which further supports the observed weak correlation between docking and experimental results.
Scheme 21: Synthesis of GS-2, GS-8, GS-9, GS-10 and S-1

**GS-2: 81% overall yield**

1. EtN (2eq), THF, rt, 18 hr, 77%
2. HN\(\text{Br}\)
3. NaHCO\(_3\), MeOH, 60 °C, 18 hr (reflux), 67%
4. Pd/C, drop Acetic acid, H\(_2\), 35 psi, 92%

**GS-8: 79.5% overall yield**

1. EtN (2eq), THF, rt, 18 hr, 77%
2. HN\(\text{Br}\)
3. NaHCO\(_3\), MeOH, 60 °C, 18 hr (reflux), 67%
4. Pd/C, drop Acetic acid, H\(_2\), 35 psi, 92%
**GS-9 : 77% overall yield**

1. **2**
   - **Reagents & Conditions:** Et$_3$N (2eq), THF
   - **Yield:** rt, 18 hr, 77%

2. **3**
   - **Reagents & Conditions:** NaHCO$_3$, MeOH
   - **Yield:** 60 °C, 18 hr (reflux), 69%

3. **5**
   - **Reagents & Conditions:** Pd/C, 1 drop Acetic acid
   - **Yield:** H$_2$, 35 psi, 92%

---

**GS-10 : 79% overall yield**

1. **2**
   - **Reagents & Conditions:** Et$_3$N (2eq), THF
   - **Yield:** rt, 18 hr, 77%

2. **3**
   - **Reagents & Conditions:** NaHCO$_3$, MeOH
   - **Yield:** 60 °C, 18 hr (reflux), 67%

3. **5**
   - **Reagents & Conditions:** Pd/C, 1 drop Acetic acid
   - **Yield:** H$_2$, 35 psi, 92%

---

**S-1 : 79% overall yield**

1. **2**
   - **Reagents & Conditions:** Et$_3$N (2eq), THF
   - **Yield:** rt, 18 hr, 77%

2. **3**
   - **Reagents & Conditions:** NaHCO$_3$, MeOH
   - **Yield:** 60 °C, 18 hr (reflux), 67%

3. **5**
   - **Reagents & Conditions:** Pd/C, 1 drop Acetic acid
   - **Yield:** H$_2$, 35 psi, 92%

---

4. **S-1**
   - **Reagents & Conditions:** THF, HCl
   - **Yield:** 80 °C, 18 h, 85%
Chapter 4

Experimental

4.1 General Procedures

All commercially available compounds were purchased from Aldrich Chemical Company or Acros and used as received unless otherwise specified. Anhydrous solvents were purchased from commercial sources and were used without additional purification except for: (i) Acetone (Me$_2$CO) which was further dried over 3 Å molecular sieves; and (ii) Tetrahydrofuran (THF) which was further distilled under nitrogen over sodium-benzophenone. Normal-phase flash and gravity column chromatography were performed using silica gel (200-425 mesh 60 Å pore size) and ACS grade solvents (Fisher). TLC analyses were performed on silicagel 250 μm fluorescent TLC plates (Baker-flex, Silica Gel IB-F from VWR International, LLC). All $^1$H NMR and $^{13}$C NMR spectra were obtained on either a Varian Inova-600 spectrometer at 600 MHz or a Unity-400 spectrometer at 400 MHz. Peak locations were referenced using either tetramethylsilane (TMS) or residual nondeuterated solvent as an internal standard. Proton coupling constants are expressed in Hertz. All NMR spectra were obtained at room temperature. Spin multiplicity for $^1$H NMR are reported using the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broadened, dd = doublet doublet,
dt = doublet triplet, dq = doublet quartet. All mass spectrometry data were obtained using an Esquire Ion trap mass spectrometer.

**4.2 Synthesis of Secondary Amine Derivative 6 or Synthon A**

**4.2.1 N-benzylglycine methyl ester 3**

\[
\text{O} \quad \text{Br} \quad + \quad \text{H}_2\text{N} \quad \text{Et}_3\text{N} (2\text{eq}), \text{THF} \quad \text{rt, 18 hr} \quad \text{O} \quad \text{H} \quad \text{N} \quad \text{O} \quad 77\%
\]

To a solution of methylbromoacetate 2 (7.66 g, 50 mmol, 4.74 mL) in 50 mL THF, triethylamine (13.9 mL, 10.1 g, 100 mmol) was added under argon, followed by addition of benzyl amine 2 (52 mmol, 5.69 mL). The solution was stirred for 18 h at room temperature. The reaction mixture was filtered to remove precipitated triethyl ammonium bromide and concentrated *in vacuo* to yield crude N-benzylglycine methyl ester 3. The crude product was purified via flash column chromatography (1:1, EtOAc: hexane) to give pure N-benzyl glycine methyl ester 3 as a yellow oily residue (8.37 g, 47 mmol, 93%). TLC \( R_f = 0.33 \) [EtOAc: hexane (1:1)]. \(^1\text{H} \text{NMR} (400 \text{ MHz}, \text{CDCl}_3)\): \( \delta = 2.03 \) (bs, NH), 3.37 (2 H, s), 3.67 (3 H, s), 3.76(2 H, s), 7.29 (5 H, m). \(^{13}\text{C} \text{NMR} (100 \text{ MHz}, \text{CDCl}_3)\): \( \delta = 49.9, 51.4, 53.1, 126.8, 127.8, 127.9, 139.2, 172.5 \). The spectral data of known compound 3 is identical to the reference [24].
4.2.2 Tertiary amine derivative 5

To a solution of N-benzylglycinemethyl ester 4 (1 g, 5.57 mmol) in methanol (5 mL), excess NaHCO$_3$ (0.56 g, 6.7 mmol) was added under argon. To this suspension bromomethyl propionate 4 (0.93 g, 5.57 mmol, 0.61 mL) in 2 mL methanol was added over 10 min at 50 °C. The mixture was stirred at 60 °C for 18 h. The solution was concentrated in vacuo and the residue was dissolved in a mixture of H$_2$O/EtOAc. The aqueous phase was extracted with EtOAc (2x 10 mL), and the combined organic phase was extracted with 1N HCl (3x 10 mL). The combined aqueous phase was washed with Et$_2$O before neutralizing with 6N NaOH. The free amines were extracted with EtOAc (4x 20 mL). The combined organic phase was washed with H$_2$O, brine, dried over anhydrous Na$_2$SO$_4$ and evaporated to dryness under vacuum. The residue was purified using flash chromatography (1:2, EtOAc: hexane) to obtain 5 (0.89 g, 3.3 mmol, 67%) as a clear oil. TLC $R_f = 0.56$ [EtOAc: hexane (1:2)]. $^1$H NMR (600 MHz, CDCl$_3$): $\delta = 2.51$ (2 H, t, $J = 7.2$ Hz), 3.04 (2 H, t, $J = 7.2$ Hz), 3.40 (2 H, s), 3.66 (3 H, s), 3.68(3 H, s), 3.81 (2 H, s), 7.24 (1 H, m), 7.30 (4 H, m) (see A1, appendix A). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta =$ 33.5, 49.8, 51.6, 51.8, 53.9, 58.0, 127.5, 128.5, 129.0, 170.0, 171.9, 173.0 (see A2, appendix A). Electrospray MS calculated for C$_{14}$H$_{19}$NO$_4$ [M+Na]$^+$ 288.1; found 288.3 (see A3, appendix A).
4.2.3 Secondary amine derivative 6 or Synthon A

![Chemical Structure]

To a solution of compound 5 (0.47 g, 1.77 mmol) in ethyl acetate (15 mL), 1-2 drops of acetic acid was added. To this suspension 10% Pd/C (50 mg) was added and stirred at room temperature for 24 h under a hydrogen atmosphere (35 psi). After completion the reaction mixture was filtered through a pad of celite which was washed extensively with MeOH. The solvents were evaporated under vacuum to obtain compound 6 (0.28 g, 1.62 mmol, 92%) a white solid, which was used directly in the next step for the coupling with synthon B (17,19,21,23). TLC \( R_f = 0.22 \) [EtOAc: hexane (1:1)]. \(^1\)H NMR (200 MHz, CDCl\(_3\)): \( \delta = 3.74 \) (3 H, s), 3.70 (3 H, s), 3.47 (2 H, s), 3.23 (bs, NH), 2.94 (2 H, t, \( J = 6.6 \) Hz), 2.56 (2 H, t, \( J = 6.6 \) Hz) (see A4, appendix A). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \( \delta = 34.60, 44.90, 50.18, 50.72, 52.17, 172.45, 173.12 \) (see A5, appendix A). Electrospray MS calculated for C\(_7\)H\(_{13}\)NO\(_4\) [M+H]\(^+\) 176.2; found 176.4 (see A6, appendix A).
4.3 Synthesis of GS-2

4.3.1 Phenyl alkyl tertiary amine 24 or Coupling of two Synthons A and B

According to a known procedure, a solution of phenol (0.94 g, 10 mmol) in acetonitrile (25 ml) was added dropwise to a stirred mixture of 1, 3-dibromopropane (10.0 g, 50 mmol, 5.05 mL), K$_2$CO$_3$ (2.07 g, 15 mmol) and acetonitrile (25 mL) at room temperature. After the addition was completed the reaction mixture was stirred at room temperature for 2 h and then heated to 60 °C for 18 h. The mixture was filtered and concentrated in vacuo. The residue was dissolved in mixture of H$_2$O/EtOAc, the organic phase was washed with 2N NaOH (3x25 mL), H$_2$O, brine, dried over anhydrous Na$_2$SO$_4$ and concentrated under in vacuo. The residue was purified via column chromatography (1:30, EtOAc: petroleum ether) to give 17 as a yellow oil (1.40 g, 6.5 mmol, 65%). Partially purified 17 (synthon B) was taken to next step without further purification.

To a stirred solution of secondary amine derivative (0.02 g, 0.09 mmol) and K$_2$CO$_3$ (0.03 g, 0.2 mmol) in dry acetone, phenoxypropylbromide 17 (0.03 g, 0.13 mmol) obtained from the previous step was added slowly and the reaction mixture was stirred at 50 °C for 48 h. The mixture was filtered and concentrated in vacuo. The residue was
dissolved in H₂O/EtOAc. The organic phase was extracted with 1N HCl (4x5 mL). The combined aqueous phase was neutralized with 2N NaOH to pH about 7-8. The volume was reduced to one fourth of its original volume under vacuum and extracted with EtOAc. The combined organic phase was washed with H₂O, brine, dried over anhydrous Na₂SO₄ and evaporated to dryness under vacuum. Chromatography using hexanes-EtOAc (2:1, v/v) afforded the title compound as a light yellow oil (0.02 g, 0.07 mmol, 72%). TLC R₅ = 0.43 [EtOAc: hexane (1:2)]. ¹H NMR (600 MHz, CD₃OD): δ = 1.87 (2 H, p, J = 6.6 Hz), 2.46 (2 H, m, J = 7.2 Hz, J = 6.6 Hz), 2.76 (2 H, m, J = 7.2 Hz, J = 6.6 Hz), 2.93 (2 H, m, J = 7.2 Hz, J = 6.6 Hz), 3.40 (2 H, s), 3.61 (3 H, s), 3.66 (3 H, s), 3.99 (2 H, t, J = 6.6 Hz), 6.89 – 6.88 (m, 3 H), 7.25 – 7.23 (m, 2 H) (see A7, appendix A). ¹³C NMR (150 MHz, CD₃OD): δ = 27.3, 32.6, 49.8, 50.5, 50.7, 50.9, 54.2, 65.3, 114.3, 120.3, 129.2, 159.3, 172.1, 173.4 (see A8, appendix A). Electrospray MS calculated for C₁₆H₂₃NO₅ [M+H]⁺ 310.2; found 310.5 (see A9, appendix A).

4.3.2 Target compound GS-2

A solution of compound 24 (100 mg, 0.31 mmol) in THF (1 mL), water (2 mL) and 1 N HCl (0.7 mL) was stirred at 80 °C for 18 h. After completion the solvents were evaporated under vacuum to obtain GS-2 (81 mg, 0.26 mmol, 83%) as a light yellow oil.
$^1$H NMR (400 MHz, CD$_3$OD): $\delta = 2.27$ (2 H, m), 2.89 (2 H, t, $J = 6.8$ Hz), 3.56-3.52 (2 H, m), 3.63-3.62 (2 H, m), 4.12 (2 H, t, $J = 5.6$ Hz), 4.24 (1 H, s), 4.3 (1 H, s), 6.95 – 6.93 (m, 3 H), 7.27 – 7.26 (m, 2 H) (see A10, appendix A). $^{13}$C NMR (150 MHz, CD$_3$OD): $\delta =$ 23.9, 28.0, 50.7, 53.3, 53.5, 64.7, 114.4, 121.0, 129.3, 158.6, 166.7, 171.1 (see A11, appendix A). Electrospray MS in negative ion mode calculated for C$_{14}$H$_{20}$NO$_5$ [M-H]$^-$ 280.1; found 280.0 (see A12, appendix A).

4.4 Synthesis of GS-8

4.4.1 Phenyl alkyl tertiary amine 25 or Coupling of two Synthons A and B

According to a known procedure, 26 a solution of 3-fluorophenol 18 (0.5 g, 4.5 mmol) in acetone (15 ml) was added drop wise to a stirred mixture of 1, 3-dibromopropane 12 (4.5 g, 22 mmol, 2.3 mL), K$_2$CO$_3$ (0.92 g, 6.7 mmol) and acetonitrile (10 mL) at room temperature. After the addition was completed, the reaction mixture was stirred at room temperature for 2 h and then heated to 60 °C for 18 h. The mixture was filtered and concentrated in vacuo. The residue was dissolved in mixture of H$_2$O/EtOAc, the organic phase was washed with 2N NaOH (3x15 mL), H$_2$O, brine, dried over anhydrous Na$_2$SO$_4$ and concentrated under in vacuo. The residue was purified via column chromatography (1:30, EtOAc: petroleum ether) to give 19 as a yellow oil (0.65 g, 2.8
mmol, 63%). Partially purified 19 (synthon B) was taken to next step without further purification.

To a stirred solution of secondary amine derivative 6 (0.35 g, 2 mmol) and K$_2$CO$_3$ (0.5 g, 4.0 mmol) in dry acetone, fluoro phenoxypropylbromide 19 (0.70 g, 2.9 mmol) obtained from the previous step was added slowly and the reaction mixture was stirred at 50 °C for 48 h. The mixture was filtered and concentrated *in vacuo*. The residue was dissolved in H$_2$O/EtOAc. The organic phase was extracted with 1N HCl (4x5 mL). The combined aqueous phase was neutralized with 2N NaOH to pH about 7-8. The volume was reduced to one fourth of its original volume under vacuum and extracted with EtOAc. The combined organic phase was washed with H$_2$O, brine, dried over anhydrous Na$_2$SO$_4$ and evaporated to dryness under vacuum. Chromatography using hexanes-EtOAc (2:1, v/v) afforded the title compound as a yellow oil (0.46 g, 1.4 mmol, 70%). TLC $R_f$ = 0.42 [EtOAc: hexane (1:2)]. $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ = 1.91 (2 H, p, $J$ = 6.6 Hz), 2.48 (2 H, m, $J$ = 6.6 Hz, $J$ = 7.2 Hz), 2.82 (2 H, t, $J$ = 6 Hz ), 3.00 (2 H, t, $J$ = 6.6 Hz), 3.41 (2 H, s), 3.64 (3 H, s), 3.68 (3 H, s), 3.99 (2 H, t, $J$ = 6.6 Hz), 6.94 – 6.88 (m, 3 H), 7.28 – 7.26 (m, 1 H) (see A13, appendix A). $^{13}$C NMR (150 MHz, CD$_3$OD): $\delta$ = 27.1, 32.6, 49.8, 50.4, 50.7, 50.8, 54.2, 65.8, 101.6, 106.9, 110.3, 130.1, 162.6, 165.0, 172.1, 173.4 (see A14, appendix A). Electrospray MS calculated for C$_{16}$H$_{22}$FNO$_5$ [M+H]$^+$ 328.16; found 328.5 (see A15, appendix A).
4.4.2 Target compound GS-8

A solution of 25 (100 mg, 0.30 mmol) in THF (1 mL), water (2 mL) and 1 N HCl (0.7 mL) was stirred at 80 °C for 18 h. After completion the solvents were evaporated under vacuum to obtain GS-8 (83 mg, 0.25 mmol, 85%) as a yellow oil. $^1$H NMR (400 MHz, CD$_3$OD): $\delta = 2.28$ (2 H, m), 2.90 (2 H, t, $J = 7.2$ Hz), 3.53 (2 H, t, $J = 7.6$ Hz), 3.62 (2 H, t, $J = 6.8$ Hz), 4.13 (2 H, t, $J = 5.6$ Hz), 4.25 (2 H, s), 6.79 – 6.67 (m, 3 H), 7.30 – 7.24 (m, 1 H) (see A16, appendix A). $^{13}$C NMR (150 MHz, CDCl$_3$): $\delta = 23.8$, 28.0, 50.8, 53.2, 53.6, 65.3, 102.2, 107.5, 107.6, 103.3, 130.3, 130.4, 167.4, 172.3 (see A17, appendix A). Electrospray MS calculated for C$_{14}$H$_{13}$FNO$_5$ [M+H]$^+$ 300.1; found 300.0 (see A18, appendix A).
4.5 Synthesis of GS-9

4.5.1 Phenyl alkyl tertiary amine 26 or Coupling of two Synthons A and B

According to a known procedure, a solution of 3-chlororophenol 20 (1.3 g, 10 mmol) in acetonitrile (25 ml) was added drop wise to a stirred mixture of 1, 3-dibromopropane 12 (10.0 g, 50 mmol, 5.05 mL), K$_2$CO$_3$ (2.07 g, 15 mmol), and acetonitrile (25 mL) at room temperature. After the addition was completed, the reaction mixture was stirred at room temperature for 2 h and then heated to 60 °C for 18 h. The mixture was filtered and concentrated in vacuo. The residue was dissolved in mixture of H$_2$O/EtOAc, the organic phase was washed with 2N NaOH (3x25 mL), H$_2$O, brine, dried over anhydrous Na$_2$SO$_4$ and concentrated under in vacuo. The residue was purified via column chromatography (1:30, EtOAc: petroleum ether) to give 21 as a yellow oil (1.6 g, 6.5 mmol, 65%). Partially purified 21 (synthon B) was taken to next step without further purification.

To a stirred solution of secondary amine derivative 6 (0.3 g, 2.0 mmol) and K$_2$CO$_3$ (0.5 g, 4.0 mmol) in dry acetone, chloro phenoxypropylbromide 21 (0.7 g, 2.9 mmol) obtained from the previous step was added slowly and the reaction mixture was stirred at 50 °C for 48 h. The mixture was filtered and concentrated in vacuo. The residue
was dissolved in H₂O/EtOAc. The organic phase was extracted with 1N HCl (4x5 mL). The combined aqueous phase was neutralized with 2N NaOH to pH about 7-8. The volume was reduced to one fourth of its original volume under vacuum and extracted with EtOAc. The combined organic phase was washed with H₂O, brine, dried over anhydrous Na₂SO₄ and evaporated to dryness under vacuum. Chromatography using hexanes-EtOAc (2:1, v/v) afforded the title compound 26 as a yellow oil (0.51 g, 1.5 mmol, 75%). TLC Rᵢ = 0.40 [EtOAc: hexane (1:2)]. ¹H NMR (600 MHz, CDCl₃): δ = 1.87 (2 H, p, J = 6.6 Hz), 2.44 (2 H, m, J = 6.6 Hz, 7.2 Hz), 2.78 (2 H, m, J = 6.6 Hz, 7.2 Hz), 2.96 (2 H, m, J = 6.6 Hz, J = 7.2 Hz), 3.39 (2 H, s), 3.62 (3 H, s), 3.66 (3 H, s), 3.95 (2 H, t, J = 6 Hz), 6.89 – 6.74 (m, 3 H), 7.17 – 7.15 (m, 1 H) (see A19, appendix A). ¹³C NMR (150 MHz, CDCl₃): δ = 27.6, 33.4, 49.9, 50.5, 51.7, 51.8, 54.9, 65.9, 113.2, 114.9, 120.9, 130.4, 132.45, 159.9, 171.9, 173.1. ) (see A20, appendix A). Electrospray MS calculated for C₁6H₂₂ClNO₅ [M+H]⁺ 344.13; found 344.5 (see A21, appendix A).

4.5.2 Target compound GS-9

A solution of compound 26 (100 mg, 0.30 mmol) in THF (1 mL), water (2 mL) and 1 N HCl (0.7 mL) was stirred at 80 °C for 18 h. After completion the solvents were
evaporated under vacuum to obtain \textbf{GS-8} (81 mg, 0.25 mmol, 80\%) as a yellow oil. $^1$H NMR (600 MHz, CD$_3$OD): $\delta = 2.27$ (2 H, m), 2.90 (2 H, t, \(J = 7.2\) Hz), 3.53 (2 H, m, \(J = 7.2\) Hz, \(J = 7.8\) Hz), 3.62 (2 H, m, \(J = 6.6\) Hz, \(J = 7.2\) Hz), 4.13 (2 H, t, \(J = 5.4\) Hz), 4.2 (2 H, s), 7.00 – 6.90 (m, 3 H), 7.25 – 7.22 (m, 1 H) (see \textbf{A22}, appendix A). $^{13}$C NMR (150 MHz, CDCl$_3$): $\delta = 23.8, 28.0, 50.8, 53.2, 53.6, 65.2, 113.0, 114.8, 121.1, 130.4, 134.7, 159.4, 167.4, 172.3$ (see \textbf{A23}, appendix A). Electrospray MS calculated for C$_{14}$H$_{19}$ClNO$_5$ \([M+H]^+\) 316.1; found 316.5 (see \textbf{A24}, appendix A).

4.6 Synthesis of GS-10

4.6.1 Phenyl alkyl tertiary amine 27 or Coupling of two Synthons A and B

![Chemical Structure](image)

According to a known procedure,\textsuperscript{26} a solution of \textit{m}-cresol \textbf{22} (1.0 g, 10 mmol) in acetonitrile (25 ml) was added drop wise to a stirred mixture of 1, 3-dibromopropane \textbf{12} (10.0 g, 50 mmol, 5.05 mL), K$_2$CO$_3$ (2.0 g, 15 mmol), and acetonitrile (25 mL) at room temperature. After the addition was completed, the reaction mixture was stirred at room temperature for 2 h and then heated to 60 °C for 18 h. The mixture was filtered and concentrated \textit{in vacuo}. The residue was dissolved in mixture of H$_2$O/EtOAc, the organic phase was washed with 2N NaOH (3x15 mL), H$_2$O, brine, dried over anhydrous Na$_2$SO$_4$.
and concentrated under in vacuo. The residue was purified via column chromatography (1:30, EtOAc: petroleum ether) to give 23 as a yellow oil (1.5 g, 6.6 mmol, 66%).

Partially purified 23 (synthon B) was taken to next step without further purification

To a stirred solution of secondary amine derivative 6 (0.3 g, 2.0 mmol) and K$_2$CO$_3$ (0.5 g, 4.0 mmol) in dry acetone, methyl phenoxypyropylbromide 23 (0.6 g, 2.9 mmol) obtained from the previous step was added slowly and the reaction mixture was stirred at 50 °C for 48 h. The mixture was filtered and concentrated in vacuo. The residue was dissolved in H$_2$O/EtOAc. The organic phase was extracted with 1N HCl (4x5 mL). The combined aqueous phase was neutralized with 2N NaOH to pH about 7-8. The volume was reduced to one fourth of its original volume under vacuum and extracted with EtOAc. The combined organic phase was washed with H$_2$O, brine, dried over anhydrous Na$_2$SO$_4$ and evaporated to dryness under vacuum. Chromatography using hexanes-EtOAc (2:1, v/v) afforded the title compound 27 as a yellow oil (0.45 g, 1.4 mmol, 70%). TLC $R_f = 0.40$ [EtOAc: hexane (1:2)]. $^1$H NMR (400 MHz, CD$_3$OD): $\delta =$ 1.89 (2 H, p, $J = 6.6$ Hz), 2.32 (3 H, s), 2.47 (2 H, m, $J = 6.6$ Hz, 7.2 Hz), 2.80 (2 H, m, $J = 6.6$ Hz, 7.2 Hz), 2.99 (2 H, m, $J = 6.6$ Hz, $J = 7.2$ Hz), 3.40 (2 H, s), 3.64 (3 H, s), 3.68 (3 H, s), 3.97 (2 H, t, $J = 6$ Hz), 6.75 – 6.68 (m, 3 H), 7.16 – 7.13 (m, 1 H) (see A25, appendix A). $^{13}$C NMR (150 MHz, CDCl$_3$): $\delta =$ 21.7, 27.8, 33.4, 49.9, 50.7, 51.6, 51.8, 54.9, 65.5, 111.5, 115.5, 121.5, 129.3, 139.6, 159.2, 172.0, 173.1 (see A26, appendix A). Electrospray MS calculated for C$_{17}$H$_{25}$NO$_5$ [M+H]$^+$ 324.2; found 324.6 (see A27, appendix A).
4.6.2 Target compound GS-11

![Chemical structure of GS-11](image)

A solution of compound 27 (100 mg, 0.30 mmol) in THF (1 mL), water (2 mL) and 1 N HCl (0.7 mL) was stirred at 80 °C for 18 h. After completion the solvents were evaporated under vacuum to obtain GS-11 (82 mg, 0.25 mmol, 80%) as a yellow oil material. $^1$H NMR (600 MHz, CD$_3$OD): $\delta = 2.26$ (2 H, m), 2.30 (3 H, s), 2.94 – 2.87 (2 H, m), 3.56 – 3.52 (2 H, m), 3.68 – 3.61 (2 H, m), 4.1 (2 H, m), 4.23 (1 H, s), 4.31 (1 H, s), 6.76 – 6.71 (m, 3 H), 7.14 – 7.10 (m, 1 H) (see A28, appendix A). $^{13}$C NMR (150 MHz, CD$_3$OD): $\delta = 20.3, 23.9, 26.0, 50.7, 53.3, 54.7, 64.8, 111.3, 115.1, 121.8, 129.1, 140.0, 158.6, 169.0, 172.4$ (see A29, appendix A). Electrospray MS calculated for C$_{15}$H$_{22}$NO$_5$ [M+H]$^+$ 296.2; found 296.5 (see A30, appendix A).

4.7 Synthesis of S-1

![Chemical structure of S-1](image)
A solution of compound 6 (50 mg, 0.30 mmol) in THF (1 mL), water (2 mL) and 1 N HCl (0.7 mL) was stirred at 80 °C for 18 h. After completion the solvents were evaporated under vacuum to obtain S-1 (40 mg, 0.22 mmol, 70%) as a yellow oil. $^1$H NMR (600 MHz, DMSO): $\delta = 2.73$ (2 H, m), 3.14 (2 H, m), 3.88 (2 H, s), 9.21 (NH, bs) (see A31, appendix A). $^{13}$C NMR (150 MHz, CDCl3): $\delta = 30.2, 42.4, 46.95, 168.2, 172.0$ (see A32, appendix A). Electrospray MS calculated in negative ion mode for C$_5$H$_8$NO$_4$ [M+H]$^+$ 148.0; found 148.0 (see A1, appendix A).

4.8 Enzymatic assay

The ASADHs from S. pneumoniae and V. cholerae were cloned, expressed, and purified following our published literature procedures. After concentration the enzyme was stored in 50 mM HEPES (pH 7) containing 1 mM EDTA and dithiothreitol (DTT) at -20 °C. ASADH catalyzes the formation of aspartyl semialdehyde (ASA) by reductive dephosphorylation of aspartyl phosphate as shown in Figure 1-1. ASADH is most conveniently assayed in the “reverse” biosynthetic direction because of the instability of the substrate aspartyl phosphate. This non-physiological reaction (Figure 4-1) is followed by monitoring the increase in the absorbance of NADPH at 340 nm.
**Figure 4-1.** Assay procedure for ASADH. The production of NADPH was monitored spectrophotometrically at 340 nm.

All the kinetic analyses of the synthesized bisubstrate enzyme inhibitors were conducted by our group member Bharani Thangavelu. The assay was performed as follows: 120 mM (pH 8.6) CHES buffer, 200 mM KCl, 1 mM ASA, 1.5 mM NADP and 20 mM phosphate were added to a 96-well plate at room temperature. The reaction was initiated by adding 30 µL of enzyme ASADH solution to the mixture of assay buffer and substrates.

The synthesized bisubstrate analogues (GS-9, GS-11, GS-8 and GS-2) were then tested for inhibition. To determine the inhibition constant ($K_i$) of each bisubstrate inhibitor, compounds were incorporated into each well in a specific row with the concentrations varied by serial dilution. The initial velocities and substrate concentrations (Figure 4-2) were fitted to the Dixon equation\(^\text{28}\) to determine $K_i$ values (Figure 4-3).
Figure 4-2. Enzyme assay by various concentrations of bisubstrate analogue GS-8 (○ uninhibited reaction; □ 4 mM; Δ 2 mM; ◊ 1 mM; ● 0.5 mM; ■ 0.25 mM; ▲ 0.125 mM; ♦ 0.0625 mM). The kinetic data represents the increase in the inhibitory effect as the concentration of the inhibitor increases.

Figure 4-3. A Dixon Plot of 1/Vmax versus Inhibitor (GS-8) concentration [I].
References


Appendix A

Supporting information for chapter 4

A1. $^1$H NMR of $N$-benzyl tertiary amine derivative 5 .................................................. 63

A2. $^{13}$C NMR NMR of $N$-benzyl tertiary amine derivative 5 ........................................ 64

A3. Mass spectrum (ESI-MS) of $N$-benzyl tertiary amine derivative 5 ($m/z = 288.3$ $[M+Na]^+$) ......................................................................................................................... 65

A4. $^1$H NMR of Secondary amine derivative 6 or Synthon A ............................................ 66

A5. $^{13}$C NMR of Secondary amine derivative 6 or Synthon A .......................................... 67

A6. Mass spectrum (ESI-MS) of Secondary amine derivative 6 or Synthon A ($m/z = 176.4$ $[M+H]^+$) ......................................................................................................................... 68

A7. $^1$H NMR of Phenyl alkyl tertiary amine 24 .................................................................. 69

A8. $^{13}$C NMR of Phenyl alkyl tertiary amine 24 ................................................................. 70

A9. Mass spectrum (ESI-MS) of Phenyl alkyl tertiary amine 24 ($m/z = 310.0$ $[M+H]^+$) . 71

A10. $^1$H NMR of Target compound GS-2 ........................................................................... 72

A11. $^{13}$C NMR of Target compound GS-2 ......................................................................... 73

A12. Mass spectrum (ESI-MS) of Target compound GS-2 ($m/z = 280.0$ $[M-H]^-$) ....... 74

A13. $^1$H NMR of Phenyl alkyl tertiary amine 25 ................................................................. 75
A14. $^{13}$C NMR of Phenyl alkyl tertiary amine 25 ..............................................................76

A15. Mass spectrum (ESI-MS) of Phenyl alkyl tertiary amine 25 ($m/z = 328.5 \ [\text{M+H}^+]$) ..............................................................77

A16. $^{1}$H NMR of Target compound GS-8 ..............................................................78

A17. $^{13}$C NMR of Target compound GS-8 ..............................................................79

A18. Mass spectrum (ESI-MS) of Target compound GS-8 ($m/z = 300.0 \ [\text{M+H}^+]$) ....80

A19. $^{1}$H NMR of Phenyl alkyl tertiary amine 26 ..............................................................81

A20. $^{13}$C NMR of Phenyl alkyl tertiary amine 26 ..............................................................82

A21. Mass spectrum (ESI-MS) of Phenyl alkyl tertiary amine 26 ($m/z = 344.5 \ [\text{M+H}^+]$) ..............................................................83

A22. $^{1}$H NMR of Target compound GS-9 ..............................................................84

A23. $^{13}$C NMR of Target compound GS-9 ..............................................................85

A24. Mass spectrum (ESI-MS) of Target compound GS-9 ($m/z = 316.5 \ [\text{M+H}^+]$) ....86

A25. $^{1}$H NMR of Phenyl alkyl tertiary amine 27 ..............................................................87

A26. $^{13}$C NMR of Phenyl alkyl tertiary amine 27 ..............................................................88

A27. Mass spectrum (ESI-MS) of Phenyl alkyl tertiary amine 27 ($m/z = 324.6 \ [\text{M+H}^+]$) ..............................................................89

A28. $^{1}$H NMR of Target compound GS-11 ..............................................................90

A29. $^{13}$C NMR of Target compound GS-11 ..............................................................91
A30. Mass spectrum (ESI-MS) of Target compound GS-11 ($m/z = 296.5 \ [M+H]^+$)........92

A31. $^1$H NMR of Target compound S-1 .................................................................................................................93

A32. $^{13}$C NMR of Target compound S-1 ................................................................................................................94

A 33. Mass spectrum (ESI-MS) of Target compound S-1($m/z = 148.1 \ [M+H]^+$).................95
A1. $^1$H NMR of N-benzyl tertiary amine derivative 5

![NMR spectrum of N-benzyl tertiary amine derivative 5]
A2. \[^{13}\text{C}\] NMR NMR of \(N\)-benzyl tertiary amine derivative 5

![Carbon-13 NMR spectrum of N-benzyl tertiary amine derivative 5]
A3. Mass spectrum (ESI-MS) of N-benzyl tertiary amine derivative 5 \((m/z = 288.3 \ [M+Na]^+)\)

![Mass spectrum of N-benzyl tertiary amine derivative 5](image)
A4. $^1$H NMR of Secondary amine derivative 6 or Synthon A

![Chemical Structure](hm-i-45pure.esp)
A5. $^{13}$C NMR of Secondary amine derivative 6 or Synthon A
A6. Mass spectrum (ESI-MS) of Secondary amine derivative 6 or Synthon A ($m/z = 176.4$ [M+H]−)
A7. $^1$H NMR of Phenyl alkyl tertiary amine 24

Chemical Shift (ppm)
A8. $^{13}$C NMR of Phenyl alkyl tertiary amine 24
A9. Mass spectrum (ESI-MS) of Phenyl alkyl tertiary amine 24 ($m/z = 310.0$ [M+H]$^+$)
A10. $^1$H NMR of Target compound GS-2
A11. $^{13}$C NMR of Target compound GS-2
A12. Mass spectrum (ESI-MS) of Target compound GS-2 ($m/z = 280.0$ [M-H]$^-$)
A13. $^1$H NMR of Phenyl alkyl tertiary amine 25

Chemical Shift (ppm)
A14. $^{13}$C NMR of Phenyl alkyl tertiary amine 25
A15. Mass spectrum (ESI-MS) of Phenyl alkyl tertiary amine 25 ($m/z = 328.5$ [M+H]$^+$)
A16. $^1H$ NMR of Target compound GS-8

Chemical Shift (ppm)
A17. $^{13}$C NMR of Target compound GS-8
A18. Mass spectrum (ESI-MS) of Target compound GS-8 ($m/z = 300.0$ [M+H]$^+$)
A19. $^1$H NMR of Phenyl alkyl tertiary amine 26

Chemical Shift (ppm)
A20. $^{13}$C NMR of Phenyl alkyl tertiary amine 26
A21. Mass spectrum (ESI-MS) of Phenyl alkyl tertiary amine 26 \((m/z = 344.5 \,[M+H]^+)\)
A22. $^1$H NMR of Target compound GS-9
A23. $^{13}$C NMR of Target compound GS-9
A24. Mass spectrum (ESI-MS) of Target compound GS-9 ($m/z = 316.5$ [M+H]$^+$)
A25. $^1$H NMR of Phenyl alkyl tertiary amine 27
A26. $^{13}$C NMR of Phenyl alkyl tertiary amine 27

![Chemical Structure](image)

![NMR Spectrum](image)
A27. Mass spectrum (ESI-MS) of Phenyl alkyl tertiary amine 27 \( (m/z = 324.6 \ [M+H]^+) \)
A28. $^1$H NMR of Target compound GS-11
A29. $^{13}$C NMR of Target compound GS-11
A30. Mass spectrum (ESI-MS) of Target compound GS-11 (m/z = 296.5 [M+H]+)
A31. $^1$H NMR of Target compound S-1
A32. $^{13}$C NMR of Target compound S-1
Mass spectrum (ESI-MS) of Target compound S-1 ($m/z = 148.1$ [M+H]$^+$)