Development of Bacterial Quorum Sensing Inhibitors and Molecular Probes

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ABSTRACT

Bacterial quorum sensing is regarded as a novel target for the design of antimicrobials. Based on lead structures identified from HTS, 39 analogues have been synthesized and evaluated in Vibrio harveyi. Potent inhibitors with IC_{50} values at single-digit micromolar concentrations for AI-2 mediated quorum sensing have been identified. On the second project, post-synthesis modifications of DNA provide easy functionalizations for expanded applications such as aptamer selection. A CBT-modified thymidine analogue (CBT-TTP) has been synthesized and used for enzymatic incorporation into DNA. Post-synthesis modifications through condensation with 1,2-aminothiol for installation of a boronic acid moiety or a fluorophore have been achieved. On the third project, H_{2}S has been recognized as an important gas-transmitter and its concentration is relevant to a variety of diseases. A novel fluorescent probe (DNS-Az) has been developed for quantitation of H_{2}S in aqueous solutions. This probe has been used to measure H_{2}S concentrations in the blood.

INDEX WORDS: Bacterial quorum sensing inhibitors, AI-2, V. harveyi, dUTP analogue incorporation, DNA modification, Bioorthogonal reaction, Hydrogen sulfide, Fluorescent probe
DEVELOPMENT OF BACTERIAL QUORUM SENSING INHIBITORS
AND MOLECULAR PROBES

by

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December 2012
DEDICATION

I want to dedicate this dissertation to my grandma (Weiying Ma), my parents (Bin Peng and Tianzhen Qi) and my husband (Chao Yang). It was your love and support that made my life so wonderful. You are and will always be the most important people in my life.
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1 DESIGN AND SYNTHESIS OF NOVEL SMALL-MOLECULE INHIBITORS OF BACTERIAL QUORUM SENSING

Abstract:

The development of new antimicrobial agents is of great importance due to the evolution of drug resistance. Bacterial quorum sensing has received much attention in recent years because of its relevance to pathological events such as biofilm formation and virulence factor expression. Based on the structures of two lead inhibitors (IC$_{50}$: 35-55 µM) against AI-2 mediated quorum sensing identified through virtual screening, we have synthesized 39 analogs and examined their inhibitory activities. Twelve of these new analogs showed equal or better inhibitory activities compared with the lead inhibitors. The best compound showed an IC$_{50}$ of about 6 µM in a whole cell assay using *Vibrio harveyi* as the model organism. The structure-activity relationship has been discussed.

1.1 Introduction

1.1.1 Bacterial quorum sensing pathways

It is known that bacteria can coordinate community wide activities upon environmental stimulation, behave like multi-cellular organisms in some sense, and thus adapt themselves to the changing environmental conditions by taking advantage of an intercellular communication process known as quorum sensing. Quorum sensing is regulated by the production and detection of small signaling molecules called autoinducers (AIs). When the environmental concentration of AIs, which is correlated to the bacterial population density, reaches a certain threshold, quorum sensing is activated. The activation of AI receptors can then modulate the expression of a variety of genes and consequently control the behavior of bacterial processes such as bioluminescence, biofilm formation, virulence factor expression, conjugation, sporulation, and swarming motility.
Several major types of small molecules are used as autoinducers in bacterial quorum sensing. For instance, acyl homoserine lactones (AHLs) are mostly used by Gram-negative bacteria\textsuperscript{7} and autoinducing peptides (AIPs) are used in Gram-positive bacteria.\textsuperscript{9} Among all autoinducers reported, autoinducer-2 (AI-2) seems to mediate quorum sensing in a remarkably wide range of bacteria including Gram-negative bacteria, such as \textit{H. influenzae}, \textit{V. harveyi}, \textit{E. coli}, and \textit{S. typhimurium}, and Gram-positive bacteria, such as \textit{B. subtilis}, \textit{B. anthracis}, and \textit{B. burgdorferi}.\textsuperscript{10} As a result, it was proposed that AI-2 could serve as an “universal signal”, which affects interspecies communications among bacteria.\textsuperscript{10} The causative agent of the disease cholera, human pathogen \textit{Vibrio cholerae}, also possesses the AI-2 mediated quorum sensing pathway.\textsuperscript{11,12} Related research has indicated that quorum sensing can control virulence factor expression and biofilm formation in \textit{V. cholerae}.\textsuperscript{11,13}

Because quorum sensing is involved in the regulation of pathologically relevant events, it is conceivable that inhibitors of quorum sensing could have therapeutic applications. In addition, selective inhibition of quorum sensing pathways without killing bacteria is less likely to exert evolutionary pressure for bacteria to undergo rapid mutation and develop drug resistance. Furthermore, quorum sensing inhibitors are important research tools in mechanistic studies. Recent years have seen an increasing interest in quorum sensing inhibitor/antagonist and agonist development.\textsuperscript{1,3,14-33}

### 1.1.2 Inhibition of AI-2 mediated bacterial quorum sensing

AI-2 mediated quorum sensing is observed in a broad range of bacteria, including Gram-positive and Gram-negative bacteria. AI-2 constitutes a group of compounds that can be inter-converted to each other upon hydration and binding with boric acid. The key intermediate in the biosynthesis of AI-2, 4,5-dihydroxy-2,3-pentanedione (DPD, 4) is synthesized by enzymes MTAN (SAH/S’-methylthioadenosine nucleosidase or SAHN) and LuxS from S-adenosyl-L-homocysteine (SAH).\textsuperscript{34} Scheme 2.1 shows the biosynthesis of AI-2 and three examples of the AI-2 family of compounds. SAH is hydrolyzed by MTAN to adenine and S-ribosyl-L-homocysteine (SRH), which is then cleaved by S-ribosylhomocysteinase (LuxS) to
form Hcy and DPD. Among the different forms of AI-2, compound 5 is the active species in regulating quorum sensing in *V. harveyi* through binding to its receptor, LuxP.34, 35

Scheme 1.1 Biosynthesis of DPD and different forms of AI-2

Inhibition of AI-2 mediated bacterial quorum sensing has been studied extensively. Inhibitors against the AI-2 pathway include those that target AI-2 synthesis (MTAN or LuxS) and the AI-2 receptor (LuxP). Based on the structures of substrates to these important enzymes, SAH mimics such as immucil-lin-A analogues have been reported as AI-2 inhibitors targeting MTAN.33 SRH analogues have also been reported as AI-2 inhibitors targeting LuxS.30, 36, 37 In the case of AI-2, different forms of AI-2 molecules are used in different bacteria. For example, S-THMF-borate (Compound 5) is recognized by LuxP in *V. harveyi*,34 and R-THMF is recognized by LsrB in *S. typhimurium* and *E. coli*.38 Despite the universal function of AI-2 in bacteria, only a few inhibitors were reported for AI-2 mediated bacterial quorum sensing.26, 31, 32, 39 Based on the structure of AI-2, our group has screened a series of boronic acids and aromatic diols in *V. harveyi*. A group of single-digit micromolar inhibitors have been identified, including boronic acids 7-1140 and pyrogallol analogues 12-1641 shown in Figure 1.1.
1.1.3 Identification of lead Al-2 inhibitors through virtual screening

Due to the fact that Al-2 mediated quorum sensing regulates gene expression in a variety of bacteria, the development of selective and potent inhibitors targeting the Al-2 pathway would be of great interest in both therapeutics and research purposes. The crystal structure of the Al-2-receptor (LuxP) complex in *V. harveyi* has been solved by Bassler and co-workers.\(^34\) In the crystal structure (Figure 1.2), the borate moiety is bound to the positively charged side chains Arg 215 and Arg 310 through hydrogen bonding. The hydroxyl groups of S-THMF-borate are stabilized by Trp 82 and Gln 77.

Figure 1.2 Crystal structure of LuxP complexed with Al-2.
A. Ribbon diagram of LuxP (PDB entry: 1JX6) complexed with ligand AI-2 (S-THMF-borate, white sticks) reproduced using the Pymol program; B. A schematic illustration of the interactions of S-THMF-borate with LuxP reproduced by using the HBPLUS and the Ligplot program.

Using this crystal structure of LuxP complexed with AI-2, a previous study in our group was conducted to find small molecules that might bind LuxP and thus could act as AI-2 inhibitors. Approximately, 1.7 million compounds from 14 commercial databases were screened by docking into the binding site of LuxP. The top 1000 structures were then scored using a combination of ChemScore, PLP, ScreenScore, ChemGauss, and ShapeGauss. 27 of the 42 hits resulted from the virtual screening were evaluated in a whole-cell based assay using V. harveyi as the model organism. Two lead structures (Figure 1.3), KM-03009 (16, IC₅₀ 35 μM) and SPB-02229 (17, IC₅₀ 55 μM) were identified to exhibit potent inhibition while showing no obvious cytotoxicity at concentrations twice that of their IC₅₀ values. When re-docked these two structures into the binding site of V. harveyi LuxP, it was suggested that KM-03009 could interact with residues Ser79, Arg215, Thr266, and Arg310 through hydrogen bonding, and Tyr81, Trp82, Asn159, Ile211, Phe206, and Ser265 through hydrophobic interactions. SPB-02229 may form hydrogen bonds with Asn159, Arg215, Thr266 and Arg310 and hydrophobic interactions with Tyr81, Trp82, Ile211, Phe206, and Ser265, a similar pattern as observed for KM-03009.
Figure 1.3 Proposed docking results of two lead compounds with V. harveyi LuxP AI-2 binding site.
A. Structure of KM-03009 and proposed docking interactions; B. Structure of SPB-02229 and proposed docking interactions.

The potency of compounds 16 and 17 determined through the whole-cell based assay has confirmed the successful discovery of inhibitors through virtual screening. The structures of these two compounds are unique and could be used as probes for mechanistic studies. In the current study, the structures of these two lead compounds are analyzed and optimized for the design of a series of new antagonists.
1.2 Results and discussion

1.2.1 Design and synthesis of analogues based on lead structures

In order to further improve potency and achieve an initial understanding of the structure-activity relationship, we have analyzed the structures of the two lead compounds (KM-03009 and SPB-02229). Though these were not especially potent inhibitors, they represented the first AI-2 inhibitors at that time and the structural scaffold was novel and small, which allows for easy modification. In the crystal structure of *V. harveyi* LuxP-AI-2 complex, the positively charged side chains of Arg 215 and Arg 310 were known to interact with three of the four borate oxygen atoms and thus stabilize the anionic tetrahedral boron. The two hydroxyl groups and the furanoyl oxygen are also involved in hydrogen bonding with Trp 82, Gln 77 and Asn 159. Compounds KM-03009 and SPB-02229 seem to interact with LuxP by using the sulfone group at the position of the borate portion of the natural ligand. Specifically, the two oxygen atoms of the sulfone group can interact with Arg 215 and Arg 310, mimicking the borate oxygen atoms.45 The aryl ring is involved in some hydrophobic interactions. Based on these findings and comparison of these two lead compounds with the other inactive hit compounds, it seems that the sulfone group should be directly attached to an aryl group and the thioamide group should be separated from the sulfone group by one atom.45 With the above information in hand, we were interested in modifying the structure of the two lead compounds by changing both ends (R¹ and R², Figure 1.4), while retaining the middle skeleton of the structure. In addition, analogs were also prepared to examine the importance of the sulfone moiety (part B) and the thioamide/thioester structure (part C).

![Figure 1.4 Optimization of the lead structure](image-url)
For part A of the structure (Figure 1.4), the following aryl groups were used: thiophenyl, pyridinyl, phenyl, or substituted phenyl groups including phenyl groups bearing a simple substituent or extended by connecting to an additional aryl group. The impact of the polarity and the size of the substitution on the phenyl group were studied. The effects of substituents at different positions (para- or meta-) of the phenyl ring were also explored. For Part B of the structure, the effect of changing the sulfone to a sulfoxide group was examined. For part C, the influence of replacing the thiocarbonyl by a carbonyl group was explored. In optimizing part D, the terminal functional group of the structure, different classes of compounds were synthesized and compared, including amides, alkylamides, esters, acids, isoxazole and hydroxylamine (Schemes 1.2-1.6).

![Scheme 1.2 Synthesis of compounds 19-21](image)

Scheme 1.2 describes the synthesis of amide and thioamide compounds 19-21. Specifically, aryl-sulfonyl amides (20) were synthesized in two steps from aryl thiols (18) and the corresponding α-
haloacetamides through substitution in the presence of potassium carbonate under reflux, followed by oxidation with oxone at room temperature.\textsuperscript{1} Thiation of arylsulfonyl amides (20) was accomplished by using Lawesson’s reagent in THF\textsuperscript{2} with yields in the range of 25-60%.

![Scheme 1.3 Synthesis of compounds 22-26](image)

The ester, thioester and acid analogs were synthesized as described in Scheme 1.3. Specifically, the synthesis of arylsulfonyl esters (23) was very similar to that of arylsulfonyl amides (20) except for that o-xylene was used in the thiation reaction as the solvent instead of THF because esters are less reactive toward the Lawesson’s reagent and requires higher temperature.\textsuperscript{2,3} The sulfonyl acetic acids (25) were synthesized through hydrolysis of the corresponding methyl esters (23) at 50 °C under basic conditions. Arylsulfenyl acetate (26) was synthesized in high yield through the oxidation of arylthioacetate (22) using m-CPBA in DCM at 0 °C.\textsuperscript{4}
Compounds with extended phenyl rings were synthesized using the procedure shown in Scheme 1.4. Specifically, Suzuki coupling reaction was used for the addition of an extra aryl ring to give 27. Conversion of the ester group to a thioester (28) was accomplished using Lawsson's reagent as described above.
Compounds 30-32 and 34-38 were synthesized using the procedures described in Schemes 1.5 and 1.6. The sulfonyl methyl isoxazoles (32) were synthesized in two steps from the reaction between aryl thiols (18) and bromomethyl isoxazole (30) followed by oxidation using oxone. The synthesis of sulfonyl \( N \)-hydroxyethylamines (38) was completed in five steps. The substitution reaction between an aryl thiol (18) and bromoacetaldehyde acetal (33) was accomplished in the presence of sodium ethoxide (1 equivalent). Acidic hydrolysis of the dimethyl acetal (34) yielded the aldehyde (35),\(^6\) which was then converted to oxime (36). Sulfone (37) was prepared through oxidation using oxone and then the oxime moiety was reduced to \( N \)-hydroxyethylamine (38) using NaCNBH\(_3\) at room temperature.\(^7\)

1.2.2 Evaluation of synthetic analogues in \( V. \) harveyi

All compounds synthesized were evaluated for their ability to inhibit Al-2-mediated quorum sensing following literature procedures.\(^8\)\(^-\)\(^12\) In doing so, the MM32 strain of \( V. \) harveyi was used. As discussed above, \( V. \) harveyi produces bioluminescence upon quorum sensing and the intensity of bioluminescence is controlled by the level of Al-2 stimulation.\(^13\) The MM32 strain lacks the LuxN receptor, which is re-
quired to respond to Al-1\textsuperscript{12}, and LuxS, which catalyzes the biosynthesis of DPD. Therefore, the bioluminescence can only be produced if a proper amount of DPD is added to the culture because there is no endogenous Al-2, and the level of Al-1 does not interfere with the Al-2 inhibition assay.

Inhibition of luminescence production by the synthesized compounds was studied in the presence 5 µM of DPD, which was chosen to ensure that luminescence production stays in a sensitive region as described previously.\textsuperscript{8} DMSO, which is used to dissolve the compounds for making the stock solutions, was found to have significant influence on the bioluminescence.\textsuperscript{8} Therefore, a constant concentration of DMSO (0.4% in volume) was maintained in the final test solutions so as to exclude its possible side effect on the results. A 96-well plate reader was used for the luminescence determination in an inhibitor concentration range from 400 to 0 µM. The IC\textsubscript{50} values were then calculated according to the inhibition curves.

Since the MM32 strain does not respond to Al-1, we also utilized the BB886 strain of \textit{V. harveyi}, which lacks the Al-2 receptor, to test the Al-1 inhibition activities of the synthesized compounds and to check their selectivity toward Al-2-mediated quorum sensing. Before the inhibition test, the two strains were checked for their specificity for Al-2 and Al-1 by incubation in the presence of boric acid/DPD and cell-free medium from MM32 (Lacks luxS enzyme which produces Al-2), respectively. The results confirmed that the MM32 strain did not respond to Al-1 and the BB886 strain did not respond to Al-2. Among all the compounds tested, 12 compounds (Compounds 24a-f, 23.g, 27a-c, and 28a-b) were observed to have significant inhibition activities against Al-2 mediated quorum sensing with IC\textsubscript{50} at or below 40 µM and 4 compounds (27a-b, 28a-b) have IC\textsubscript{50} in the single digit micromolar range. Among the 12 active compounds, five compounds (24c-d, 27a, 27c, and 28b) showed good selectivity towards Al-2 with the IC\textsubscript{50} values for Al-1 inhibition above 200 µM.

In analyzing the Al-2 inhibition results, we were interested in achieving some basic understanding of the structural features, which are essential and/or beneficial for Al-2 inhibition. The first thing that we
examined was whether the thioamide structure (part C, Figure 1.4) was important. From the compounds listed in Table 1.1, one can see that the “thio” structure feature was important for activity, but not essential. For example, the only difference between KM-03009 and 20a was that one was the thioamide (KM-03009) and the other one was a simple amide. However, their activities differ by over ten-fold with the thioamide compound being more active. The situations are similar when comparing 20b vs. 21b, 20c vs. 21c, and 20d vs. 21d.

\[
\begin{align*}
\text{Table 1.1 Structures and activities of amides and thioamides} \\
\begin{array}{|c|c|c|c|c|}
\hline
\text{Compound} & R^1 = & R^2 = & X = & \text{IC}_{50} \text{ for AI-2 (µM)} & \text{IC}_{50} \text{ for AI-1 (µM)} \\
\hline
\text{KM-03009} & \text{Thiophen-2-yl} & H & S & 35\pm3 & 71\pm5 \\
20a & \text{Thiophen-2-yl} & H & O & >400 & -- \\
20b & \text{Phenyl} & H & O & >400 & >400 \\
21b & \text{Phenyl} & H & S & 89\pm10 & 158\pm22 \\
21c & \text{Thiophen-2-yl} & \text{Ethyl} & O & >400 & >400 \\
21c & \text{Thiophen-2-yl} & \text{Ethyl} & S & 138\pm25 & 143\pm18 \\
20d & \text{Phenyl} & \text{Ethyl} & O & >400 & >400 \\
21d & \text{Phenyl} & \text{Ethyl} & S & 91\pm14 & >400 \\
\hline
\end{array}
\end{align*}
\]

Next we examined the comparison between thioesters and esters (Table 1.2). The same conclusions can be drawn, i.e., “thiation” is very important for AI-2 activities in this series with the thioesters having higher or equal activities compared with regular esters. For example, the only difference between 23a and 24a is that 23a is an ester and 24a is a thioester and yet their IC\textsubscript{50} values differ by about 6-fold. The situations are similar when comparing between 23b and 24b, and 23c and 24c. However,
there are also other cases, such as 23d vs. 24d and 23g vs. 24e, where the thioesters and the esters have similar activities.

![Chemical structure](image)

<table>
<thead>
<tr>
<th>Table 1.2 Structures and activities of esters and thioesters</th>
<th></th>
<th></th>
<th></th>
<th>IC₅₀ for Al-2 (µM)</th>
<th>IC₅₀ for Al-1 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td>R¹ =</td>
<td>R² =</td>
<td>X =</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23a</td>
<td>Thiophen-2-yl</td>
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<td>O</td>
<td>124±16</td>
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<td>Ethyl</td>
<td>O</td>
<td>170±30</td>
<td>&gt;400</td>
</tr>
<tr>
<td>24b</td>
<td>Phenyl</td>
<td>Ethyl</td>
<td>S</td>
<td>33±4</td>
<td>96±20</td>
</tr>
<tr>
<td>23c</td>
<td>Thiophen-2-yl</td>
<td>Isopropyl</td>
<td>O</td>
<td>127±21</td>
<td>&gt;400</td>
</tr>
<tr>
<td>24c</td>
<td>Thiophen-2-yl</td>
<td>Isopropyl</td>
<td>S</td>
<td>34±2</td>
<td>&gt;400</td>
</tr>
<tr>
<td>23d</td>
<td>Phenyl</td>
<td>Isopropyl</td>
<td>O</td>
<td>38±15</td>
<td>217±101</td>
</tr>
<tr>
<td>24d</td>
<td>Phenyl</td>
<td>Isopropyl</td>
<td>S</td>
<td>33±4</td>
<td>&gt;400</td>
</tr>
<tr>
<td>23e</td>
<td>Thiophen-2-yl</td>
<td>Methyl</td>
<td>O</td>
<td>&gt;400</td>
<td>&gt;400</td>
</tr>
<tr>
<td>24f</td>
<td>Phenyl</td>
<td>Methyl</td>
<td>O</td>
<td>&gt;400</td>
<td>&gt;400</td>
</tr>
<tr>
<td>23g</td>
<td>4-Bromophenyl</td>
<td>Ethyl</td>
<td>O</td>
<td>17±3</td>
<td>11±1</td>
</tr>
<tr>
<td>24e</td>
<td>4-Bromophenyl</td>
<td>Ethyl</td>
<td>S</td>
<td>14±2</td>
<td>35±10</td>
</tr>
<tr>
<td>23h</td>
<td>4-Bromophenyl</td>
<td>Methyl</td>
<td>O</td>
<td>81±3</td>
<td>115±35</td>
</tr>
<tr>
<td>23i</td>
<td>3-Bromophenyl</td>
<td>Ethyl</td>
<td>O</td>
<td>163±22</td>
<td>319±23</td>
</tr>
<tr>
<td>23j</td>
<td>4-Methoxyphenyl</td>
<td>Ethyl</td>
<td>O</td>
<td>87±6</td>
<td>79±15</td>
</tr>
<tr>
<td>24f</td>
<td>4-Methoxyphenyl</td>
<td>Ethyl</td>
<td>S</td>
<td>26±4</td>
<td>33±4</td>
</tr>
<tr>
<td>23k</td>
<td>Propyl</td>
<td>Ethyl</td>
<td>O</td>
<td>&gt;400</td>
<td>&gt;400</td>
</tr>
</tbody>
</table>
In comparing between esters and amides (Part D), higher inhibition activities of the ethyl and isopropyl esters were observed compared to their corresponding amides. For example, thioesters 24a-d (Table 1.2, between 22 and 34 µM) have lower IC₅₀ values compared to their corresponding thioamides such as KM-03009 (35 µM) and 21b-d (around 100 µM), though it is hard to say whether some of these small differences are meaningful given the intrinsic fluctuation of whole cell assay results. For the substituents of part D of the ester series, there is essentially no difference between ethyl esters and isopropyl esters, but methyl esters are obviously weaker inhibitors. For example, compounds 23e and 23f are inactive toward AI-2. However, the same trend was not observed with the amide compounds. One thing worth mentioning is that the isopropyl ester compounds (23c, d and 24c, d) seem to show the highest selectivity between AI-1 and AI-2 inhibition.

As for Part A, the aryl ring of the structure seems to be an important component for activities because the replacement of the aryl ring by an alkyl group led to a significant decrease in the activity. This becomes very clear when the IC₅₀ values of compounds with an alkyl chain at the Part A position such as 23k (Table 1.2, ester, >400 µM) and 24g (Table 1.2, thioester, 122 µM) are compared with most of the other ethyl esters (around 150 µM for esters and 30 µM for thioesters). A heterocycle, such as the thio-phenyl group, is slightly better than a phenyl group. For example the IC₅₀ values of compounds 23a/24a (thiophenyl) are slightly lower than that of compounds 23b/24b (phenyl). Additional substituents on the aryl ring seem to favor inhibition activities. For example, adding a bromo or a methoxyl group at the para-position of the phenyl ring resulted in significantly improved activities from an IC₅₀ of 170 µM (23b) to 17 µM (23g) and 87 µM (23j). However, if the substituent is on the meta-position, no improvement was observed. A case in point is compound 23i, which has an IC₅₀ of 163 µM.
Table 1.3 Structures and activities of biaryl compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ar =</th>
<th>X =</th>
<th>IC_{50} for AI-2 (µM)</th>
<th>IC_{50} for AI-1 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>27a</td>
<td>Phenyl</td>
<td>O</td>
<td>8.7±2.9</td>
<td>293±108</td>
</tr>
<tr>
<td>28a</td>
<td>Phenyl</td>
<td>S</td>
<td>5.7±0.8</td>
<td>32±2</td>
</tr>
<tr>
<td>27b</td>
<td>4-Methylphenyl</td>
<td>O</td>
<td>8.2±2.3</td>
<td>66±32</td>
</tr>
<tr>
<td>28b</td>
<td>4-Methylphenyl</td>
<td>S</td>
<td>5.6±1.3</td>
<td>&gt;400</td>
</tr>
<tr>
<td>27c</td>
<td>H2N</td>
<td>O</td>
<td>31±2</td>
<td>&gt;200</td>
</tr>
<tr>
<td>27d</td>
<td></td>
<td>O</td>
<td>158±43</td>
<td>&gt;400</td>
</tr>
<tr>
<td>27e</td>
<td></td>
<td>O</td>
<td>&gt;400</td>
<td>&gt;400</td>
</tr>
</tbody>
</table>

Because of the promising results with para-substituted phenyl group in part A, we synthesized additional compounds with a bulky substituent at the para-position (Table 1.3). Several points are readily noticeable in this series of compounds. First, the “biaryl” compounds tend to be more active than the single aryl compounds unless the second aryl group has ring heteroatom and/or a substitution. For example, biaryl compounds 27a, b (8.2-8.7 µM) and 28a, b (5.6-5.7 µM) all have IC_{50} values in the single digit micromolar range, while their corresponding “single aryl ring” analogs compounds 23b (170 µM) and 24b (33 µM) have much lower activities. However, with an isoquinolinyl (27c) or dihydrobenzodioxinyl (27d) group as the second ring, the IC_{50} values are over 150 µM. Second, a methyl group on the additional phenyl ring does not seem to make much difference. However, when a bulky group is attached, the molecule starts to lose its activity. This point is demonstrated by the activity change from 27a to 27e,
which has a quinoline as the second ring. Third, as for the selectivity in inhibition against Al-2 and Al-1, a phenyl group extended by a second phenyl ring seems to improve the selectivity in biaryl compound 27a (about 34 fold), whereas a polar substituent on the phenyl ring seems to decrease the selectivity for Al-2 (e.g., 23g-j and 24e-f). Fourth, the effect of “thiation” of the carbonyl group in the biaryl series seems to be less significant compared to those with only one aryl group. For example, the IC₅₀ of biaryl compounds 27a-b (esters, 8.2-8.7 µM) are close to those of compounds 28a-b (thioesters, about 5.6-5.7 µM). Overall, the introduction of a second aryl ring is advantageous for improved activity and selectivity.

The results of inhibition evaluation also revealed that reducing the sulfone group to a sulfoxide seems to result in diminished activities. For example, compound 23g (sulfone) has an IC₅₀ of 17 µM, while the corresponding sulfoxide (26a, Table 1.4) has an IC₅₀ of 48 µM. The possible reason for the activity difference may be due to the better structure mimicry of the borate group in Al-2 by a sulfone group than a sulfoxide. Additionally, a charged or polar terminal group (part D) also results in significantly reduced activities (25a, b, 32a, b, 38a, b)

<table>
<thead>
<tr>
<th>Table 1.4 Structures and activities of other compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compound</strong></td>
</tr>
<tr>
<td>26a</td>
</tr>
<tr>
<td>25a</td>
</tr>
<tr>
<td>25b</td>
</tr>
<tr>
<td>32a</td>
</tr>
<tr>
<td>32b</td>
</tr>
</tbody>
</table>
In order to exclude potentially misleading results caused by possible cytotoxicity, the effect of selected synthesized active inhibitors with IC$_{50}$ values below 150 µM (21c, 21d, 23a, 24a, 24b, 23c, 24c, 23d, 24d, 23g, 24e, 23h, 23j, 24f, 27a, 28a, 27b, 28b, 27c, and 26a,) on bacterial growth was tested on the MM32 strain of *V. harveyi* by following procedures published earlier. The results showed that none of these compounds has cytotoxicity at concentrations, which are twice the IC$_{50}$ value. That means the decrease of bioluminescence production observed was solely due to the inhibition of quorum sensing.

To further explore the influence of the compounds on the growth of bacteria and to normalize the bioluminescence to cell density, especially during the same time point in which we assayed for the inhibition of fluorescence, a plate counting test was performed for the following compounds: KM-03009, 27a, 27b, 27c, 28a and 28b, using the MM32 strain of *V. harveyi*. The bacteria culture incubated for 5 hours in the presence of a compound at a concentration, which was close to its IC$_{50}$ was diluted to 1:100 and 1:1000, and plated onto fresh LM plates and incubated at 30 °C for 24-48 hr. The colonies appeared were counted and compared to the blank (bacteria incubated without any inhibitor). The number of CFU (colony forming unit) of the bacteria incubated in the presence of an inhibitor at about the IC$_{50}$ concentration was about the same as that of the blank. For example, the number of colonies appeared in the presence of the inhibitors ranged from 80-170% of that of the controls (without any inhibitors), while the bioluminescence intensity of these experiments with inhibitors at IC$_{50}$ concentrations was only 30-60% of the controls. Such results further suggested that the compounds indeed inhibited the luminescence production (quorum sensing), not bacteria growth.
1.2.3 Structure-activity relationship of synthetic inhibitors

Aimed at achieving further understanding of the structure-activity relationship, we also conducted a Comparative Molecular Field Analysis (CoMFA). The CoMFA methodology is a 3D Quantitative Structure-Activity Relationship (3D-QSAR) technique by analyzing the steric and electrostatic fields. In this case all compounds were optimized by the semi-empirical MOPAC/AM1 method and then assigned with AM1BCC charges. These conformations were then aligned with compound 28a (the most potent compound) for CoMFA computation as depicted in Figure 1.5.

![Figure 1.5 Molecular alignments of all compounds](image)

CoMFA results suggest that the steric and electrostatic fields contribute 56% and 44% to AI-2 inhibitory activities, respectively. Statistically, the resulting standard error (SE) of 0.207, noncross-validated correlation coefficient ($r^2$) of 0.931, cross-validated coefficient ($q^2$) of 0.922 and F value of 76.72 confirm the reliability of our CoMFA model. Table 1.5 shows that comparison of the calculated
pIC$_{50}$ and experimentally determined pIC$_{50}$ values using the CoMSIA model developed and Figure 1.6 shows the schematic correlation of such data.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Experimental pIC$_{50}$</th>
<th>Predicted pIC$_{50}$</th>
<th>Residual</th>
</tr>
</thead>
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<tr>
<td>KM-03009</td>
<td>4.46</td>
<td>3.87</td>
<td>0.59</td>
</tr>
<tr>
<td>SPB-02229</td>
<td>4.26</td>
<td>4.44</td>
<td>-0.18</td>
</tr>
<tr>
<td>20a</td>
<td>3.00</td>
<td>3.22</td>
<td>-0.22</td>
</tr>
<tr>
<td>20b</td>
<td>3.00</td>
<td>3.25</td>
<td>-0.25</td>
</tr>
<tr>
<td>21b</td>
<td>4.05</td>
<td>3.87</td>
<td>0.18</td>
</tr>
<tr>
<td>20c</td>
<td>3.00</td>
<td>3.13</td>
<td>-0.13</td>
</tr>
<tr>
<td>21c</td>
<td>3.86</td>
<td>3.91</td>
<td>-0.05</td>
</tr>
<tr>
<td>20d</td>
<td>3.00</td>
<td>3.30</td>
<td>-0.30</td>
</tr>
<tr>
<td>21d</td>
<td>4.04</td>
<td>3.71</td>
<td>0.33</td>
</tr>
<tr>
<td>23a</td>
<td>3.91</td>
<td>3.91</td>
<td>0.00</td>
</tr>
<tr>
<td>24a</td>
<td>4.66</td>
<td>4.32</td>
<td>0.33</td>
</tr>
<tr>
<td>23b</td>
<td>3.77</td>
<td>3.62</td>
<td>0.15</td>
</tr>
<tr>
<td>24b</td>
<td>4.48</td>
<td>4.55</td>
<td>-0.07</td>
</tr>
<tr>
<td>23c</td>
<td>3.90</td>
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<td>4.38</td>
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<tr>
<td>23d</td>
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<td>4.32</td>
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</tr>
<tr>
<td>23e</td>
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<td>-0.01</td>
</tr>
<tr>
<td>23f</td>
<td>3.00</td>
<td>3.12</td>
<td>-0.12</td>
</tr>
<tr>
<td></td>
<td>23g</td>
<td>24e</td>
<td>23h</td>
</tr>
<tr>
<td>---</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>4.77</td>
<td>4.85</td>
<td>4.09</td>
</tr>
<tr>
<td></td>
<td>4.51</td>
<td>4.94</td>
<td>3.76</td>
</tr>
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</tr>
</tbody>
</table>
Figure 1.6 Experimental versus predicted pIC₅₀ for CoMFA 3D-QSAR model

For clarity in presentation, two 3D contour maps are shown in Figure 1.7. In the steric contour map, a high density of green and yellow colors around the R₁ substituent (Figure 1.4) suggests that a moderate bulky aromatic ring, such as diphenyl ring, is favorable; in the meanwhile the yellow color around the R₂ substituent also indicates a less bulky group here is desired. In the electrostatic contour map, a red color around the sulfonyl moiety and the R₂ substituent suggests that the less electronegative substituents in these positions could enhance the biological activity. The CoMFA model may provide useful insight into the future design of novel AI-2 inhibitors.

Figure 1.7 3D contour maps around compound 28a as the result of a CoMFA analysis of the AI-2 inhibitory activities.
Regions where substitution enhances (green) or reduces (yellow) the inhibitory affinity (left); the color coding indicates regions where electronegative substituents would enhance (blue) or reduce (red) the inhibitory activities (right).

Though the studies presented give an initial understanding of the structure-activity relationship, it should be noted that whole cell bacterial assays have certain intrinsic experimental variations that may not allow us to draw firm conclusions related to structural variations that only resulted in small changes in activities. When the same compound was tested using an entirely new batch of bacteria from the same source, IC_{50} variations of up to 1-3 fold were observed in some extreme cases. Though such variations are common in whole cell bacterial assay, one does need to be careful in drawing quantitative conclusions. The results presented can be viewed as a qualitative trend.

1.3 Experimental section

General information

All reagents were purchased from Acros and Aldrich. Boronic acids were provided by Frontier Scientific, Inc. DPD was synthesized following literature procedures.\textsuperscript{20, 21} \textsuperscript{1}H-NMR and \textsuperscript{13}C-NMR spectra were recorded at 400 and 100 MHz, respectively, on a Bruker 400 NMR spectrometer. Mass spectral analyses were performed by the mass spectrometry facilities at Georgia State University.

1.3.1 Synthesis and characterization of small molecule analogues

General Experimental Procedure for Substitution Reaction of Thiol and Haloacetamides, Haloacetates or 5-(Bromomethyl)isoxazole. Representative Procedure for Substitution of Thiophene-2-thiol (18a) and 2-iodoacetamide (Schemes 1.2 and 1.3, reaction a; Scheme 1.5, reaction b): A mixture of thiophene-2-thiol (18a, 690 mg, 5.94 mmol, 2.2 equiv.), 2-iodoacetamide (499 mg, 2.7 mmol, 1.0 equiv.) and potassium carbonate (560 mg, 4.05 mmol, 1.5 equiv.) in acetone (20 mL) was heated under
reflux for 6 h (TLC). The solution was filtered and solvent was evaporated. Dichloromethane (50 mL) was added to dissolve the residue. The solution was washed with saturated NaHCO₃ (8 mL × 2), H₂O (5 mL × 2) and brine (10 mL), and dried over Na₂SO₄. Evaporation of solvent afforded the crude product which was purified by column chromatography (Hex/EtOAc, 2:1) to give 2-(thiophen-2-ylthio)acetamide (19a, 229 mg, 49%) as light yellow oil. The crude product can also be used in the next step without purification.

**Ethyl 2-(phenylthio)acetate (22b).** Yellow oil; yield 64%; ¹H NMR (CD₂OD) δ 7.39-7.40 (d, J = 7.2 Hz, 2H), 7.28-7.31 (t, J = 7.2 Hz, 7.68 Hz, 2H), 7.20-7.24 (t, J = 6.8 Hz, 7.6 Hz, 1H), 4.08-4.13 (t, J = 7.2 Hz, 2H), 3.68 (s, 2H), 1.16-1.19 (t, J = 7.2 Hz, 3H); GC-MS, m/z 196 (M⁺).

**Ethyl 2-(4-bromophenylthio)acetate (22g).** Colorless oil; yield 87%; ¹H NMR (CDCl₃) δ 7.41-7.43 (d, J = 8.4, 2H), 7.26-7.30 (d, J = 8.4 Hz, 2H) , 4.14-4.19 (q, J = 7.2 Hz, 2H), 3.61 (s, 2H), 1.21-1.25 (t, J = 7.2 Hz, 3H); ¹³C NMR (CDCl₃) δ 169.4, 134.2, 132.1, 131.5, 121.0, 61.7, 36.6, 14.1; MS (ES⁺), m/z 275.0 (M⁺)⁺.

**Methyl 2-(4-bromophenylthio)acetate (22h).** Colorless oil; yield 56%; ¹H NMR (CDCl₃) δ 7.42-7.44 (d, J = 8.4 Hz, 1H), 7.26-7.29 (d, J = 8.8 Hz, 1H), 3.72 (s, 3H), 3.63 (s, 2H), 13C NMR (CDCl₃) δ 169.9, 134.1, 132.1, 131.5, 121.1, 52.6, 36.4; MS (ES⁺), m/z 282.9 (M+23)⁺.

**Ethyl 2-(4-methoxyphenylthio)acetate (22j).** Colorless oil; yield 99%; ¹H NMR (CDCl₃) δ 7.41-7.43 (d, J = 8.8 Hz, 2H), 6.83-6.86 (d, J = 8.8 Hz, 2H) , 4.11-4.17 (q, J = 7.2 Hz, 2H), 3.79 (s, 3H), 3.51 (s, 2H), 1.20-1.23 (t, J = 7.2 Hz, 3H); ¹³C NMR (CDCl₃) δ 169.9, 159.6, 134.2, 124.9, 114.6, 61.3, 55.3, 38.6, 14.1; MS (ES⁺), m/z 227.1 (M+1)⁺.
Ethyl 2-(propylthio)acetate (22k). Colorless oil; yield 77%; $^1$H NMR (CDCl$_3$) δ 4.17-4.22 (q, $J = 7.2$ Hz, 2H), 3.21 (s, 2H), 2.60-2.64 (t, $J = 14.8$ Hz, 2H), 1.59-1.68 (m, $J = 7.2$ Hz, 2H), 1.27-1.31 (t, $J = 7.2$ Hz, 3H), 0.98-1.01 (t, $J = 7.2$ Hz, 3H); $^{13}$C NMR (CDCl$_3$) δ 170.6, 61.2, 34.6, 33.6, 22.3, 14.1, 13.3; MS (ES$^+$), m/z 163.1 (M+1)$^+$. 

5-(Phenylthiomethyl)isoxazole (31b). Yellow oil; yield 87%; $^1$H NMR (CDCl$_3$) δ 8.15 (s, 1H), 7.25-7.38 (m, 5H), 6.07 (s, 1H), 4.19 (s, 2H); GC-MS, m/z, 191 (M$^+$).

General Experimental Procedure for Oxone Oxidation Reaction. Representative Procedure for Oxidation of 2-(Thiophen-2-ythio)acetamide (19a) (Schemes 1.2 and 1.3, reaction b; Scheme 1.5, reaction c, Scheme 1.6, reaction d): To a stirred solution of 2-(thiophen-2-ythio)acetamide (19a, 229 mg, 1.32 mmol, 1.0 equiv.) in MeOH (5.4 mL) and THF (5.4 mL) was added the solution of oxone (1.12 g, 1.82 mmol, 1.38 equiv.) in H$_2$O (7.2 mL) dropwise. The mixture was stirred at room temperature overnight. The solution was filtered and solvent was removed. Then dichloromethane (50 mL) was added and the solution was washed with H$_2$O (8 mL), brine (8 mL × 2), and dried over Na$_2$SO$_4$. Evaporation of solvent afforded the crude product which was purified by column chromatography (Hex/EtOAc 2:1 – 1:1) to give 2-(thiophen-2-ylsulfonyl)acetamide (20a, 241 mg, 89%) as white solid.

2-(Thiophen-2-ylsulfonyl)acetamide (20a). White solid; yield 44% for two steps; $^1$H NMR (CD$_3$OD): δ 7.97 (s, 1H), 7.78 (s, 1H), 7.23-7.24 (d, 1H), 4.23 (s, 2H); $^{13}$C NMR (CD$_3$OD): δ 164.4, 139.5, 135.0, 127.7, 62.4; MS (ES$^+$), m/z 206 (M+1)$^+$, 228 (M+Na)$^+$. 


2-(Phenylsulfonyl)acetamide (20b). White solid; yield 89% in two steps. $^1$H NMR ((CD$_3$)$_2$CO) $\delta$ 7.95-7.97 (m, 2H), 7.73-7.77 (m, 1H), 7.63-7.67 (m, 2H), 6.71-7.18 (br, 2H), 4.17 (s, 2H); $^{13}$C NMR ((CD$_3$)$_2$CO) $\delta$ 162.4, 139.9, 133.8, 129.1, 128.3, 61.4; MS (ES$^+$), m/z 200 (M+1)$^+$, 222 (M+Na)$^+$.  

$N,N$-Diethyl-2-(thiophen-2-ylsulfonyl)acetamide (20c). Light yellow crystals; yield 78% in 2 steps; $^1$H NMR (CDCl$_3$) $\delta$ 7.74-7.76 (m, $J = 4.8$ Hz, 2H), 7.15-7.18 (t, $J = 4.8$ Hz, 1H), 4.29 (s, 2H), 3.45-3.50 (q, $J = 7.2$ Hz, 2H), 3.34-3.40 (q, $J = 7.2$ Hz, 2H), 1.20-1.24 (t, $J = 7.2$ Hz, 3H), 1.11-1.14 (t, $J = 7.2$ Hz, 3H), $^{13}$C NMR (CDCl$_3$) $\delta$ 160.6, 139.6, 135.6, 135.0, 128.0, 61.0, 43.3, 41.1, 14.4, 13.0; MS (ES$^-$), m/z 260.0 (M-1)$^-$; m.p. = 89-90 °C.  

$N,N$-Diethyl-2-(phenylsulfonyl)acetamide (20d). White crystals 72% for 2 steps; $^1$H NMR (CDCl$_3$) $\delta$ 7.92-7.94 (d, $J = 7.2$ Hz, 2H), 7.65-7.69 (t, $J = 7.2$ Hz, 1H), 7.55-7.59 (t, $J = 7.6$ Hz, 2H), 4.21 (s, 1H), 4.16-4.21 (q, $J = 7.2$ Hz, 2H), 1.21-1.25 (t, $J = 7.2$ Hz, 3H), $^{13}$C NMR (CDCl$_3$) $\delta$ 160.6, 139.0, 134.3, 129.2, 128.8, 60.0, 43.3, 41.0, 14.4, 12.9; MS (ES$^-$), m/z 255.0 (M-1)$^-$; m.p. = 84-85 °C.  

Ethyl 2-(thiophen-2-ylsulfonyl)acetate (23a). Colorless oil; yield 71% in two steps; $^1$H NMR (CDCl$_3$) $\delta$ 7.80-7.81 (dd, $J = 1.2$ Hz, 3.6 Hz, 4.8 Hz, 1H), 7.76-7.78 (dd, $J = 1.2$ Hz, 4.0 Hz, 4.8 Hz, 1H), 7.18-7.20 (q, $J = 1.2$ Hz, 3.6 Hz, 4.0 Hz, 1H), 4.22 (s, 1H), 4.16-4.21 (q, $J = 7.2$ Hz, 2H), 1.21-1.25 (t, $J = 7.2$ Hz, 3H); $^{13}$C NMR (CDCl$_3$) $\delta$ 162.3, 139.3, 135.4, 135.1, 128.0, 62.5, 62.0, 13.9; MS (ES$^+$), m/z 235 (M$^+$), 257 (M+Na)$^+$.  

Ethyl 2-(phenylsulfonyl)acetate (23b). White solid; yield 71%; $^1$H NMR (CDCl$_3$) $\delta$ 7.95-7.97 (d, $J = 7.6$ Hz, 2H), 7.68-7.72 (t, $J = 7.2$ Hz, 1H), 7.57-7.61 (t, $J = 7.2$ Hz, 7.6 Hz, 2H), 4.17 (s, 1H), 4.12-4.15 (q, $J =
7.2 Hz, 2H), 1.17–1.26 (t, J = 7.2 Hz, 3H); $^{13}$C NMR (CDCl$_3$) δ 162.3, 138.7, 134.3, 129.2, 128.6, 62.4, 61.0, 13.9; MS (ES$^-$), m/z, 229 (M+1)$^-$, 251 (M+Na)$^+$; m.p. = 43-44 °C.

Isopropyl 2-(thiophen-2-ylsulfonyl)acetate (23c). Colorless oil; yield 95% in two steps; $^1$H NMR (CDCl$_3$) δ 7.69-7.71 (m, 2H), 7.10 – 7.12 (t, J = 4.0 Hz, 1H), 4.94- 4.97 (m, J = 6.0 Hz, 1H) 4.11 (s, 2H), 4.15 – 4.20 (d, J = 6.0 Hz, 6H). $^{13}$C NMR (CDCl$_3$) δ 161.7, 139.4, 135.3, 134.8, 127.8, 70.6, 62.3, 21.5; MS (ES$^-$), m/z, 247 (M−1)$^-$.  

Isopropyl 2-(phenylsulfonyl)acetate (23d). Colorless oil; yield 92% in two steps; $^1$H NMR (CDCl$_3$) δ 7.92-7.94 (d, J = 8.0 Hz, 2H), 7.64 – 7.68 (m, 1H), 7.54-7.58 (m, 2H), 4.91- 4.97 (m, J = 6.0 Hz, 1H) 4.07 (s, 2H), 4.13, 4.14 (d, J = 6.0 Hz, 6H). $^{13}$C NMR (CDCl$_3$) δ 161.8, 138.8, 134.2, 129.1, 128.5, 70.4, 61.2, 21.4; MS (ES$^-$), m/z 241 (M−1)$^-$.  

Methyl 2-(thiophen-2-ylsulfonyl)acetate (23e). Colorless oil; yield 73% in two steps; $^1$H NMR (CDCl$_3$) δ 7.76-7.68 (m, 2H), 7.17-7.19 (t, J = 4.4 Hz, 1H), 4.21 (s, 1H), 3.75 (s, 3H); MS (ES$^-$), m/z 221 (M+1)$^-$, 243 (M+Na)$^+$.  

Methyl 2-(phenylsulfonyl)acetate (23f). Colorless oil; yield 80% in two steps; $^1$H NMR (CDCl$_3$) δ 7.71-7.83 (m, 2H), 7.62 – 7.68 (t, J = 7.2 Hz, 1H), 7.51-7.55 (m, J = 7.2 Hz, 8.0 Hz, 2H), 4.07 (s, 1H), 3.64 (s, 3H); MS (ES$^-$), m/z 213 (M−1)$^-$.  

Ethyl 2-(4-bromophenylsulfonyl)acetate (23g). Colorless crystal; yield 90%; $^1$H NMR (CDCl$_3$) δ 7.81-7.83 (d, J = 8.8 Hz, 2H), 7.72-7.74 (d, J = 8.4 Hz, 2H), 4.14-4.19 (q, J = 7.2 Hz, 2H), 4.11 (s, 2H), 1.21-
1.24 (t, J = 7.2 Hz, 3H); $^{13}$C NMR (CDCl$_3$) δ 162.2, 137.6, 132.5, 130.2, 129.8, 62.5, 60.9, 13.9; MS (ES$^+$), m/z 328.9 (M+23)$^+$; m.p. = 49-50 °C.

**Methyl 2-{(4-bromophenylsulfonyl)acetate (23h)}.** White crystals; yield 88%; $^1$H NMR (CDCl$_3$) δ 7.81-7.83 (d, J = 8.8 Hz, 2H), 7.72-7.75 (d, J = 8.8 Hz, 2H), 4.13 (s, 2H), 3.73 (s, 3H); $^{13}$C NMR (CDCl$_3$) δ 162.7, 137.6, 132.6, 130.1, 129.9, 60.6, 53.2; MS (ES$^+$), m/z 314.9 (M+23)$^+$; m.p. = 78-79 °C.

**Ethyl 2-{(3-bromophenylsulfonyl)acetate (23j)}.** Colorless crystals; yield 75% in two steps; $^1$H NMR (CDCl$_3$) δ 8.11 (s, 1H), 7.90-7.92 (d, J = 9.2 Hz, 1H), 7.82-7.84 (d, J = 7.9 Hz, 1H), 7.47-7.51 (t, J = 7.9 Hz, 1H), 4.15-4.21 (q, J = 7.1 Hz, 2H), 4.15 (s, 2H); $^{13}$C NMR (CDCl$_3$) δ 162.0, 140.4, 137.3, 131.5, 130.7, 127.2, 123.1, 62.5, 60.9, 13.8; MS (ES$^+$), m/z 329.1 (M+23)$^+$; m.p. = 36-37 °C.

**Ethyl 2-{(4-methoxyphenylsulfonyl)acetate (23j)}.** Colorless oil; yield 71%; $^1$H NMR (CDCl$_3$) δ 7.86-7.88 (d, J = 8.8 Hz, 2H), 7.02-7.04 (d, J = 9.2 Hz, 2H), 4.13-4.18 (q, J = 6.8 Hz, 2H), 4.09 (s, 2H), 3.89 (s, 3H), 1.20-1.24 (t, J = 6.8 Hz, 3H); $^{13}$C NMR (CDCl$_3$) δ 164.2, 162.6, 130.8, 130.2, 114.3, 62.3, 61.2, 55.7, 13.9; MS (ES$^+$), m/z 281.0 (M+23)$^+$.

**Ethyl 2-{(propylsulfonyl)acetate (23k)}.** Colorless oil; yield 84%; $^1$H NMR (CDCl$_3$) δ 4.25-4.31 (q, J = 7.2, 2H), 3.95 (s, 2H), 3.22-3.26 (m, J = 8.0 Hz, J = 2.4, 2H), 1.90-1.95 (m, J = 7.6 Hz, J = 2.4 Hz, 2H), 1.31-1.35 (t, J = 7.2 Hz, 3H), 1.10-1.13 (t, J = 7.2 Hz, 3H); $^{13}$C NMR (CDCl$_3$) δ 163.1, 62.6, 57.4, 55.2, 15.7, 13.9, 13.0; MS (ES$^+$), m/z 317.0 (M+23)$^+$.

**5-{(Thiophen-2-ylsulfonyl)methyl}isoxazole (32a).** Yellow solid, yield 69% in two steps; $^1$H NMR (CDCl$_3$) δ 8.25-8.26 (d, J = 1H), 7.53-7.77 (dd, J = 1.2 Hz, 1.6 Hz, 5.2 Hz, 1H), 7.57-7.58 (dd, J = 1.6 Hz, 4.0 Hz),
7.13-7.16 (q, $J = 4.0$ Hz, 5.2 Hz, 1H), 7.45-7.46 (d, 1H), 4.69 (s, 2H); $^{13}$C NMR (CDCl$_3$) δ 159.7, 150.7, 137.9, 135.6, 135.4, 128.2, 105.6, 55.0; MS (ES$^+$), m/z 230 (M+1)$^+$. 

5-{Phenylsulfonylmethyl}isoxazole (32b). Yellow solid, yield 62%; $^1$H NMR (CDCl$_3$) δ 8.22 (s, 1H), 7.77-7.79 (d, $J = 8.0$ Hz, 2H), 7.66-7.70 (t, $J = 7.2$ Hz, 2H), 7.26-7.56 (t, $J = 7.6$ Hz, 2H), 6.38 (s, 1H), 4.60 (s, 2H); $^3$C NMR (CDCl$_3$) δ 159.7, 150.6, 137.6, 134.6, 129.4, 128.4, 105.4, 53.8; MS (ES$^+$), m/z 224 (M+1)$^+$, 246(M+Na)$^+$, m.p. = 87-88 °C.

2-{Phenylsulfonyl}acetaldehyde oxime (37b). White solid; yield 84%; $^1$H NMR ((CD$_3$)$_2$CO, mixture of isomers, ratio 1/1) δ 10.69 (s, 0.5H), 10.49 (s, 0.5 H), 7.66-7.98 (m, 5H), 7.33 (t, 0.5H), 6.82 (t, 0.5H), 4.37 (d, 1H), 4.11(d, 1H); MS (ES$^+$), m/z 200 (M+1)$^+$, 222(M+Na)$^+$; m.p. = 91-94 °C.

**General Experimental Procedure for Thiation Reaction of Amides. Representative Procedure for Thiation of 2-{(Thiophen-2-yl)sulfonyl}acetamide (20a) (Scheme 1.2, reaction c):** To a solution of 2-{(thiophen-2-yl)sulfonyl}acetamide (20a, 33 mg, 0.17 mmol, 1.0 equiv.) in anhydrous THF (5 mL) was added Lawesson’s reagent (67 mg, 0.17 mmol, 1.0 equiv.) at room temperature under N$_2$; then the reaction was heated at reflux for 2 h. After solvent was evaporated, the residue was dissolved in dichloromethane (50 mL). The solution was washed with H$_2$O (5 mL), brine (5 mL), and dried over Na$_2$SO$_4$. Evaporation of solvent afforded the crude product which was purified by column chromatography (Hex/EtOAc/Acetone 1:1:0.01) to give 2-{(Phenylsulfonyl)ethanethioamide (KM-03009, 22 mg, 61%) as white solid.

2-{(Thiophen-2-yl)sulfonyl}ethanethioamide (KM-03009). White solid, yield 38%; $^1$H NMR ((CD$_3$)$_2$CO) δ 9.13 (br, 1H), 8.70 (br, 1H), 8.05-8.06 (dd, $J = 1.2$ Hz, 4.8 Hz, 1H), 7.77-7.79 (dd, $J = 1.2$ Hz,
4.0 Hz), 7.26-7.28 (dd, J = 4.0 Hz, 4.8 Hz, 1H), 4.66 (s, 2H); \(^{13}\)C NMR ((CD\(_3\))\(_2\)CO) \(\delta\) 192.7, 139.0, 135.5, 135.2, 127.9, 70.5; MS (ES\(^+\)), m/z 222 (M+1), 244 (M+Na); m.p. = 154-155 °C.

2-(Phenylsulfonyl)ethanethioamide (21b). White solid; yield 61%; \(^1\)H NMR ((CD\(_3\))\(_2\)CO) \(\delta\) 8.61-9.12 (br, 2H), 7.94-7.96 (m, 2H), 7.74-7.78 (m, 1H), 7.63-7.67 (m, 2H), 4.60 (s, 2H); \(^{13}\)C NMR ((CD\(_3\))\(_2\)CO) \(\delta\) 192.9, 138.6, 134.1, 129.0, 128.7, 69.1; MS (ES\(^+\)), m/z 216 (M+1), 238 (M+Na); m.p. = 165-166 °C.

\(N,N\)-Diethyl-2-(thiophen-2-ylsulfonyl)ethanethioamide (21c). Light yellow solid; yield 25%; \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.76-7.77 (d, J = 5.0 Hz, 1H), 7.69-7.71 (d, J = 3.8 Hz, 1H), 7.16-7.18 (m, J = 3.8 Hz, J = 5.0 Hz, 1H), 4.80 (s, 2H), 3.92-3.97 (q, J = 7.2 Hz, 2H), 3.84-3.89 (q, J = 7.2 Hz, 2H), 1.29-1.32 (t, J = 7.2 Hz, 3H), 1.25-1.29 (t, J = 7.2 Hz, 3H); \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\) 185.0, 138.2, 136.1, 134.9, 127.7, 69.3, 48.2, 47.6, 13.3, 10.8; MS (ES\(^+\)), m/z 278.2 (M+1), m.p. = 98-99 °C.

\(N,N\)-Diethyl-2-(phenylsulfonyl)ethanethioamide (21d). White solid, yield 30%; \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.88-7.90 (d, J = 7.2 Hz, 2H), 7.66-7.70 (t, J = 7.4 Hz, 1H), 7.54-7.58 (t, J = 8.0 Hz, 2H), 4.73 (s, 2H), 3.91-3.96 (q, J = 7.2 Hz, 2H), 3.86-3.90 (q, J = 7.2 Hz, 2H), 1.31-1.33 (t, J = 7.2 Hz, 3H), 1.25-1.28 (t, J = 7.2 Hz, 3H); \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\) 185.2, 137.8, 134.2, 129.2, 128.8, 68.4, 48.1, 47.6, 13.3, 10.8; MS (ES\(^+\)), m/z 272.2 (M+1), m.p. = 103-105 °C.

General Experimental Procedure for Thiation Reaction of Esters. Representative Procedure for Thiation of Ethyl 2-(Thiophen-2-ylsulfonyl)acetate (23a) (Scheme 1.3, reaction c; Scheme 1.4, reaction b): To a solution of ethyl 2-(thiophen-2-ylsulfonyl)acetate (23a, 50 mg, 0.22 mmol, 1.0 equiv.) in anhydrous o-xylene (2 mL) was added Lawesson’s reagent (174 mg, 0.43 mmol, 2.0 equiv.) under N\(_2\). The reaction was heated under reflux for 6 h. After solvent was evaporated, the residue was dissolved in...
dichloromethane (50 mL) and the solution was washed with H₂O (5 mL), brine (5 mL × 2), and dried over Na₂SO₄. Evaporation of solvent afforded the crude product, which was purified by column chromatography (Hex/EtOAc, 6:1) to give O-ethyl 2-(thiophen-2-ylsulfonyl)ethanethioate (24a, 18 mg, 34%) as light yellow solid.

**O-Ethyl 2-(thiophen-2-ylsulfonyl)ethanethioate (24a).** Light yellow solid; yield 34%; ¹H NMR ((CD₃)₂CO) δ 8.10 (s, 1H), 7.78 (s, 1H), 7.31 (d, 1H), 4.79 (s, 2H), 4.43-4.45 (d, J = 6.8 Hz, 2H), 1.27-1.30 (t, J = 6.8 Hz, 3H); ¹³C NMR ((CD₃)₂CO) δ 206.1, 139.4, 135.5, 135.4, 128.1, 72.3, 69.6, 12.7; MS (ES⁺), m/z 251 (M+1)⁺, 268 (M+H₂O)⁺, 273 (M+Na)⁺; m.p. = 50-51 °C.

**O-Ethyl 2-(phenylsulfonyl)ethanethioate (24b).** White solid; yield 31%; ¹H NMR ((CD₃)₂CO) δ 7.92-7.94 (m, 2H), 7.77-7.91 (m, 1H), 7.66-7.70 (m, 2H), 4.72 (s, 2H), 4.35-3.38 (q, J = 7.2 Hz, 2H), 1.18-1.22 (t, J = 7.2 Hz, 3H); ¹³C NMR ((CD₃)₂CO) δ 206.4, 139.0, 134.1, 129.1, 128.6, 71.1, 69.4, 12.5; MS (ES⁺), m/z 245 (M+1)⁺, 267 (M+Na)⁺; m.p. = 33-34 °C.

**O-Isopropyl 2-(thiophen-2-ylsulfonyl)ethanethioate (24c).** Yellow solid; yield 24%; ¹H NMR ((CD₃)₂CO) δ 8.07 – 8.09 (m, 1H), 7.77-7.78 (m, 1H), 7.31 – 7.29 (m, 1H), 5.47- 5.50 (m, J = 6.0 Hz, 1H) 4.75 (s, 2H), 1.27,1.29 (d, J = 6.0 Hz, 6H); ¹³C NMR ((CD₃)₂CO) δ 206.7, 141.0, 136.9, 136.7, 129.5, 78.5, 74.1, 21.4; MS (ES⁺), m/z 287 (M+Na)⁺; m.p. = 53-55 °C.

**O-Isopropyl 2-(phenylsulfonyl)ethanethioate (24d).** Yellow solid; yield 27%; ¹H NMR ((CD₃)₂CO) δ 7.93, 7.95 (d, J = 7.6 Hz, 2H), 7.77-7.79 (d, J = 7.2 Hz, 1H), 7.66-7.70 (t, J = 7.6 Hz, 8.0 Hz, 2H), 5.41- 5.43 (m, J = 6.4 Hz, 1H) 4.70 (s, 2H), 1.19-1.21 (d, J = 6.4 Hz, 6H); ¹³C NMR ((CD₃)₂CO) δ 206.9, 140.5, 135.4, 130.6, 130.0, 78.3, 72.8, 21.3; MS (ES⁺), m/z 257 (M-1)⁻; m.p. = 36-37 °C.
**O-Ethyl 2-(4-bromophenylsulfonyl)ethanethioate (24e).** Light yellow solid; yield 20%; $^1$H NMR (CDCl$_3$) $\delta$ 7.74-7.76 (d, $J = 8.8$ Hz, 2H), 7.70-7.72 (d, $J = 8.8$ Hz, 2H), 4.52 (s, 2H), 4.40-4.45 (q, $J = 7.2$ Hz, 2H), 1.29-1.33 (t, $J = 7.2$ Hz, 3H); $^{13}$C NMR (CDCl$_3$) $\delta$ 204.3, 137.0, 132.4, 130.3, 129.6, 71.4, 69.7, 13.3; MS (ES$^+$), m/z 344.9 (M+23)$^+$; m.p. = 84-85 °C.

**O-Ethyl 2-(4-methoxyphenylsulfonyl)ethanethioate (24f).** Light yellow solid; yield 18%; $^1$H NMR (CDCl$_3$) $\delta$ 7.80-7.82 (d, $J = 9.2$ Hz, 2H), 7.00-7.02 (d, $J = 9.2$ Hz, 2H), 4.51 (s, 2H), 4.40-4.45 (q, $J = 7.2$ Hz, 2H), 3.90 (s, 3H), 1.29-1.33 (t, $J = 7.2$ Hz, 3H); $^{13}$C NMR (CDCl$_3$) $\delta$ 205.1, 164.1, 131.0, 129.7, 114.2, 71.8, 69.5, 55.7, 13.3; MS (ES$^+$), m/z 297.0 (M+23)$^+$; m.p. = 58-59 °C.

**O-Ethyl 2-(propylsulfonyl)ethanethioate (24g).** Light yellow oil; yield 9.4%; $^1$H NMR (CDCl$_3$) $\delta$ 4.56-4.62 (q, $J = 7.2$, 2H), 4.37 (s, 2H), 3.21-3.25 (m, $J = 7.9$ Hz, $J = 2.4$ Hz, 2H), 1.91-1.97 (m, $J = 7.6$ Hz, $J = 2.0$ Hz, 2H), 1.44-1.48 (t, $J = 7.2$ Hz, 3H), 1.09-1.12 (t, $J = 7.2$ Hz, 3H); $^{13}$C NMR (CDCl$_3$) $\delta$ 205.7, 69.9, 68.1, 54.4, 29.7, 16.0, 13.4, 13.0; MS (ES$^+$), m/z 233.0 (M+23)$^+$.

**Compound 28a.** Light yellow solid; yield 17%; $^1$H NMR (CDCl$_3$) $\delta$ 7.93-7.95 (d, $J = 8.6$ Hz, 2H), 7.73-7.76 (d, $J = 8.6$ Hz, 2H), 7.59-7.61 (d, $J = 6.9$ Hz, 2H), 7.46-7.50 (t, $J = 7.0$ Hz, 2H), 7.42-7.44 (t, $J = 7.1$ Hz, 1H), 4.55 (s, 2H), 4.41-4.47 (q, $J = 7.1$ Hz, 2H), 1.27-1.31 (t, $J = 7.1$ Hz, 3H); $^{13}$C NMR (CDCl$_3$) $\delta$ 204.7, 147.1, 139.0, 136.6, 129.3, 129.1, 128.8, 127.6, 127.4, 71.6, 69.6, 13.3; MS (ES$^+$), m/z 319.0 (M-1)$^+$; m.p. = 70-72 °C.

**Compound 28b.** White solid; yield 21%; $^1$H NMR (CDCl$_3$) $\delta$ 7.92-7.94 (d, $J = 8.8$ Hz, 2H), 7.74-7.76 (d, $J = 8.4$ Hz, 2H), 7.52-7.54 (d, $J = 8.0$ Hz, 2H), 7.29-7.31 (d, $J = 7.6$ Hz, 2H), 4.56 (s, 2H), 4.41-4.44 (q, $J =
7.2 Hz, 2H), 2.42 (s, 1H), 1.26-1.29 (t, J = 7.2 Hz, 3H); $^{13}$C NMR (CDCl$_3$) δ 162.4, 147.2, 138.9, 136.8, 136.0, 129.8, 129.1, 127.5, 127.2, 62.4, 61.1, 21.1, 13.9; MS (ES$^+$), m/z 357.1 (M+23)$^+$; m.p. = 98-100 °C.

**General Experimental Procedure for Cross Coupling Reaction.** Representative Procedure for Cross Coupling of Ethyl 2-(4-Bromophenylsulfonyl)acetate (23g) and Phenylboronic Acid (Scheme 1.4, reaction a): A mixture of ethyl 2-(4-bromophenylsulfonyl)acetate (23g, 188.7 mg, 0.61 mmol, 1 equiv.), phenylboronic acid (180 mg, 1.47 mmol, 2.4 equiv.), Pd(OAc)$_2$ (11 mg, 0.049 mmol, 0.08 equiv.), PPh$_3$ (39 mg, 0.147 mmol, 0.24 equiv.) and K$_2$CO$_3$ (255 mg, 1.84 mmol, 3.0 equiv.) in DMF (3.3 mL) was degassed using vacuum pump and backfilled with N$_2$. Then the reaction mixture was heated and stirred at 90 °C overnight. After cooling to room temperature, the resulting mixture was poured into 5.0 mL 1N HCl (in ice bath). Then the mixture was extracted with EtOAc (40 mL). The organic extract was washed with brine (8 mL) and dried over Na$_2$SO$_4$. Evaporation of solvents and purification of the residue by column chromatography (Hexanes:EtOAc 8:1 – 5:1) gave the pure compound (27a) as white solid (79 mg, 42%).

**Compound 27a.** White solid; 42%; $^1$H NMR (CDCl$_3$) δ 8.00-8.02 (d, J = 8.8 Hz, 2H), 7.76-7.79 (d, J = 6.8 Hz, 2H), 7.47-7.51 (t, J = 6.8 Hz, 2H), 7.44-7.46 (t, J = 7.2 Hz, 1H), 4.15-4.20 (q, J = 7.2 Hz, 2H), 4.15 (s, 2H), 1.20-1.23 (t, J = 7.2 Hz, 3H); $^{13}$C NMR (CDCl$_3$) δ 162.4, 147.2, 139.0, 137.2, 129.1, 128.8, 127.8, 127.4, 62.4, 61.1, 13.7; MS (ES$^+$), m/z 327.2 (M+23)$^+$, m.p. = 108-110 °C.

**Compound 27b.** White solid; yield 49%; $^1$H NMR (CDCl$_3$) δ 7.98-7.80 (d, J = 8.8 Hz, 2H), 7.74-7.77 (d, J = 8.4 Hz, 2H), 7.50-7.53 (d, J = 8.0 Hz, 2H), 7.29-7.31 (d, J = 8.0 Hz, 2H), 4.15-4.19 (q, J = 7.2 Hz, 2H), 4.15 (s, 2H), 2.42 (s, 1H), 1.19-1.23 (t, J = 7.2 Hz, 3H); $^{13}$C NMR (CDCl$_3$) δ 162.4, 147.2, 138.9, 136.8, 136.0, 129.8, 129.1, 127.5, 127.2, 62.4, 61.1, 21.1, 13.9; MS (ES$^+$), m/z 341.3 (M+23)$^+$; m.p. = 83-85 °C.
Compound 27c. White solid; yield 37%; $^1$H NMR ((CD$_3$)$_2$CO) $\delta$ 8.06-8.11 (m, $J = 8.4$ Hz, $J = 8.8$ Hz, 4H), 8.01-8.03 (d, $J = 8.8$ Hz, 2H), 7.86-7.89 (d, $J = 8.8$ Hz, 2H), 4.42 (s, 2H), 4.10-4.12 (q, $J = 7.2$ Hz, 2H), 1.12-1.16 (t, $J = 7.2$ Hz, 3H); $^{13}$C NMR (Acetone-d6) $\delta$ 168.4, 163.5, 146.5, 142.6, 139.8, 135.8, 130.2, 129.3, 128.7, 128.3, 62.6, 61.5, 14.2; MS (ES$^+$), m/z 370.2 (M+23)$^+$; m.p. = 181-183 °C.

Ethyl 2-(23-dihydrobenzo[b][1,4]dioxin-6-yl)phenylsulfonfyl)acetate (27d). White solid; yield 88%; $^1$H NMR (CDCl$_3$) $\delta$ 7.95-7.97 (d, $J = 8.8$ Hz, 2H), 7.70-7.72 (d, $J = 8.4$ Hz, 2H), 7.14-7.15 (t, $J = 2.4$ Hz, 1H), 7.11-7.13 (m, $J = 8.4$ Hz, $J = 2.4$ Hz, 1H), 6.96-6.98 (d, $J = 8.4$ Hz, 1H), 4.31 (s, 4H), 4.14 (s, 2H), 4.14-4.17 (q, $J = 7.2$ Hz, 2H), 1.19-1.23 (t, $J = 7.2$ Hz, 3H); $^{13}$C NMR (CDCl$_3$) $\delta$ 162.4, 146.6, 144.4, 143.9, 136.6, 132.3, 129.1, 127.2, 120.5, 117.9, 116.2, 64.5, 64.4, 62.4, 61.1, 13.9; MS (ES$^+$), m/z 385.3 (M+23)$^+$; m.p. = 116-118 °C.

Ethyl 2-(4-(isoquinolin-4-yl)phenylsulfonfyl)acetate (27e). Light yellow solid; yield 30%; $^1$H NMR (CDCl$_3$) $\delta$ 9.33 (s, 1H), 8.50 (s, 1H), 8.11-8.14 (d, $J = 8.4$ Hz, 2H), 8.08-8.11 (d, $J = 7.6$ Hz, 1H), 7.80-7.82 (d, $J = 8.4$ Hz, 1H), 7.73-7.77 (m, $J = 8.4$ Hz, 2H), 7.67-7.72 (t, $J = 8.4$ Hz, 2H), 4.20-4.25 (q, $J = 7.2$ Hz, 2H), 4.23 (s, 2H), 1.24-1.28 (t, $J = 7.2$ Hz, 3H); $^{13}$C NMR (CDCl$_3$) $\delta$ 162.4, 153.1, 143.5, 142.8, 138.3, 133.6, 131.2, 130.8, 128.9, 128.1, 127.7, 124.0, 62.5, 61.0, 13.9; MS (ES$^+$), m/z 356.0 (M+1)$^+$; m.p. = 123-125 °C.

Experimental Procedure for m-CPBA Oxidation of Ethyl 2-(4-Bromophenylthio)acetate (23g). **(Scheme 1.3, reaction v):** A solution of m-CPBA (110 mg, 75% pure, 0.48 mmol, 1 equiv.) in DCM (3.5 mL) was added dropwise to a stirred solution of ethyl 2-(4-bromophenylthio)acetate (132 mg, 0.48 mmol, 1 equiv.) at 0 °C. Then the reaction mixture was stirred at 0 °C for 25 min. The reaction solution was filtered and diluted with DCM (10 mL) and was washed with saturated NaHCO$_3$ (8 mL × 2), H$_2$O (8 mL) and brine (10 mL). The organic layer was dried over Na$_2$SO$_4$. Then the solvent was removed under
vacuum to give the crude product, which was purified by column chromatography (Hex/EtOAc 3:1) to
give pure ethyl 2-(4-bromophenylsulfanyl)acetate (26a, 119 mg, 85%) as colorless crystals.

**Ethyl 2-(4-bromophenylsulfanyl)acetate (26a).** Colorless crystals, yield 85%; $^1$H NMR (CDCl$_3$) $\delta$
7.68-7.70 (d, $J = 8.4$ Hz, 2H), 7.56-7.59 (d, $J = 8.8$ Hz, 2H), 4.14-4.20 (q, $J = 7.2$ Hz, 2H), 3.83-3.86 (d, $J = 13.6$ Hz, 1H), 3.65-3.69 (d, $J = 13.6$ Hz, 1H), 1.22-1.25 (t, $J = 7.2$ Hz, 3H); $^{13}$C NMR (CDCl$_3$) $\delta$ 164.4, 142.2, 132.6, 126.3, 125.8, 62.1, 61.5, 14.0; MS (ES$^+$), m/z 290.9 (M+1)$^+$; m.p. = 62-63 °C.

**General Experimental Procedure for Hydrolysis of Esters. Representative Procedure for Hydrolysis of Methyl 2-(Thiophen-2-ylsulfonyl)acetate (22e) (Scheme 1.3, reaction d):** To a stirred
solution of methyl 2-(thiophen-2-ylsulfonyl)acetate (22e, 115 mg, 0.52 mmol, 1 equiv.) in MeOH (5 mL)
and THF (1 mL) was added a solution of NaOH (209 mg, 5.22 mmol, 10 equiv.) in H$_2$O (1 mL) dropwise.
The reaction mixture was heated and stirred at 50 °C for 5 h. Then the organic solvents were evaporated
and the mixture was diluted with 10 mL H$_2$O. The solution was adjusted to pH 10 with NaOH (5 M
aqueous solution) and extracted with DCM (10 mL × 3). The aqueous phase was acidified to pH 2, and
further extracted with DCM (30 mL × 3). The organic phase was dried over Na$_2$SO$_4$, and the solvent was
removed to get 2-(thiophen-2-ylsulfonyl)acetic acid (25a) as white solid (78 mg, 72%).

**2-(Thiophen-2-ylsulfonyl)acetic acid (25a).** White solid; yield 72%; $^1$H NMR (CD$_3$OD) $\delta$ 7.97 –
7.98 (d, $J = 4.8$ Hz, 1H), 7.79-7.81 (d, $J = 4.0$ Hz, 1H), 7.22-7.24 (m, $J = 4.0$ Hz, $J = 4.8$ Hz, 1H), 4.36 (s, 2H);
$^{13}$C NMR (CD$_3$OD) $\delta$ 165.7, 141.2, 136.6, 136.5, 129.2, 62.9; MS (ES$^+$), m/z 205 (M-1)$^+$; m.p. = 124-126 °C.
2-(Phenylsulfonyl)acetic acid (25b). White solid; yield 86%; $^1$H NMR (CD$_3$OD) δ 7.96-7.98 (d, $J = 7.6$ Hz, 2H), 7.71-7.75 (t, $J = 7.6$ Hz, 1H), 7.61-7.65 (t, $J = 8.0$ Hz, 2H), 4.29 (s, 2H); $^{13}$C NMR (CD$_3$OD) δ 165.7, 140.7, 135.4, 130.4, 129.7, 61.6; MS (ES$^-$), m/z 200 (M$-$1$)^-$; m.p. = 105-107 °C.

Experimental Procedure for the Synthesis of 5-(Bromomethyl)isoxazole (30) (Scheme 1.5, reaction a): A mixture of 5-methylisoxazole (29, 622 mg, 0.6 mL, 7.48 mmol, 1.0 equiv.), NBS (1.33 g, 7.48 mmol, 1.0 equiv.) and 2,2’-azobis(2-methyl-propionitrile) (AIBN, 25 mg, 0.15 mmol, 2% equiv.) in carbon tetrachloride (40 mL) was heated at reflux under irradiation with a tungsten light for 4.5 h. After the solvent was evaporated, ethyl acetate was added (40 mL) and the solution was washed with H$_2$O (5 mL × 2), brine (10 mL), and dried over Na$_2$SO$_4$. Evaporation of solvent afforded the crude product, which was purified by column chromatography (Hex/EtOAc, 5:1) to give 5-(bromomethyl)-isoxazole (30, 480 mg, 40%) as colorless oil.

5-(Bromomethyl)isoxazole (30). Colorless oil; yield 40%; $^1$H NMR (CDCl$_3$) δ 8.22 (s, 1H), 6.33 (s, 1H), 4.50 (s, 2H).

General Experimental Procedure for Substitution Reaction Between Thiols and 2-Bromo-1,1-dimethoxyethane (33). Representative Procedure for Substitution Between Thiophene-2-thiol (18a) and 2-Bromo-1,1-dimethoxyethane (33) (Scheme 6, reaction a): A mixture of absolute ethanol (3 mL) and sodium (152 mg, 6.6 mmol, 1.2 equiv.) in a dry flask was heated to reflux until the solution was clear and cooled in an ice bath (under nitrogen). Thiophene-2-thiol (18a, 767 mg, 0.6 mL, 6.6 mmol, 1.2 equiv.) was added at 0 °C under N$_2$ and the reaction was stirred for another 15 min at 0 °C. Then 2-bromo-1,1-dimethoxyethane (33, 97%, 930 mg, 0.67 mL, 5.5 mmol, 1.0 equiv.) was added and the reaction was heated at reflux for 2 h. The solution was filtered and solvent was evaporated. Ethyl acetate (50 mL) was
added and the solution was washed with H₂O (8 mL × 2), saturated NaCl (8 mL), and dried over Na₂SO₄. Evaporation of solvent gave the crude product which was purified by chromatography (Hex/EtOAc, 20:1) to give 2-(2,2-dimethoxyethylthio)-thiophene (34a, 538 mg, 48%) as colorless oil.

**2-(2,2-Dimethoxyethylthio)thiophene (34a).** Colorless oil; yield 48%; ¹H NMR (CDCl₃) δ 7.35-7.37 (dd, J = 1.2 Hz, J = 5.2 Hz), 7.17-7.19 (dd, J = 1.2 Hz, J = 3.6 Hz, 1H), 6.97-6.99 (d, J = 5.6 Hz, 1H), 3.52-3.54 (t, J = 5.6 Hz, 1H), 3.37 (s, 6H), 2.99-3.00 (d, J = 5.6 Hz, 2H); ¹³C NMR (CDCl₃) δ 134.0, 134.0, 129.6, 127.5, 103.3, 53.5, 40.9.

**(2,2-Dimethoxyethyl)(phenyl)sulfane (34b).** Colorless oil; yield 59%; ¹H NMR (CDCl₃) δ 7.40-7.42 (m, 2H), 7.28-7.33 (m, 2H), 7.21-7.23(t, 1H), 4.54-4.57 (t, J = 5.6 Hz, 1H), 3.39 (s, 1H), 3.14-3.15 (d, 5.6 Hz); ¹³C NMR (CDCl₃) δ 136.1, 129.5, 129.1, 129.0, 126.3, 103.2, 53.6, 36.5.

**General Experimental Procedure for Hydrolysis of Aldehyde Dimethyl Acetals (34).**

**Representative Procedure for Hydrolysis of 2-(2,2-dimethoxyethylthio)-thiophene (34a) (Scheme 1.6, reaction b):** To a solution of 34a (113 mg, 0.55 mmol) in acetone (0.6 mL) was added 1% HCl (0.6 mL); then the reaction was heated under reflux for 1.5 h. Solvent was evaporated and ethyl acetate (50 mL) was added. The organic layer was washed with saturated NaHCO₃ (8 mL), H₂O (8 mL × 3), brine (8 mL), and dried over Na₂SO₄. The evaporation of solvent afforded the crude product, which was purified by column chromatography (Hex/EtOAc 6:1) to give 2-(thiophen-2-ylthio)acet-aldehyde (35a, 44 mg, 51%) as light yellow oil.
2-(Thiophen-2-ylthio)acetaldehyde (35a). Light yellow oil; yield 51%; \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 9.63-9.65 (t, \(J = 3.2\) Hz, 1H), 7.40-7.43 (m, 1H), 7.19-7.20 (m, 1H), 7.99-7.04 (m, 1H), 3.46-3.49 (m, \(J = 3.2\) Hz, 2H); MS (ES\(^+\)), m/z 159 (M+1) \(^+\).

2-(Phenylthio)acetaldehyde (35b). Colorless oil, yield 94%; \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 9.54-9.55 (t, \(J = 3.2\) Hz, 1H), 7.25-7.38 (m, 5H), 3.60-3.61 (d, \(J = 3.2\) Hz, 2H); MS (ES\(^+\)), m/z 153 (M+1) \(^+\).


Representative Procedure for Synthesis of 2-(thiophen-2-ylthio)acetaldehyde oxime (36a) (Scheme 1.6, reaction c): A mixture of 2-(thiophen-2-ylthio)acetaldehyde (35a, 144 mg, 0.91 mmol, 1.0 equiv.), hydroxylamine hydrochloride (95 mg, 1.37 mmol, 1.5 equiv.) and potassium bicarbonate (137 mg, 1.37, 1.5 equiv.) in MeOH (14 mL) was stirred at room temperature overnight. The solution was filtered and solvent was evaporated. Then ethyl acetate (50 mL) was added and the solution was washed with H\(_2\)O (10 mL \(\times\) 2), saturated NaCl (8 mL), and dried over anhydrous Na\(_2\)SO\(_4\). Evaporation of solvent afforded crude product which was purified by chromatography (Hex/EtOAc, 6:1) to give 2-(thiophen-2-ylthio)acetaldehyde oxime (36a, 106 mg, 67%) as light yellow oil.

2-(Thiophen-2-ylthio)acetaldehyde oxime (36a). Light yellow oil; yield 67%; \(^1\)H NMR ((CD\(_3\))\(_2\)CO, mixture of isomers, ratio 1/1.5) \(\delta\) 9.94, 10.31 (s, 1H), 6.81-7.59 (m, 4H), 3.53-3.55, 3.72-3.74 (d, 2H); MS (ES\(^+\)), m/z 174 (M+1) \(^+\).

2-(Phenylthio)acetaldehyde oxime (36b). Colorless oil; yield 91%; \(^1\)H NMR ((CD\(_3\))\(_2\)CO, mixture of isomers, ratio 1/1) \(\delta\) 10.49 (s, 0.5 H), 10.50 (s, 0.5H), 7.66-7.98 (m, 5H), 7.33 (t, 0.5 H), 6.82 (t, 0.5 H), 4.36-4.37 (d, 1H), 4.12-4.13 (d, 1H); MS (ES\(^+\)), m/z 168 (M+1) \(^+\).
General Experimental Procedure for the Reduction Reaction. Representative Procedure for Reduction of \(N\)-(2-(thiophen-2-ylsulfonyl)ethyl)-hydroxylamine (38a) (Scheme 1.6, reaction e): To a solution of 2-(thiophen-2-ylsulfonyl)acetaldehyde oxime (37a, 46 mg, 0.22 mmol, 1.0 equiv.) in MeOH (2 mL) was added sodium cyanoborohydride (37 mg, 0.59 mmol, 2.68 equiv.); then the solution of 4.0 M HCl in 1.4-dioxane (0.2 mL) was added in three portions to maintain \(\text{pH} = 3.0\) during the reaction time (3.5 h). Then the solution of 5 N NaOH was added to adjust the pH to 11.0 and then solvent was evaporated. Dichloromethane (50 mL) was added and the solution was washed with saturated NaCl (8 mL \(\times\) 2), and dried over anhydrous Na\(_2\)SO\(_4\). Evaporation of solvent afforded the crude product, which was purified by column chromatography (Hex/EtOAc, 2:3) to give \(N\)-(2-(thiophen-2-ylsulfonyl)ethyl)-hydroxylamine (38a, 14 mg, 30%) as white solid.

\(N\)-(2-(Thiophen-2-ylsulfonyl)ethyl)hydroxylamine (38a). White solid, 30% in two steps; \(^1\)H NMR (CDCl\(_3\)) \(\delta 7.73\)−7.77 (m, 2H), 7.17-7.19 (m, 1H), 7.53-7.56 (t, \(J = 6.4\) Hz, 2H), 3.37-3.40 (t, \(J = 6.4\) Hz, 2H); \(^{13}\)C NMR (CDCl\(_3\)) \(\delta 140.2, 134.3, 128.1, 54.7, 47.6\); MS (ES\(^+\)), \(m/z\) 208 (M+1)

\(N\)-(2-(phenylsulfonyl)ethyl)hydroxylamine (38b). White solid; yield 38%; \(^1\)H NMR (CDCl\(_3\)) \(\delta 7.93\)−7.95 (m, 2H), 7.67-7.71 (m, 1H), 7.57-7.61 (m, 2H), 5.50 (br, 1H), 3.42-3.45 (t, \(J = 6.0\) Hz, 2H), 3.33-3.36 (t, \(J = 6.0\) Hz, 2H); \(^{13}\)C NMR (CDCl\(_3\)) \(\delta 139.3, 133.9, 129.4, 128.0, 53.1, 47.3, 135.8, 129.1, 128.5, 126.0, 57.0, 30.0\); MS (ES\(^+\)), \(m/z\) 202 (M+1); m.p. = 67-68 °C.

1.3.2 Whole cell Al-2 inhibition assay in V. harveyi

All compounds tested were of high purity (about 98% with impurities being mostly solvents) as judged by \(^1\)H-NMR. The NMR spectra of all final compounds are provided in the Supplemental Section. MM32 and BB886 strains of \(V.\) harveyi were purchased from ATCC (MM32 #BAA-1121, BB886 #BAA-
The quorum sensing assays were performed by following literature. In the AI-1 bioassays, cell-free culture (5% of the test medium volume) from BB886 with OD$_{600}$ of 0.8 - 1.2 was added as the source of AI-1. Kanamycin (50 µg/mL) was used as antibiotics in incubation and inoculation.

1.3.3 Cytotoxicity assay in V. harveyi

Cell growth test (MM32)

Cell growth test was performed according to literature protocol. MM32 strain of V. harveyi (ATCC #BAA-1121) was grown for 16 h with aeration (175 rpm) at 30 °C in 2 mL of AB medium with antibiotics (kanamycin 50 µg/mL and chloramphenicol 10 µg/mL). Then this bacterial culture was diluted 100-fold with 20 mL AB medium in a 250 mL flask, tested compounds were added and incubated at 30 °C (175 rpm). The OD$_{600}$ values were determined every 20 min. The doubling time was calculated based on the OD$_{600}$ values.

Plate counting test (MM32)

Plate counting test was performed on the basis of the MM32 test. MM32 bacteria were streak-seeded on fresh LM plates and then cultured in the presence of kanamycin 50 µg/mL and chloramphenicol 10 µg/mL. Colonies appeared after overnight incubation at 30 °C. Single colonies were picked from the LM plates and were grown for 16 h with aeration (175 r.p.m.) at 30 °C in 2 mL of Autoinducer Bioassay (AB) medium with antibiotics (kanamycin 50 µg/mL and chloramphenicol 10 µg/mL). Then the solution was diluted to OD$_{600}$ 0.7 and the bacteria preinoculum was grown in AB-Fe medium with 1.2 mM of iron to a OD600 of 1.0-1.1 with shaking at 30 °C (175 r.p.m.) for 1-1.5 h. The resulting inoculum culture was then diluted 5000-fold in fresh AB medium. Solutions of the test compounds in AB medium at concentrations ranging from 0 to 400 µM were prepared in 96-well plates. To these solutions, freshly synthesized DPD solution (pH 7) was added for a final concentration of 5 µM. Boric acid was added to give a final concentration of 1 mM. After addition of bacteria in AB medium, the
micro plates were covered with a non-toxic plate sealer and incubated at 30 °C with aeration for 3-5 h. Light production was measured every hour using a Perkin-Elmer luminescence microplate reader. The bacteria solution incubated for 5 h in the presence of a compound at a concentration, which was close to its IC₅₀ was diluted to 1:100 and 1:1000, and plated onto fresh LM plates and incubated at 30 °C for 24-48 h. The colonies appeared were counted. The concentration of bacteria, which yielded 30-300 colonies was considered valid and CFU was recorded. The CFU (colony forming unit) ratio (CFU₁₂/CFU_blank) and the luminescence ratio (Luminescence₁₂/Luminescence_blank) were compared.

1.4 Conclusions

In this study, structure optimization was performed with two hit compounds obtained from virtual screening. Thirty nine new analogs were successfully synthesized and tested. Among all the synthesized analogs, 12 were found to show good inhibition activity with IC₅₀ values below 40 µM, 4 of which showed single digit micromolar IC₅₀ values, while 6 out of the 12 possess good selectivity toward AI-2 mediated quorum sensing. Overall, the following structural features are beneficial to AI-2 inhibition activities: a sulfone group (part B), “thiation” of the carbonyl group of part C, a hydrophobic group of modest size in part D, and a biphenyl system in part A.

1.5 Acknowledgment

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2 A METHOD FOR RAPID AND SPECIFIC DNA POST-SYNTHESIS MODIFICATION

Abstract:

Nucleic acids have been used in a number of applications such as therapeutic agents and nanomaterials. Post-synthesis modification of DNA is an important way of functionalizing DNA molecules for extended applications. In this research, a method that enzymatically incorporates a cyanobenzothiazole (CBT)-modified thymidine has been described. The side chain handle CBT can undergo a rapid and site-specific cyclization reaction with 1,2-aminothiols to afford DNA functionalization in aqueous solution. Another key advantage of this method is the formation of a single stereo/regioisomer in the process, which allows for precise control of DNA modification to yield a single component.

2.1 Introduction

2.1.1 Therapeutic applications of nucleic acids

As one of the most important biomolecules, nucleic acids such as DNA and RNA are known to be involved in the storage and transferring of genetic information. On the other hand, small interfering RNA (siRNA)\(^{22}\) and microRNA (miRNA)\(^{23}\) were found to play important roles in post-translational gene regulations. In 1977, Paterson and co-workers found that an exogenous oligonucleotide could inhibit translation in a cell-free system.\(^ {24}\) Later Stephenson and Zamecnik showed that a short synthetic DNA complementary to the Rous sarcoma virus 35S RNA could inhibit viral replication in the culture.\(^ {25}\) These “antisense” nucleic acids interfere with the translation by binding with the “sense” mRNA strand, either sterically obstructing the ribosomes or forming a DNA-RNA hybrid, which causes the selective cleavage of the target mRNA by RNase H.\(^ {26}\) The high specificity and low toxicity have suggested that antisense mechanism could be especially useful in diagnostics and therapeutics.\(^ {26-28}\) Fomivirsen, which is an
antisense drug inhibiting cytomegalovirus (CMV) replication, has been approved by US FDA in 1998 for the treatment of CMV retinitis.\textsuperscript{29, 30}

Besides antisense therapy, nucleic acid aptamers are emerging as promising therapeutic agents.\textsuperscript{31-33} Nucleic acid aptamers are DNA or RNA molecules that form complex three-dimensional structures and could bind to specific targets with high affinity. Aptamers are obtained from combinatorial nucleic acid libraries through in vitro selection process termed SELEX (Systematic Evolution of Ligands by Exponential Enrichment).\textsuperscript{34-36} In this process, nucleic acid strands in the library bound with the target were separated from the unbound to form an enriched library, which is subject to amplification and subsequently another round of selection. Repeated positive selection process and counter-selection could ensure the binding affinity and specificity. The target of aptamer range from small molecules such as theophylline,\textsuperscript{37} flavin mononucleotide (FMN)\textsuperscript{38} and adenosine triphosphate (ATP),\textsuperscript{39} proteins such as thrombin,\textsuperscript{34} streptavidin\textsuperscript{40} and reverse transcriptase\textsuperscript{41} to complex systems such as cell surface.\textsuperscript{42} These aptamers bind to their targets selectively with dissociation constants in the nanomolar to micromolar range. Compared to antibodies which are relatively unstable and are raised from time-consuming in vivo approaches, nucleic acid aptamers exhibit advantages such as low-cost solid phase synthesis and easy chemical modifications. Therefore, aptamers show great potential in the application of diagnostics and therapeutics. Pegaptanib, which is an RNA aptamer against vascular endothelial growth factor (VEGF)-165, has been approved by US FDA for the treatment of neovascular age-related macular degeneration (AMD).\textsuperscript{43}

In addition to the therapeutic applications of nucleic acids in antisense and aptamer therapy, the unique physical and chemical characteristics of DNA as genomic materials also make them very useful for various other applications. For example, DNA has been explored for the application in nanoarchitectures,\textsuperscript{44} nanorobots,\textsuperscript{45} nanocomputing,\textsuperscript{46} reaction encoding,\textsuperscript{47} and nanosensing.\textsuperscript{48} There is a growing interest in the exploration of nucleic acids as therapeutic agents and novel nano-materials.
2.1.2 DNA post-synthesis modifications through bioorthogonal reactions

Although research results and commercial availability of nucleic acid drugs are evidence for the promising potential of nucleic acids as therapeutic agents, the clinical success has not been satisfactory. First, nucleic acids are prone to degradation by nucleases in the biological environment. RNA molecules are also unstable under basic conditions. Second, they show extremely poor membrane permeability due to the highly charged backbone. These two properties decreased the bioavailability of unmodified nucleic acids. Third, much less functional groups for binding are found in nucleic acids compared to proteins and peptides. This might be one reason for the inefficient aptamer selection process. In addition, for many applications, nucleic acids must be equipped with specific functionalities, such as fluorescent markers, biotin, thiols, sugars, proteins, positively charged peptides (octaarginines), and boronic acid for endowing different properties. Therefore, tremendous efforts have been made to modify nucleic acids in order to improve their bioavailability and functionality.

Typical strategies for nucleic acid modifications are shown in Figure 2.1. These strategies include 1) changing the 2-hydroxyl group on RNA to fluoro, amino or methoxyl groups or using methylene bridges to lock the ribose structure (locked nucleic acids or LNA) in order to avoid degradation of RNA molecules; 2) backbone modifications such as using phosphorthioate instead of phosphodiester linkage to avoid nuclease degradation; and 3) conjugation with other molecules. Oligonucleotide conjugation could be done at 5'- or 3'- termini during or after solid-phase synthesis because of their easy accessibility. Recent research has found that modifications on C5' of thymine or C7'-of adenine could be well tolerated by DNA polymerases, especially polymerases from the family B to form high-density functionalized DNA molecules.
Figure 2.1 Strategies for nucleic acid modification

In our work of preparing modified DNA, we are especially interested in building boronic acid-functionalized DNA libraries for aptamer selection. It is well known that boronic acid as a Lewis acid undergoes reversible covalent interactions with nucleophiles, especially 1,2- and 1,3-diol structures, which are commonly found on saccharides (Figure 2.1). Previous work from our group has identified a bis-boronic acid that could selectively recognize Sialyl Lewis X (sLex) expressed on cancer cell surface.

Scheme 2.1 Boronic acid-diol interaction
Glycosylation is one of the most important protein post-translational modifications and is involved in a variety of biological processes such as cell signaling and cell-cell interactions.\textsuperscript{58} Altered protein glycosylation patterns are implicated in a number of diseases such as diabetes\textsuperscript{59} and cancer.\textsuperscript{60, 61} Carbohydrate-based biomarkers have been identified for many diseases.\textsuperscript{62-65} However, protein glycosylation is not directly encoded in the genome and is controlled by various factors. This has made glycosylation extremely complicated and difficult to study. Due to their high binding affinity and specificity, aptamers could be a useful tool for the study of glycosylation and thus possible diagnostic and therapeutic agents for glycosylation-related diseases. Considering the intrinsic affinity of boronic acid to carbohydrates, it is conceivable that when DNA is modified with a boronic acid moiety, the aptamer selection might be gravitate toward the glycosylation site and therefore for specific differentiation of the glycosylation patterns.

Recent advances in bioorthogonal reactions (or “click” chemistry)\textsuperscript{49} such as Huisgen 1,3-dipolar cycloaddition has brought new strategies for DNA modification. Specifically, the labs of Seela\textsuperscript{66} and Carell\textsuperscript{67} first reported the functionalization of DNA nucleobases through copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction, which was developed by Sharpless\textsuperscript{68} and Meldal.\textsuperscript{69} This protocol is highly efficient and specific, and results in almost quantitative incorporation of different labels into the DNA. While these seminal reports have allowed easy DNA modification, the CuAAC method has the issue of copper-catalyzed cleavage of DNA.\textsuperscript{69} In addition, Cu(I) also poses stability problems for some other functional groups. For example, boronic acid was reported to be degraded during post-synthetic functionalization of DNA using CuAAC.\textsuperscript{70} Copper-free chemistry has thus been used recently by different researchers, including strain-promoted azide-alkyne cycloaddition,\textsuperscript{71} Staudinger ligation,\textsuperscript{72, 73} Diels-Alder reaction,\textsuperscript{74, 75} hydrazone\textsuperscript{76} and oxime\textsuperscript{77} formation, and native peptide ligation.\textsuperscript{51, 78} Although these methods provide alternative ways for copper-free DNA labeling, they still possess some limitations, e.g,
low reaction rate, the need of other reagents/organic solvents for functionalization, as well as the possibility of generating stereo/regioisomers.

In the previous studies our group has successfully demonstrated the synthesis and enzymatic incorporation of C₅-boronic acid-modified TTPs (B-TTP) into DNA⁷⁹⁻⁸¹ (Figure 2.2). A series of B-TTPs (Compounds 43-48) have been synthesized and successfully incorporated into DNA enzymatically through primer extension or polymerase chain reactions (PCR).

![Figure 2.2 Incorporation of boronic acid moiety into DNA through pre-functionalized B-TTP](image)
To explore solid-phase synthesis of modified DNA and to avoid Cu(I)-mediated degradation of specific boronic acid we have also synthesized deoxyuridine phosphoramidite (49) bearing DIFO, a strained alkyne handle to incorporate functional groups such as boronic acids through copper-free post-synthesis modification (Figure 2.3). It was found that the copper-free “click” chemistry could be used reliably for DNA decoration. In addition to the ability to attach boronic acid moieties, other functional groups could be incorporated. Therefore, DNA molecules with a “general” handle is superior to the pre-functionalized ones due to versatility and synthetic simplicity.

Figure 2.3 Incorporation of boronic acid moiety into DNA through solid-phase synthesis and copper-free post-synthesis modification

Although the previous work paved the way for enzyme-catalyzed functionalization with a boronic acid and the large scale synthesis of single stranded boronic acid-modified DNA for further applications, several limitations still need to be addressed, including the complex synthesis of different B-TTP moieties, Cu(I)-mediated degradation of specific boronic acid functional groups, the potential problems
during enzymatic recognition as well as multiple stereoisomers generated due to lack of symmetry. In an attempt to circumvent these restrictions, and most importantly, to develop a novel platform for the rapid and specific post-synthesis modification of DNA that can address some limitations of current methods, we are further interested in the strategy of enzyme-catalyzed (such as PCR) synthesis of DNA with a “general handle”, which can undergo a rapid and specific post-synthesis functionalization. Specifically, we need an approach that allows (1) high enzymatic incorporation efficiency of the “general handle” modified nucleotide; (2) fast, site-specific, and chemoselective post-synthesis modification with its reactive partner in aqueous solution; (3) high yield of a single stereochemically pure product. The last point is especially important and remains a major obstacle for further application in DNA modification, such as aptamer selection. Because even if the yield of the desired isomer is 90% at a single base, the synthesis of a 90-mer DNA would have the possibility of generating multiple isomers with random distribution of regio-/stereo-isomers at different positions. In the present research, a method that meets the above-mentioned criteria for post-synthesis modification of DNA is described.

2.2 Results and discussion

2.2.1 Synthesis of CBT-modified deoxyuridine (CBT-TTP) and labeling agents and kinetic study of the cycloaddition reaction

To start this project, we first explored the well-developed repertoire of biocompatible reactions to find the appropriate reaction partners for highly reaction selectivity, absence of stereoisomers, and compatibility with biological systems. The condensation of 1,2-aminothiol with 2-cyanobenzothiazole (CBT) was chosen. This reaction, known as the last step of the bio-synthesis of luciferin, has drawn much attentions recently, largely due to the rejuvenation work of Rao and Chin. In employing this reaction for post-synthesis DNA labeling, we have the option of either incorporating a 1,2-aminothiol or CBT into the nucleotide. We chose the pathway of making the CBT-modified nucleotide, which does not
involve protection/deprotection during the synthesis. In addition, the 1,2-aminothiol also renders the click-labeling agent good solubility in aqueous solution. Based on the known fact that 5-position modification of deoxyuridine can be tolerated by polymerases and reverse transcriptases and our own successful experience in developing a series of 5-position boronic-acid functionalized deoxyuridines, we plan to synthesize a CBT-modified dUTP analogue (CBT-TTP, 50) for enzymatic incorporation into the DNA strand (Figure 2.4).

CBT-TTP (50) was prepared in 8 steps. Specifically, commercially available 1,4-benzoquinone (51) undergoes Michael addition reaction with L-cysteine ethyl ester (52). Oxidative cyclization and ring-shrinking reaction yields ethyl 6-hydroxybenzo[d]thiazole-2-carboxylate (55) in two steps. Propargylation, followed by ammonolysis and dehydration provided a modified CBT with terminal alkyne moiety 58. Subsequent Sonogashira reaction yielded CBT-T 59. Triphosphorylation was accomplished by
the classical one-pot three-step method\textsuperscript{93} (Scheme 2.2) to provide CBT-TTP (60) in 1.3\% overall isolated yield, which was then used for enzymatic incorporation.

\begin{center}
\includegraphics[width=\textwidth]{scheme2_2.png}
\end{center}

\textbf{Scheme 2.2 Synthesis of Compound 50 (CBT-TTP)}

The labeling agent, boronic acid-modified cysteine analogue (Cys-BA, 65) was synthesized in 4 steps (Scheme 2.3). According to literature procedures,\textsuperscript{92} commercial L-cysteine was protected with acetone and Boc protecting groups to form compound 62, which is then linked to the boronic acid moiety through amide bond formation. Subsequent de-protection steps yield Cys-BA (65) in 55\% overall isolated yield.
Before the incorporation study, we studied the reaction profile. CBT has been reported to react rapidly and specifically with 1,2-aminothiol with a second-order rate constant of $9 \text{ M}^{-1}\text{s}^{-1}$. We reasoned that CBT-TTP would proceed in the same fashion. To confirm this, kinetic study using literature methods was performed for the reaction between the precursor CBT-T (59, Scheme 2.2) and cysteine. The reaction mixture was stirred at room temperature and monitored by HPLC at different time points. The second-order rate constant was determined to be $22 \text{ M}^{-1}\text{s}^{-1}$ (Figure 2.5) under near physiological conditions (phosphate buffer, pH = 7.4)).
2.2.2 Enzymatic incorporation and post-synthesis labeling of CBT-TTP

2.2.2.1 Primer extension and click labeling

Initially, the incorporation of CBT-TTP into DNA was studied through primer extension. Specifically, using CBT-TTP and the Klenow fragment, was conducted using a short sequence of 21-mer oligonucleotide (nt) Template-1 (5’-GGTTCCAGCAACCCGCTA-3’) and a 14-mer FAM labeled primer (5’-(FAM)-TAGCGGGTGTGGCTGG-3’) (Figure 2.6), which have been successfully used in our previous incorporation studies of different functionalized DNA.\(^\text{79,80,88}\) Klenow fragment (3’-5’ exo-) was used to avoid cleavage of the template. The primer was designed in such a way that the first incorporated base would be a T, so there are two possible scenarios in the extension: either fully extended product or no extension at all. The obtained DNA products were studied using polyacrylamide gel electrophoresis (PAGE). As shown in Figure 2.6A, negative controls without dTTP (Lane 1, Figure 2.6A), without Klenow fragment (Lane 2, Figure 2.6A), and primer plus template only (Lane 3, Figure 2.6A) showed no full length DNA sequence. Instead, annealing products were observed. Negative control with primer only (Lane 4, Figure 2.6A) did not show annealing product due to the absence of template. On the other hand,
primer extension product using CBT-TTP (Lane 6, Figure 2.6A) in place of dTTP gave a similar full-length DNA band as that of positive control with Klenow fragment and natural dNTPs only (Lane 5, Figure 2.6A). Such results indicated that synthesized CBT-TTP could be recognized similarly as dTTP by the Klenow fragment, and incorporated into DNA.

After demonstrating the successful incorporation of CBT-TTP, we further explored the feasibility of post-synthesis modification of DNA. The same 21-nt template and 14-nt primer without FAM label (5'-TAGCGGGTTGCTGG-3') were used. Synthesized Cys-BA was used for the labeling of CBT-DNA. As shown from Figure 2.6B, fully extended product was observed using both dNTPs (Lane 1, Figure 2.6B) and CBT-TTP (Lane 2, Figure 2.6B). However, after being treated with Cys-BA (1 mM, final concentration (50 equiv.), 30 min, supplemented with tris(2-carboxyethyl)phosphine (TCEP, 1 mM final concentration)) with the primer extension product, only CBT-DNA21 (CBT incorporated DNA product, 21nt) showed the post-synthesis product as expected (Lane 4, Figure 2.6B). The reduced mobility of Cys-BA “click”-labeled CBT-DNA21 indicated the reaction, which is presumably due to the interaction between boronic acid and polyacrylamide matrix.\(^{70,80}\) The result was further supported by the band with different mobility after treating the Cys-BA “click” labeled CBT-DNA\(_{21}\) with H\(_2\)O\(_2\) (1 mM final concentration, 1 h) (Lane 5, Figure 2.6B), which is due to the well-known oxidation reaction of converting phenyl boronic acid functional group into phenol group.\(^{93}\) As a control, the mobility for the dNTPs-DNA\(_{21}\) (extended DNA product using dNTPs, 21nt) band did not change after treating with Cys-BA (Lane 3, Figure 2.6B), indicating no reactions as expected.
A. Primer extension using CBT-TTP catalyzed by Klenow fragment (3'-5'exo-), 20% PAGE analysis; B. Post-synthesis labeling using “click” reagent Cys-BA, 20% PAGE analysis. 1) dNTPs-DNA₂₁, 2) CBT-DNA₂₁, 3) dNTPs-DNA₂₁ + Cys-BA, 4) CBT-DNA₂₁ + Cys-BA, 5) Cys-BA “Click” labelled CBT-DNA₂₁ treated with H₂O₂;

Understandably, mobility studies alone would not be enough to prove the post-synthesis modifications. MALDI-MS was used to further examine the DNA products. Specifically, dNTP-DNA₂₁ treated with Cys-BA (Lane 3, Figure 2.6B) had the same peak with m/z of 6518 (Figure 2.7B, calculated [M+H]+: 6519, for MALDI spectrum please see Appendix) as the original DNA product. Such results indicate that Cys-BA does not interfere/react with dNTP-DNA₂₁, as expected. In contrast, full extension of the primer using CBT-TTP instead of dTTP yielded a CBT-DNA₂₁ (Lane 2, Figure 2.6B) with m/z of 6716 (Figure 2.7A, calculated [M+H]+: 6717) in MALDI-MS. When treated with Cys-BA, CBT-DNA₂₁ was converted to a “click” labeled product (Lane 4, Figure 2.6B) with m/z of 6917 (Figure 2.7C, calculated [M-2H₂O+H]+: 6917), corresponding to the Cys-BA “click” labeled CBT-DNA₂₁ product. After treating the “click” product (Lane 4, Figure 2.6B) with H₂O₂, a product (Lane 5, Figure 2.6B) with m/z of 6929 (Figure 2.7D, calculated [M+H]+: 6927), corresponding to the boronic acid oxidation product, was observed as expected. Such results confirmed the intended click-modifications.
Figure 2.7 MALDI spectra of DNA products.

A. dNTP-DNA; B. CBT-DNA; C. boronic acid modified CBT-DNA; D. boronic acid modified CBT-DNA treated with H₂O₂.

Although C₅-position of thymine moiety is not involved in base paring, we were still interested in the effect of a CBT and boronic acid moiety on the thermostability of a DNA duplex. This was investigated through a thermodenaturation study. Double stranded DNA₂₁ prepared through Klenow fragment (3′-5′-exo) was purified using Millipore Amicon 3k membrane filter and used for the thermostability test in PBS buffer. Considering the reversible denaturation of dsDNA, the UV absorbance of the cell was recorded while the temperature was decreasing by a rate of 0.5 °C/min. UV melting curves are shown in Figure 2.8. The results (Table 2.1) have suggested that the incorporation of one CBT
or boronic acid moiety in a 21-bp DNA duplex only slightly decreased its stability ($T_m$ decreased from 73.00 °C to 70.96 °C and 70.24 °C, respectively).

After successful incorporation of one CBT moiety into DNA using Klenow fragment-catalyzed primer extension reaction, we further explored the feasibility of incorporating multiple CBT moieties. Thus, 21-nt Template-2 (5'-TCAGTCACCACCGGCTA-3') and Template-3 (5'-CACGACACCAGCAACCGCTA-3') were designed to incorporate two and three CBT-TTP, respectively. To our surprise, the primer extension reaction catalyzed by Klenow fragment was unsuccessful due to multiple incomplete bands, even at elevated temperature or longer reaction time (results not shown).

Knowing family B polymerases are relatively more tolerance to modified TTP,55,94 a family B polymerase

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**Figure 2.8 UV-melting curves of DNA duplexes.**

A. dNTPs-DNA$_{21}$, B. CBT-DNA$_{21}$, C. dNTPs-DNA$_{21}$ + Cys-BA.

**Table 2.1 Melting temperatures ($T_m$) of DNA duplexes**

<table>
<thead>
<tr>
<th>DNA/Rounds</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Average</th>
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<tr>
<td>dNTPs-DNA$_{21}$</td>
<td>72.97</td>
<td>73.02</td>
<td>73.02</td>
<td>72.97</td>
<td>73.00 ± 0.03</td>
</tr>
<tr>
<td>CBT-DNA$_{21}$</td>
<td>70.94</td>
<td>70.97</td>
<td>70.97</td>
<td>70.97</td>
<td>70.96 ± 0.02</td>
</tr>
<tr>
<td>CBT-DNA$_{21}$ + Cys-BA</td>
<td>69.87</td>
<td>70.92</td>
<td>69.92</td>
<td>70.92</td>
<td>70.24 ± 0.59</td>
</tr>
</tbody>
</table>
(KOD XL) from Thermococcus kodakaraensis, which is a mixture of the natural form and an exo- mutant, was thus chosen instead for the reaction. As is shown in Figure 2.9A, the electrophoretic mobility of modified DNA21 (lanes 2, 3 and 4) was further decreased with the incorporation of more CBT moieties. This clearly indicated the successful incorporation of multiple CBT-TTP into DNA through enzyme-catalyzed reactions. This was further confirmed by MALDI analysis of the incorporated product using Template-2 and Template-3 (calculated [M+H]⁺ for Template-2: 6929, found: 6930; calculated [M+H]⁺ for Template-3 7135, found: 7136, for MALDI spectrum please see Appendix). The extension product using Template-3 was further used for post-synthesis modification by Cys-BA. As is shown in Figure 2.9B, the incorporated product (lane 2) could be successfully labeled by Cys-BA, showing a slower-moving band (lane 3), which is consistent with the phenomena observed when using the template with one CBT moiety incorporation.

![Figure 2.9 Incorporation and post-synthesis labeling of multiple CBT moieties.](image)

A. Primer extension using CBT-TTP catalyzed by KOD-XL DNA polymerase, 20% PAGE analysis. 1) Template-1 with dTTP, 2) Template-1 with CBT-TTP, 3) Template-2 with CBT-TTP, 4) Template-3 with CBT-TTP; B. Post-synthesis labeling using extension product of Template-3 amd “click” reagent Cys-BA, 20% PAGE analysis, 1) dNTPs-DNA21, 2) CBT-DNA21, 3) CBT-DNA21 + Cys-BA.
2.2.2.2  **PCR amplification and click labeling**

Encouraged by the successful incorporation of CBT-TTP into short DNA sequences, we further investigated the PCR amplification of a longer 90-mer DNA strand. A 90nt ssDNA template (5'-CCTTCGTTGCTGGCAGAGCCAGTCAGACGCAGCTGACGTGCCAAGCACTATGACGGACACCCTT CAGAATTGCACCA-3') was used in the PCR study. We first screened the incorporation using three commercially available polymerases, including a family A polymerase from *Thermus aquaticus* (Taq) and family B polymerases from *Thermococcus litoralis* (Deep Vent, exo) and *Thermococcus kodakaraensis* (KOD XL), which was used for primer extension experiments. As can be seen from the results summarized in Figure 2.10, incorporation of CBT-TTP were inefficient when using *Taq* (Lane 2, Figure 2.10) and Deep Vent (Lane 6, Figure 2.10). On the other hand, efficient incorporation was obtained by using KOD XL polymerase, similar to the primer extension experiments for multiple CBT moieties incorporation.

![Figure 2.10 Enzyme screening for PCR incorporation of CBT-TTP.](image)

Lanes 1, 3, and 5 are using dNTPs, and lanes 2, 4 and 6 are using CBT-TTP instead of dTTP. Lanes 1, 2: *Taq* polymerase, 3, 4: KOD-XL polymerase, 5, 6: Deep Vent (exo-) Polymerase.

Since experiment has indicated that KOD XL could efficiently incorporate CBT-TTP in both PCR and primer extension reactions. Therefore, further studies were then conducted using KOD XL as the
polymerase. Post-synthesis labeling of the PCR product was explored using synthesized Cys-BA and a fluorescent labeling agent Cys-FITC. As a negative control, no DNA product (Lane 1, Figure 2.10A) was observed without using dTTP or CBT-TTP. When the appropriate nucleotides are used, dNTPs-DNA$_{90}$ (DNA product using dNTPs, 90nt, Lane 2, Figure 2.10A) and CBT-DNA$_{90}$ (CBT incorporated DNA product, 90nt, Lane 3, Figure 2.10A) were obtained. This also indicated that CBT moiety-containing DNA chains could be used as templates for amplification. The all natural DNA 90-mer showed different mobility from that of CBT-DNA$_{90}$, presumably because of the added molecular weight of the latter. After post-synthesis modification with Cys-BA (1 mM final concentration for 30 min, supplemented with 1 mM TCEP), a new band with further reduced mobility was observed for the CBT-DNA$_{90}$ product (Lane 4, Figure 2.10A). As a control, a band with the same mobility was observed after the subjection of dNTPs-DNA$_{90}$ to the same treatment with Cys-BA (Lane 5, Figure 2.10A). Such results demonstrated the feasibility of post-synthesis click-modification with a longer 90-mer DNA with multiple CBT moieties.

Finally, to further demonstrate the generality of this approach for post-synthesis modification of DNA. 1,2-aminothiol conjugated FITC (Cys-FITC) (Figure 2.10B) was used instead of Cys-BA. The same phenomenon was observed as with Cys-BA. As can be seen from the EtBr channel in Figure 3C, which stain all DNA products, no DNA product (negative control, Lane 1, Figure 2.10B) was observed without using dTTP or CBT-TTP. On the other hand, dNTPs-DNA$_{90}$ (Lane 2, Figure 2.10B) and CBT-DNA$_{90}$ (Lane 3, Figure 2.10B) were observed with different mobilities. After post-synthesis modification with Cys-FITC (1 mM final concentration), supplemented with TCEP (1 mM final concentration) for 30 min, a new band with further reduced mobility was observed (Lane 5, Figure 2.10B). As a control, dNTPs-DNA$_{90}$ was also treated with Cys-FITC under the same conditions. However, no mobility changes were observed after treatment (Lane 4, Figure 2.10B). The successful post-synthesis modification of CBT-DNA$_{90}$ was further confirmed when imaging the same gel through the FITC channel, which only detects green fluorescein
signal. Only the band of Cys-FITC labeled CBT-DNA$_{90}$ showed FITC signal, with no observable green fluorescein signals for all the other bands as expected.

![Figure 2.11 “Click” labeling of PCR products.](image)

A. “Click” labeling of dNTPs/CBT-DNA$_{90}$ with Cys-BA (15% PAGE). 1) no dTTP, 2) dNTPs, 3) CBT-TTP, 4) DNA product after post-synthesis modification of CBT-DNA$_{90}$ (lane 3) with Cys-BA for 30 min, 5) DNA yielded after post-synthesis modification of dNTPs-DNA$_{90}$ (Lane 2) with Cys-BA for 30 min; B. “Click” labeling of dNTPs/CBT-DNA$_{90}$ with Cys-FITC (15% PAGE). 1) no dTTP, 2) dNTP, 3) CBT-TTP, 4) DNA product after post-synthesis modification of CBT-DNA$_{90}$ (lane 3) with Cys-FITC 30 min, 5) DNA yielded after post-synthesis modification of dNTPs-DNA$_{90}$ (Lane 2) with Cys-FITC for 30 min. The FITC channel only detects DNA products with green fluorescence, while EtBr channel detects all DNA products.

From experimental results obtained above, it was demonstrated that CBT-TTP could be successfully incorporated into DNA strands through enzymatic primer extension and PCR reactions. The CBT-modified DNA obtained from extension and PCR reactions could undergo rapid and specific cycloaddition with boronic acid and fluorophore-modified cysteine analogues. This is a new approach for DNA modification and could be used for DNA fluorescent labeling and aptamer selection.
2.3 Experimental section

General information

Solvents and reagents were purchased from VWR International, Oakwood Product Inc., or Sigma-Aldrich Co. and used without purification unless specified otherwise. When necessary, solid reagents were dried under high vacuum. Reactions with compounds sensitive to air or moisture were performed under argon. Solvent mixtures are indicated as volume/volume ratios. Thin layer chromatography (TLC) was run on Sorbtech W/UV254 plates (0.25 mm thick), and visualized under UV-light or by a Ce-Mo staining solution (phosphomolybdate, 25 g; Ce(SO4)2·4H2O, 10 g; conc. H2SO4, 60 mL; H2O, 940 mL) with heating. Flash chromatography was performed using Fluka silica gel 60 (mesh size: 0.040-0.063 mm) using a weight ratio of ca. 30:1 for silica gel over crude compound. 1H, 13C, and 31P-NMR spectra were recorded on a Bruker 400 spectrometer (400, 100, and 166 MHz, respectively) in deuterated chloroform (CDCl3), methanol-d4 (CD3OD), and DMSO-d6 with either tetramethylsilane (TMS) (0.00 ppm) or the NMR solvent as the internal reference. HPLC purification for CBT-TTP was carried out using a Shimadzu LC-10AT VP system with a Zobax C18 reversed-phase column (9.4 mm × 25 cm). The sample was eluted (6 mL/min) with buffer A (20 mM triethylammonium acetate, pH 6.9-7.1) and buffer B (50% acetonitrile, 20 mM triethyl ammonium acetate) with the following: 0 min 0% B; 20 min 20% B; 30 min 100% B; 38 min 100% B; 40 min 0% B; and 45 min 0% B.

DNA primers and templates were purchased from Integrated DNA Technologies. Klenow fragment (3'-5' exo), Taq. DNA polymerase, and Deep Vent (exo-) DNA polymerase were purchased from New England Biolabs. KOD-XL DNA polymerase was purchased from Novagen, Darmstadt. Reaction buffers were used as provided.

2.3.1 Synthesis of CBT-TTP

Synthesis of Ethyl 6-{prop-2-ynyloxy}benzo[d]thiazole-2-carboxylate 56
To a solution of 55 (2.23 g, 10 mmol) in DMF (30 mL) was added \( \text{K}_2\text{CO}_3 \) (2.70 g, 20 mmol) and the mixture was stirred at RT for 30 min. After addition of propargyl bromide (80% in toluene, 3.7 mL, 20 mmol), the reaction mixture was stirred at RT for 12 h before diluting with EtOAc (300 mL). Then the mixture was washed with water (50 mL × 3) and brine (50 mL), and dried over \( \text{Na}_2\text{SO}_4 \). Solvent evaporation followed by flash chromatography (Hex: EA, 4:1) gave a yellow solid (2.35 g, 90% yield). 

\[ \begin{align*}
\text{1H NMR (CDCl}_3\text{):}\  & 8.14 \ (d, J = 9.1 \text{ Hz, 1H}), \ 7.49 \ (d, J = 2.3 \text{ Hz, 1H}), \ 7.24 \ (dd, J = 2.3, 9.1 \text{ Hz, 1H}), \ 4.80 \ (s, 2H), \ 4.55 \ (q, J = 8.7 \text{ Hz, 2H}), \ 2.58 \ (s, 1H), \ 1.48 \ (t, J = 8.7 \text{ Hz);} \\
\text{13C NMR (CDCl}_3\text{):}\ & 160.7, \ 157.4, \ 156.4, \ 148.3, \ 138.4, \ 126.3, \ 117.9, \ 105.1, \ 63.0, \ 56.4, \ 14.3.
\end{align*} \]

HRMS (ESI) for \( \text{C}_{13}\text{H}_{12}\text{NO}_3\text{S} \) \([\text{M+H]}^+\) Calcd. 262.0545, Found 262.0544.

**Synthesis of 6-{Prop-2-ynyloxy}benzo[d]thiazole-2-carboxamide 57**

To a solution of ester 56 (2.35 g, 9 mmol) in EtOH 100 mL was added ammonium hydroxide 40 mL. The reaction mixture was heated to 75 °C for 5 h and then cooled to RT. Solvent was removed using a rota-evaporator, and the resulting precipitate was filtered, washed with \( \text{H}_2\text{O} \), EtOH and EtOAc to give a white solid (1.54 g, yield 73%). 

\[ \begin{align*}
\text{1H NMR (CDCl}_3\text{):}\ & 8.40 \ (s, 1H), \ 8.02 \ (d, J = 9.1 \text{ Hz, 1H}), \ 8.00 \ (s, 1H), \ 7.81 \ (d, J = 2.3Hz, 1H), \ 7.25 \ (dd, J = 2.3, 9.1 \text{ Hz, 1H}), \ 4.91 \ (d, J = 2.2 \text{ Hz, 2H}), \ 3.64 \ (t, J = 2.2 \text{ Hz, 1H}); \\
\text{13C NMR (CDCl}_3\text{):}\ & 163.7, \ 162.4, \ 157.3, \ 148.7, \ 139.9, \ 125.7, \ 118.3, \ 107.3, \ 79.8, \ 79.6, \ 57.1. \ 
\end{align*} \]

IR film 3424.7, 3301.3, 1696.0, 1600.8, 1502.0, 1399.5, 1256.1, 1230.5; HRMS (ESI) for \( \text{C}_{11}\text{H}_{9}\text{N}_2\text{O}_2\text{S} \) \([\text{M+H]}^+\) Calcd 233.0380; found 233.0387.

**Synthesis of 6-{Prop-2-ynyloxy}benzo[d]thiazole-2-carbonitrile 58**

To a suspension of amide 57 (1.1 g, 4.7 mmol) in anhydrous pyridine (25 mL) was added a solution of phosphorous oxychloride (0.85 mL, 9.4 mmol) in \( \text{CH}_2\text{Cl}_2 \) (5 mL) dropwise at 0 °C. After stirring at 0 °C for 1 h, the reaction was slowly warmed to ambient temperature and the resulting solution was
stirred for an additional 12 h. CH₂Cl₂ (200 mL) was added, and the reaction was quenched by addition of H₂O 20 mL. The organic phase was separated, washed with H₂O (20 mL × 2), brine (20 mL), and dried over Na₂SO₄. Solvent evaporation followed by flash chromatography (Hex: EtOAc, 6:1) afforded compound 58 as a light yellow solid (900 mg, yield 82%). ¹H NMR (CDCl₃): 8.13 (d, J = 9.1 Hz, 2H), 7.50 (d, J = 2.5 Hz, 1H), 7.24 (dd, J = 2.5, 9.1 Hz, 1H), 4.82 (d, J = 2.4 Hz, 2H), 2.59 (t, J = 2.4 Hz, 1H); ¹³C NMR (CDCl₃): 158.2, 147.4, 137.2, 134.0, 126.0, 118.8, 113.1, 104.7, 56.5; IR film 3255.3, 2232.2, 2126.5, 1606.5, 1551.5, 1536.5, 1470.1, 1256.8, 1230.3, 1132.0, 1049.9, 127.7. HRMS (ESI) for C₁₁H₇N₂OS [M+H]⁺ Cacld. 215.0279, Found 215.0289.

**Synthesis of CBT-T 59**

To a mixture of 5-iodo-deoxyuridine (708 mg, 2 mmol), Pd(PPh₃)₄ (230 mg, 0.2 mmol), and CuI (76 mg, 0.4 mmol) in anhydrous DMF (12 mL) under argon was added DIPEA (0.62 mL, 4 mmol) followed by a solution of compound 58 (700 mg, 3.0 mg) in DMF (4 mL). After stirring at RT overnight, DMF was evaporated, and the remaining dark gum was purified by flash chromatography (DCM: MeOH, 20:1) to afford compound 59 as a pale yellow solid (630 mg, yield 72%). ¹H NMR (CD₃OD): 8.38 (s, 1H), 8.07 (d, J = 9.2 Hz, 2H), 7.80 (d, J = 2.0 Hz, 1H), 7.33 (dd, J = 2.0, 9.2 Hz, 1H), 6.20 (t, J = 6.4 Hz), 5.05 (s, 2H), 4.38 (m, 1H), 3.92 (d, J = 3.2 Hz), 3.78 (m, 1H), 2.99 (s, 1H), 2.86 (s, 1H), 2.29 (m, 1H), 2.21 (m, 1H); ¹³C NMR (CD₃OD): 162.7, 158.3, 149.5, 146.9, 144.7, 137.2, 134.0, 124.9, 118.7, 112.6, 104.8, 97.6, 87.6, 86.8, 85.6, 79.0, 70.3, 60.9, 56.6, 40.2; IR film 3413.7, 2229.5, 1725.8, 1667.6, 1616.6, 1500.8, 1475.2, 1372.7, 1278.4, 1229.3, 1129.9, 1091.8, 1001.6; HRMS (ESI) for C₂₀H₁₆N₄O₆SNa [M+Na]⁺ Cacld 463.0683; Found 463.0674.
Synthesis of CBT-TTP 50

To a solution of CBT-T 59 (70 mg, 0.16 mmol) and proton sponge (42 mg, 0.19 mmol, pre-dried in vacuum over P₂O₅ overnight) in anhydrous trimethylphosphate (0.6 mL) was added freshly distilled POCl₃ (18 µL, 0.19 mmol) dissolved in anhydrous trimethylphosphate (0.2 mL) dropwise via a syringe with stirring under argon at 0 °C. The reaction mixture was further stirred in an ice-bath for 2 h and then a solution of tributylammonium pyrophosphate (226 mg, 0.48 mmol) and tri-n-butylamine (0.4 mL) in 1.0 mL of anhydrous DMF was added in one portion. The mixture was stirred at RT for 10 min and then triethylammonium bicarbonate solution (0.1 M, pH 8, 11 mL) was added. After stirring at RT for an additional hour, the resulting reaction mixture was transferred to a 50-mL centrifuge tube. Then EtOH (33 mL) was added followed by 3 M NaCl solution (1.0 mL). After vortexing for 10 sec, the centrifuge tube was placed at – 80 °C for 1 h, and then centrifuged at 5000 rpm for 20 min. After removing the supernatant, the resulting pellet was purified by HPLC and lyophilized to give a pale yellow powder (9 mg, 10%). ¹H NMR (D₂O): 8.57 (s, 1H), 8.05 (d, J = 6.8 Hz, 2H), 7.65 (s, 1H), 7.27 (d, J = 9.2 Hz, 1H), 6.11 (t, J = 6.4 Hz), 5.00 (s, 2H), 4.11-4.13 (m, 3H), 2.27-2.31 (m, 2H); ¹³C NMR (D₂O): 158.5, 151.5, 147.1, 146.0, 138.1, 135.7, 125.7, 119.9, 113.9, 106.2, 99.2, 88.9, 86.5, 86.4, 86.3, 79.5, 70.8, 65.8, 58.1, 58.0, 39.3, 17.5; ³¹P NMR (D₂O) -10.9, -11.5, -23.4. HRMS (ESI) for C₂₀H₁₅N₄O₁₅P₃S [M-H] Cacld. 678.9702; Found 678.9683.

2.3.2 Synthesis of cysteine-bearing labeling agents

Synthesis of L-(-)-(tert-Butoxycarbonyl)-2,2-dimethylthiazolidine-4-carboxylic acid 13

Compound 62 was prepared starting from L-cysteine hydrochloride (60) in two steps following literature procedures.⁹²
Synthesis of compound 64

To a mixture of compound 62 (836 mg, 3.2 mmol), 63 (500 mg, 2.7 mmol), and HOBt (397 mg, 2.9 mmol) in anhydrous DCM (20 mL) and DMF (20 mL) under ice-bath, was added Et3N (0.7 mL, 5.3 mmol) followed DCC (607 mg, 2.9 mmol) in one portion. The mixture was stirred at RT overnight and the resulting solid was filtered off. The filtrate was concentrated under vacuum and the residue was purified by flash chromatography (DCM: MeOH, 30:1) to afford intermediate 64. $^1$H NMR (CDCl$_3$): 7.83 (d, $J = 6.8$ Hz, 2H), 7.25 (d, $J = 6.8$ Hz, 2H), 4.82 (brs, 1H), 4.48 (s, 2H), 3.25 (m, 2H), 1.82 (s, 3H), 1.77 (s, 3H), 1.50 (s, 6H).

Synthesis of Cys-BA (65)

Compound 64 was stirred in DCM/TFA (20 mL/20 mL) at RT for another 2 h. Solvent was evaporated under vacuum and the residue was treated with diethyl ether (100 mL). The resulting precipitate was collected through filtration. After washing several times with diethyl ether, the collected precipitate was dissolved in EtOH/H$_2$O (300 mL/300 mL) with stirring for 30 mins. Evaporation of the solvent yielded compound 65 as a white solid (497 mg, 73% in two steps). $^1$H NMR (CD$_3$OD): 7.67 (d, $J = 4.0$ Hz, 2H), 7.25 (d, $J = 4.0$ Hz, 2H), 4.39 (m, 1H), 4.08 (m, 1H), 3.00 (m, 2H); $^{13}$C NMR (CD$_3$OD): 168.2, 140.7, 134.9, 127.7, 55.8, 44.2, 26.1; HRMS (ESI) for C$_{10}$H$_{15}$BN$_2$O$_3$S [M+H]$^+$ Calcd 255.0969; Found 255.0965.

2.3.3 Enzymatic incorporation and post-synthesis labeling

2.3.3.1 General procedure for Klenow fragment catalyzed primer extension using dTTP or CBT-TTP

The reaction mixtures of a final volume of 50 μL contained 21-nt template (5’-GGTTCCACCAGCAACCGCTA-3’, 20 μM), 14-nt primer or 5’-FAM 14-nt primer (5’-(FAM)-TAGCGGTTGCTGG-3’, 20 μM), 10 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl$_2$, 1 mM Dithiothreitol at pH
7.9, dATP, dCTP, dGTP, dTTP or CBT-TTP at concentrations of 200 μM and 0.5 U μL⁻¹ Klenow fragment. Reactions were performed by incubating the prepared solutions at 25 °C for 30 min. The primer extension products were analyzed by 20% PAGE.

2.3.3.2 General procedure for KOD-XL catalyzed primer extension using dTTP or CBT-TTP

The reaction mixtures of a final volume of 50 μL contained 21-nt template (Template-2: 5’-TCAGTCACCAGCAACCCGCTA-3’, Template-3: 5’-CACGACACCAGCAACCCGCTA-3’, 20 μM), 14-nt primer (5’-TAGCGGGTTGCTGG, 20 μM), 10 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂, 1 mM Dithiothreitol at pH 7.9, dATP, dCTP, dGTP, dTTP or CBT-TTP at concentrations of 200 μM and 0.5 U μL⁻¹ KOD-XL. Reactions were performed by incubating the prepared solutions at 90 °C for 1 min, 20 °C for 1 min and 66 °C for 20 min. The primer extension products were analyzed by 20% PAGE.

2.3.3.3 Post-synthesis labeling of the primer extension products CBT-DNA₂₁

The primer extension product CBT-DNA₂₁ was purified using Millipore Amicon 3 kDa spin column. 5 μL of 10 mM TCEP in PBS buffer was mixed with 25 μL of 2 mM Cys-BA in 1 x PBS buffer. The reaction was allowed to stand at RT for 10 min. Pre-purified CBT-DNA₂₁ (20 μL) was then added to the mixture, which was further incubated at 37 °C for 1 h. The negative control experiment was performed following the same procedure except using dNTPs-DNA₂₁, the primer extension product using dNTPs and other reagents. The resulting DNA products after post-synthesis modification were purified with Millipore Amicon 3 kDa spin column and analyzed by 20% polyacrylamide gel electrophoresis. For MALDI analysis, the DNA product was further purified on a Sephadex G25 column. Fractions were collected and concentrated using a Millipore Amicon 3 kDa spin column.

2.3.3.4 General procedure for PCR incorporation using dTTP or CBT-TTP

The PCR mixture of a final volume of 50 μL contained DNA 90-nt template (5’-CTTTGCTGGTCTGCCCTTCGTGAGCGGAGTCAGACGCACGCTGATCCCTGACGGAGCTGGACTATGACGGGAGCCCTT
CAGAATTCGCACCA-3', 10 nM), primer 1 (5'-TGGTGCGAATTCTGAAGGGT-3', 1 μM), primer 2 (5'-CCTCGTTGTCTGCCTTCGT-3', 1 μM), dATP, dCTP, dGTP, dTTP or CBT-TTP at a concentration of 200 μM, 0.5 U μL⁻¹ DNA polymerase and 1 x reaction buffer as provided by vender. Ten thermal cycles were conducted with melting at 90 °C for 20 s, annealing at 48 °C for 20 s, and extending at 72 °C for 30 s with initial denaturing at 90 °C for 2 min and final extension at 72 °C for 5 min. The PCR products were then analyzed by 15% PAGE.

2.3.3.5 **Post-synthesis labeling of the PCR product CBT-DNA₉₀**

Post synthesis labeling of the PCR products was performed using similar procedures as those for primer extension. Specifically, CBT-DNA₉₀ prepared from PCR was purified using Millipore Amicon 10 kDa spin column. 5 μL of 10 m M TCEP was mixed with 25 μL of 2 mM Cys-BA or Cys-FITC and allowed to stand at RT for 10 min. Pre-purified CBT-DNA₉₀ (20 μL) was then added to the mixture, which was further incubated at 37 °C for 30 min. The negative control experiment was performed following the same procedure except using dNTPs-DNA₉₀, the PCR product using dNTPs and other reagents. The resulting DNA product after post-synthesis modification was purified with Millipore Amicon 10 kDa spin column and analyzed by 15% PAGE.

2.3.4 **Kinetic study of the reaction between CBT-T and 1,2-aminothiol (L-cysteine)**

![Scheme 2.4 Reaction scheme of CBT-T with L-cysteine](image)
To a solution of CBT-T 59 in acetonitrile was added a solution of L-cysteine in PBS buffer (pH = 7.4) to afford the final concentration of CBT-T (500 μM), L-cysteine (550 μM) in a mixture of acetonitrile/PBS buffer (v/v: 1/4). The resulting mixture was stirred at room temperature and monitored by HPLC at different time points in a similar way as literature reported.95, 96 Applying similar kinetic analysis reported in the references, 1/[CBT-T] vs reaction time was plotted and the linear regression analysis of 1/[CBT-T] vs. time gave the second-order rate constant as 22 M⁻¹s⁻¹.

2.3.5 Thermodenaturization of duplex DNAs

The UV-melting temperature studies were carried out by Cary 300 UV–Vis Spectrophotometer and a temperature control module. Duplex DNA samples were prepared through Klenow fragment primer extension reactions with dTTP or CBT-TTP. Post-synthetic sample was prepared by click reaction of CBT-DNA21 with Cys-BA. All samples were cleaned using Millipore Amicon 10 kDa spin column. The samples (1 μM DNA duplexes) were dissolved in PBS buffer (pH 7.4). The samples were heated to 80 °C and cooled to 6 °C at a rate of 0.5 °C per min. 4 Rounds of melting were performed for each sample. The melting temperature data and curves are presented in Table 2.1 and Figure 2.7.

2.4 Conclusions

Nucleic acids have been used in a number of new applications including therapeutics and nanomaterial science. There is a critical need to develop chemistry that allows for the ready functionalization of nucleic acid molecules. In the present research, a novel method for post-synthesis modification of DNA is described thorough the design, synthesis, and successful enzymatic incorporation of a cyanobenzothiazole (CBT)-modified TTP. The CBT-TTP incorporated DNA products can undergo rapid post-synthesis modification via a biocompatible condensation reaction with 1,2-aminothiol conjugated boronic acid (Cys-BA) or FITC analogue (Cys-FITC). This approach provides a novel way for rapid and site-specific post-synthesis modification of DNA without the issue of regio- or stereo-isomers and the
formation of a single isomer makes this approach suitable for DNA-based aptamer selection work and other applications.

2.5 Acknowledgment

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3 A FLUORESCENT PROBE FOR FAST QUANTITATION OF HYDROGEN SULFIDE

Abstract:

Hydrogen sulfide (H₂S) has been recognized as one of the endogenous gasotransmitters, which are produced in the cell and participate in a variety of cellular functions. However, the accurate measurement of H₂S in biological systems is still a challenge. We have developed dansyl-azole (DNS-Az) as a fluorescent probe for H₂S. In the presence of trace amount of sulfide anion, DNS-Az is reduced into sulfonamide quantitatively, which is excited at 340 nm and gives a strong fluorescence at around 500 nm. It has been proved to be very selective and efficient. Detection limit is about 1 µM in 20 mM phosphate buffer (pH 7.5)/0.05% Tween-20 (Buffer/Tween) and 5 µM in commercial bovine serum. This probe has also been applied to the measurement of endogenous H₂S in blood using C57BL6/J mice model. The result obtained is consistent with the data obtained by using other methods. A new method has been developed for the quantitative analysis of H₂S in blood.

3.1 Introduction

3.1.1 Biological significance of hydrogen sulfide

Hydrogen sulfide (H₂S) is a colorless gas with a strong odor of rotten eggs. It has a density (1.54 g/L) higher than air and good water solubility (4.0 g/L at 20°C). It dissociates in aqueous solutions in two steps to HS⁻ and S²⁻ with a pKₐ of 6.9 and pKₐ of 12. Consequently, over 75% of H₂S exists as hydrosulfide (HS⁻) at physiological pH. Naturally occurring H₂S is produced from submarine hydrothermal activity and marine biologic processes. H₂S also results from industrial activities and residential waste settings such as septic or sewer systems. It is well known as a toxic gas. Although the odor threshold that can be detected by human nose ranges from 0.005-0.3 ppm, exposure to over 100 ppm of H₂S can quickly cause olfactory paralysis. Overexposure to this gas leads to unconsciousness, brain damage,
or even death. However, H₂S can be breathed at a low concentration (e.g. 20 ppm) without posing any cumulative harmful effects.

Endogenous H₂S is produced during cysteine (Cys) metabolism. Three main pathways for enzymatic H₂S synthesis have been discovered, including synthesis by cystathionine β-synthase (CBS, mainly localized in brain and liver), 102 cystathionine γ-lyase (CSE, mainly localized in liver), 103 and cysteine aminotransferase (CAT)/3-mercaptopyruvate sulfurtransferase (3MST, mainly localized in vascular endothelium and brain). 104, 105 Since H₂S is present in human brain 106, 107 and blood 108 in fairly high concentrations (10-150 μM), there have been intensive efforts in exploring the biological roles of H₂S. With the aid of various bio-analytical techniques, H₂S has been identified as one of the three gasotransmitters, 109, 110 joining nitric oxide (NO) 111 and carbon monoxide (CO). 112 H₂S regulates the respiration chain by inhibiting complex IV, cytochrome oxidase. 113, 114 It also dilates blood vessels and lowers blood pressure by acting as a smooth muscle relaxant and by opening the potassium dependent ATP channel. 115-117 The signaling mechanism of H₂S is through protein S-sulfhydration, conversion of cysteine –SH groups to –SSH. 118-121 The endogenous concentration of H₂S is closely related to numerous diseases, including cardiovascular diseases, Down syndrome, 122 and lung diseases. 123 Endogenous/exogenous H₂S was also found to show protective effects in the cardiovascular (CV) 124 and central nervous systems (CNS) 125 and to play a dose-dependent regulatory role in inflammation. 126, 127 Therefore, the regulation of H₂S levels is a possible drug development strategy. 128, 129 Slow-release H₂S donors are needed for a continuous low-level production of H₂S, and thus are useful in both research and therapeutic applications. 130-133 More H₂S donors with different mechanisms and tunable releasing rates are needed for therapeutic applications. Thus, the selective detection and quantitation of H₂S have both diagnostic and therapeutic significance.
3.1.2 Detection methods of hydrogen sulfide

Compared to other biologically important molecules, H₂S is both volatile and reactive. It is very easily oxidized by various oxidants. Therefore, the accurate detection of H₂S depends heavily on the experimental procedure, including sample preparation and detection methods. The analysis of H₂S using various methods results in markedly different conclusions. For example, endogenous concentrations of H₂S have been reported to be 50-150 µM in most publications over the past two decades;¹⁰⁶,¹²³,¹³⁴-¹³⁶ however, several recent studies have indicated that the concentration of free H₂S is in the low nanomolar range;¹³⁷,¹³⁸ which is orders of magnitude lower than previously reported. Considering this discrepancy in published data and due to the clinical relevance of H₂S, accurate measurement of its endogenous concentration is very important. Before the biological roles of H₂S had been revealed, its measurement primarily had applications in the environmental area. Sulfide detection was explored by earth scientists for water analysis.⁹⁷,¹³⁹ Nowadays, these methods have been modified for analysis in biological systems. So far there have been three types of methods developed for the measurement of H₂S. These methods include chromatographic methods, electrochemical methods, and methods using reactive colorimetric/fluorometric probes.

Gas chromatography (GC) is the most commonly employed chromatographic method.¹³⁴,¹⁴⁰-¹⁴³ Specifically, H₂S containing gas in the headspace of a sample or liberated from strongly acidic solutions of sample preserve is injected into the GC column. After separation, it is detected by photoionization detector (PID),¹⁴³ flame photometric detector (FPD),¹⁴¹,¹⁴² or pulsed flame photometric detector (PFPD).¹⁴⁰ For derived products, GC-MS¹³⁴ and high performance liquid chromatography (HPLC)¹⁴⁴ have also been employed in the detection of H₂S. Because the additional separation step provides high resolution with low background, chromatographic methods are able to selectively detect H₂S with a very low detection limit (pM-nM). However, the use of chromatographic methods is limited because of its
heavy reliance on instruments. In addition, multistep sample preparation is often required, which also adds inconvenience to the analytical procedure.

Electrochemical methods include the use of either silver/sulfide selective membrane electrodes\textsuperscript{145-149} or polarographic methods.\textsuperscript{150,151} These methods can provide nanomolar detection limits. The advantages of electrochemical methods include high sensitivity, easy sample preparation, and commercially available and inexpensive instruments. Disadvantages include long equilibration time, the requirement of frequent reconditioning due to metal sulfide deposition on electrodes, and the inherent problem of interference. The use of electrochemical methods in live systems is also limited.

Besides chromatographic and electrochemical methods, a colorimetric method, which takes advantage of the reaction between H$_2$S and $N,N$-dimethyl-$p$-phenylenediamine, has been approved by US Environmental Protection Agency (EPA) as a standard method for H$_2$S quantitation in aqueous solutions (Figure 1).\textsuperscript{152-155} This reaction uses Fe$^{3+}$ as the oxidant and forms a dye, methylene blue, as the product, which absorbs intensely at 670 nm. This absorption change is used to quantitate H$_2$S in solution. The methylene blue method has a detection limit in the nanomolar range and has been used in a number of studies for H$_2$S quantitation in biological samples. However, dimers and trimers of methylene blue are formed at concentrations higher than 30 µM, where a linear calibration curve cannot be obtained. As a result, the quantitation of H$_2$S at concentrations greater than 30 µM should be done using diluted samples.

Because of the high sensitivity and the ability to conduct analysis in live systems, fluorescent probes have found a wide variety of applications. A typical fluorescent probe functions through a selective reaction, either reversible binding or irreversible conversion, with the target molecule to form a product, which shows distinct absorption or fluorescence changes compared to the probe itself. The change in absorption or fluorescence is then used for the detection or quantitation of the target molecule. High selectivity of the sensing reaction allows for direct analysis without or with minimal
sample preparations. The reaction rate is also a key factor because a fast reaction saves experimental time and leads to efficient detection or even real-time monitoring.

Due to the biological significance of H$_2$S, the development of fluorescent probes for it fast and accurate detection has become a very actively pursued area.$^{156-158}$ In this part of dissertation we report a fluorescent probe (DNS-Az) for fast and quantitative detection of H$_2$S in aqueous solutions, including bovine serum.

3.2 Results and discussion

3.2.1 Design and synthesis of a novel fluorescent probe for hydrogen sulfide

With the idea of developing a new method that will be useful for the rapid assay of hydrogen sulfide concentrations under physiological conditions, we undertook the effort of searching for a selective chemosensing agent for hydrogen sulfide. For easy use in a biology lab, the chemosensing agent should (1) act fast (within seconds) under mild conditions, (2) be chemically stable for long-term storage, (3) be sensitive for detection under near physiological conditions, (4) show a linear concentration-signal relationship within physiologically relevant hydrogen sulfide concentration ranges for easy quantitation, (5) show minimal or no interference by other anions in the blood serum, and (6) be functional in aqueous solutions and blood plasma. Herein we report the development of a fluorescent chemoprobe and its application in the determination of hydrogen sulfide in aqueous solution, serum and whole blood.

Fluorescence is one of the most sensitive detection methods. Thus we were interested in selecting a fluorophore, which has a high quantum yield, emits at a long wavelength, and responds to hydrosulfide by fluorescent property changes. Dansyl is a commonly used fluorophore, and well known for its strong fluorescence and long emission wavelength. We were interested in designing a sulfide-sensitive agent using this fluorophore by taking advantage of the known unique reduction of an azido
group by hydrogen sulfide. We reasoned that the reduction of an azido group attached to a strongly electron-withdrawing group would occur at an accelerated rate. Because of the difference in electronegativity of the azido and amino groups and the added degree of rotational freedom for the azido group, reduction of sulfonyl azide into sulfonamide should trigger a change in the electronic properties and thus the fluorescent properties of the dansyl moiety. Therefore we synthesized dansyl-azide (DNS-Az, 67, Scheme 3.1).

![Scheme 3.1 DNS-Az (67) as a fluorescent probe for sulfide](image)

The synthesis of DNS-Az is simple with only one step from commercially available DNS-Cl. Sodium azide reacts fast with DNS-Cl to yield the product nearly quantitatively. The product (67) is purified through flash chromatography, and is ready for further evaluation for hydrogen sulfide detection.

### 3.2.2 Evaluation of DNS-Az as a fluorescent probe for hydrogen sulfide

Due to quenching effect of the azido group, DNS-Az (67) by itself is non-fluorescent. The fluorescence quantum yield of DNS-Az is 0.007 in acetonitrile (ACN). In order to evaluate DNS-Az as a fluorescent probe for hydrogen sulfide, Na₂S was used as a hydrogen sulfide source. Upon addition of Na₂S, DNS-Az solution showed a strong fluorescence enhancement. This fluorescence enhancement is due to the formation of DNS-NH₂ (68), which is the reduction product. The identification of the product
has been confirmed through NMR, HPLC and MS studies (for details please see experimental section). DNS-Az shows an absorption maxima at 358 nm ($\epsilon = 2930 \text{ M}^{-1}\text{cm}^{-1}$). When converted to DNS-NH$_2$, an 18 nm blue shift was found in the absorption spectra (Figure 3.1A). The fluorescent quantum yield of DNS-NH$_2$ is 0.37 (Figure 3.1B). The changes in absorption wavelength and fluorescence quantum yield lead to the fluorescence increase upon addition of Na$_2$S.

![Figure 3.1 Absorption spectra of DNS-Az and DNS-NH2 and quantum yield test.](image)

A. UV absorption spectra were recorded for DNS-Az (67) (solid line) and DNS-NH$_2$ (68) (dashed line) in acetonitrile at 100 µM, respectively, with acetonitrile as the blank reference; B. Fluorescence quantum yield test of compounds 67 and 68.

The magnitude of the fluorescent enhancement was solvent-dependent. When the experiments were conducted in 20 mM phosphate buffer (pH 7.5)/ACN 1:1, 150 fold of fluorescent intensity enhancement was observed with the addition 25 µM of sulfide. For a thorough understanding of the solvent effect, we tested the following solvent systems: acetonitrile, deionized water, 20 mM sodium phosphate buffer (pH 7.5), acetonitrile/water (1:1), acetonitrile/phosphate buffer (1:1), and 20 mM sodium phosphate buffer (pH 7.5) with 0.05% Tween-20 (Figure 3.2). The strongest response was observed in acetonitrile with a maximum of 130-fold fluorescent intensity increase with the addition of
10 μM of sulfide. The solvent system that gave the smallest fluorescent intensity increases (about 8 fold) was phosphate buffer or water alone. The presence of sodium phosphate buffer did not affect the fluorescence intensity (Figure 3.2). Very interestingly, addition of 0.05% of Tween-20, a commonly used additive in biological experiments as a buffer component, led to a substantial increase in the magnitude of the fluorescent intensity change of DNS-Az upon sulfide addition. In such a mixed solvent, addition of 25 μM of sulfide led to a 40-fold fluorescence enhancement. The detection limit was as low as 1 μM with a S/N of 3:1.

![Graph](image)

**Figure 3.2** DNS-Az fluorescence responses to sulfide in different solvent systems.

DNS-Az 100 μM, Na₂S 10 μM, λₑₓ = 340 nm, λₑₘ = 517 nm

The reaction time profile of DNS-Az with sulfide was tested in different solvent systems. It was found that the reaction completes very fast even in aqueous solutions. For example, the reaction between 100 μM of DNS-Az completes within 3 minutes in a mixed solvent of 20 mM phosphate buffer and ACN (Figure 3.3A, B) or a 20 mM phosphate buffer with 0.05% Tween-20 (Figure 3.3C, D).
In order to study the selectivity of this chemoprobe for sulfide, the fluorescent properties of DNS-Az in the presence of various anions were examined in buffer/Tween. No comparable response was observed from other anions (Figure 3.4). Since the detection is based on the reducing property of sulfide, other possible reducing anions, such as iodide, bromide, fluoride, bisulfite, and thiosulfate, were also tested. Totally 18 anions were screened, no obvious response was observed for most of the anions at 1 mM, a concentration that is 40 fold higher than that of sulfide. Among all the anions, only HSO₃⁻, S₂O₃²⁻, and S₂O₅²⁻ led to some fluorescent intensity increases. However, the extent of the fluorescence increase...
was far smaller than those caused by sulfide even when the concentrations of those anions were 4-fold higher than that of sulfide (Figure 3.4B).

Figure 3.4 Selectivity test for DNS-Az using different anions.

A. Comparison of fluorescence intensity changes of DNS-Az solution with the addition of sulfide and other anions; B. Fluorescence spectra of DNS-Az upon addition of various anions (DNS-Az 100 μM, HSO₃⁻, S₂O₃²⁻, and S₂O₅²⁻ 100 μM; HS⁻ 25 μM, all other anions 1 mM in 20 mM sodium phosphate buffer (pH 7.5) with 0.05% Tween-20, λₑₓ= 340 nm). Anions tested: Cl⁻, Br⁻, I⁻, F⁻, OH⁻, OAc⁻, CN⁻, N₃⁻, NO₂⁻, HCO₃⁻, HSO₃⁻, SO₄²⁻, S₂O₃²⁻, S₂O₅²⁻, S₂O₇²⁻, HPO₄²⁻, citrate.
The response of DNS-Az to other strong reducing agents, such as thiophenol, benzyl mercaptan and cysteine were also tested. Benzyl mercaptan was the only one that showed strong enough responses (about 1/5 of that of sulfide at the same concentration, Figure 3.5), which could pose an interfering problem. However, since benzyl mercaptan is rarely found in biological systems, this should not be a practical issue. DNS-Az was also found to be recalcitrant to the possible displacement reaction resulting from attack by an amino group. It showed very limited response to glycine and lysine at concentrations as high as 50 mM. This good selectivity for sulfide is very important and has indicated that the fluorescent probe DNS-Az could be used in complex biological systems for sulfide detection with minimal sample pre-treatment.

![Fluorescence response of DNS-Az to other reducing agents and selected amino acids.](image)

**Figure 3.5** Fluorescence response of DNS-Az to other reducing agents and selected amino acids.  
DNS-Az 100 μM in 20 mM phosphate buffer/0.05% Tween-20, λ<sub>ex</sub> = 340 nm, λ<sub>em</sub> = 517 nm.

A linear relationship is always important for easy and accurate analysis. Thus, hydrogen sulfide concentration-dependent study was performed using both fluorometer and micro-plate reader. The fluorescence response of DNS-Az to sulfide was studied in a variety of solvent systems, including ACN, a 1:1
mixed solvent of 20 mM phosphate buffer with ACN and buffer/Tween. It was found that DNS-Az reacts with sulfide essentially quantitatively in both organic solvents and aqueous solutions. The fluorescence intensity showed a reproducible linear relationship in buffer/Tween against hydrogen sulfide in all solvent system tested. When sulfide concentration is higher than that of DNS-Az, the plot was found to reach a plateau (results not shown), which means that the stoichiometry of this reaction was 1:1. Figure 3.6 shows a triplicated calibration curve obtained in Buffer/Tween using a 96-well plate and a microplate reader.

![Figure 3.6 Hydrogen sulfide calibration curve in buffer/Tween.](image)

Fluorescence was determined using 96-well plates: DNS-Az 200 μM, Na₂S 0-100 μM in buffer/Tween (excitation filter 340 nm, emission filter 535 nm).

DNS-Az was found to be relatively stable under room light. However, it was found that prolonged UV radiation (>5 min) enhanced the fluorescence intensity of DNS-Az (Figure 3.7). As a result, fluorescence intensity should be recorded using freshly made reaction mixture in order to obtain reproducible results. The observed increase in fluorescence most likely is due to reactions involving nitrene generated under UV radiation. However, under normal experimental conditions, this is not an issue.
Figure 3.7 Fluorescence stability of DNS-Az under UV radiation.

A. Fluorescence stability of DNS-Az (25 μM) under UV radiation at 340 nm in 20 mM phosphate buffer/0.5% Tween; B. fluorescence change of DNS-Az (25 μM) at 457 nm over time under UV radiation at 340 nm in 20 mM phosphate buffer/0.5% Tween

Thus far, all the selectivity and linearity studies suggest that DNS-Az (67) can be used for the determination of sulfide concentrations in a biological sample. Considering the physiological significance of H₂S levels in the blood, DNS-Az was evaluated in commercially available bovine serum for a further test. Upon addition of sulfide, the solution of DNS-Az also showed very significant fluorescent intensity increases. Though bovine serum showed background fluorescence (Figure 3.8, 0s), it was negligible compared to the strong fluorescence of DNS-NH₂ (68) generated from the reaction. It should be noted that the reaction went very fast in bovine serum (complete within seconds, Figure 3.8). This is very important considering the fast metabolism and evaporation of hydrogen sulfide in biological systems. This unprecedented fast response could provide the possibility of quantitative detection without any pre-treatment of samples.
DNS-Az 100 μM, H2S 30 μM, λ\text{ex}=340\text{ nm}, \lambda\text{em}=493\text{ nm}.

In order to further evaluate DNS-Az as a fluorescent probe in blood serum, a triplicated calibration curve was obtained in bovine serum using a 96-well plate and a microplate reader. An excellent linear relationship was also obtained in bovine serum (Figure 3.9). This calibration curve covers the range of reported endogenous levels of hydrogen sulfide, indicating that this probe is very suitable for the detection of sulfide in biological samples. Due to the changes in the micro-environment and the viscosity of the medium, fluorescence intensity and emission wavelength is different when using different medium. For the samples that the medium is not readily available for calibration curve generation, a standard addition procedure could be used for accurate measurement. Overall, the results suggest that the anions and biological substrates normally encountered in the blood do not pose a problem in the quantitative detection of sulfide in a biological sample.

For a simple application, DNS-Az was used to test the time dependent decay of hydrogen sulfide in bovine serum. The extremely fast response of NDS-Az toward sulfide allows accurate determination of real-time sulfide concentrations in bovine serum. When DNS-Az was added at several different time points after the addition of Na2S into bovine serum, the concentration of sulfide at each time points
could be determined by comparing the fluorescence readings to a standard curve. The result is shown in Figure 3.9B. It was found that sulfide has a very short half-life ($t_{1/2} \sim 5$ min). This is due to fast metabolism of hydrogen sulfide in the serum.

![Figure 3.9 Detection of hydrogen sulfide in bovine serum.](image)

**Figure 3.9 Detection of hydrogen sulfide in bovine serum.**

A. Calibration curve of sulfide in bovine serum, fluorescence was determined using 96-well plates: DNS-Az 200 μM, Na$_2$S 0-100 μM in commercial bovine serum (excitation filter 340 nm, emission filter 535 nm); B. Time dependent decay of sulfide in bovine serum, sulfide 100 μM (initial concentration), DNS-Az (200 μM) was added at 0, 30, 60, 120, 300 and 660 s after addition of sulfide, fluorescence intensity was tested using a fluorometer 3 min after the addition of DNS-Az, $\lambda_{ex}= 340$ nm, $\lambda_{em}= 493$ nm.

### 3.2.3 Determination of hydrogen sulfide concentration in mouse blood

Encouraged by the promising results obtained in phosphate buffer and bovine serum, we applied this fluorescent chemoprobe to the determination of hydrogen sulfide concentrations in blood using C57BL6/J mouse model (Figure 3.10). A standard addition procedure was used in the experiment. Zero points were obtained through treatment of blood with ZnCl$_2$ (1 mM final concentration). Standard Na$_2$S solutions were added into blood samples to a series of concentrations of 0, 10, 50, and 100 μM. The re-
The results obtained are shown in Table 3.1. The average sulfide concentration in mouse blood ($n = 5$) was determined to be $31.9 \pm 9.4 \, \mu M$, very similar to previous reported values in mouse plasma (34.1 \, \mu M). This has confirmed that our fluorescent chemoprobe can indeed be used into the detection of hydrogen sulfide in real biological samples and the result is comparable with those obtained using current method.
Detection was performed using an internal standard method using C57BL6/J mice (n=5), determined using 96-well plates (DNS-Az 200 μM, excitation filter 360 nm, emission filter 528 nm). Zero point was obtained by trapping sulfide with ZnCl₂ and calibration curve was obtained by using an internal standard method. (Hydrogen sulfide 0, x, x+10, x+50, and x+100 μM).

A. Sulfide concentration in mouse 1 (25.0 μM);
B. Sulfide concentration in mouse 2 (30.2 μM);
C. Sulfide concentration in mouse 3 (26.1 μM);
D. Sulfide concentration in mouse 4 (48.4 μM);
E. Sulfide concentration in mouse 5 (28.9 μM)

### Table 3.1 Detection of hydrogen sulfide in mouse blood

<table>
<thead>
<tr>
<th>Subject #</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂S (μM)</td>
<td>25.0</td>
<td>30.2</td>
<td>26.1</td>
<td>48.4</td>
<td>28.9</td>
<td>31.9 ± 9.4</td>
</tr>
</tbody>
</table>

### 3.3 Experimental section

General information

All reagents were purchased from Aldrich. Thermometers were not calibrated. ¹H NMR and ¹³C NMR spectra were recorded on a Bruker Avance 400 MHz NMR spectrometer. Mass spectral analysis was performed on an ABI API 3200 (ESI-Triple Quadrupole). HPLC was performed on a Hewlett Packard Series 1100 HPLC (column: Agilent Prep-C18 5 μm, 4.6 × 250 mm). UV-Vis absorption spectra were rec-
ordered on a Shimadzu PharmaSpec UV-1700 UV-Visible spectrophotometer. Fluorescence spectra were recorded on a Shimadzu RF-5301PC fluorometer. 96-Well plates were read and recorded on a PerkinElmer 1420 multi-label counter.

3.3.1 Synthesis and characterization of DNS-Az and DNS-NH₂

Synthesis and characterization of DNS-Az (67)

A suspension of 66 (250 mg, 0.93 mmol) in 15 mL of EtOH was added dropwise into a stirred solution of sodium azide in 7 mL of a mixed solvent (H₂O/EtOH, 1:1). Then the reaction mixture was stirred at room temperature for 3 hr. The organic solvent was evaporated in vacuum, and the aqueous solution was extracted by DCM. The combined organic layers was washed with brine and then dried over MgSO₄. Solvent evaporation gave the crude product, which was purified by flash chromatography to give 67 (107 mg, 42%) as a light yellow oil. ¹H NMR (400 MHz, DMSO-d₆): 8.68-8.66 (d, J = 8.4 Hz, 1H), 8.40-8.38 (m, 1H), 8.07-8.05 (d, J = 8.0 Hz, 1H), 7.77-7.73 (m, 2H), 7.75 (s, 2H), 7.36 (d, J = 7.8 Hz), 7.34, (d, J = 7.8 Hz), 2.86 (s, 6H); ¹³C NMR (100 MHz, CDCl₃): 152.2, 133.7, 132.7, 130.1, 130.1, 129.7, 129.3, 123.0, 118.8, 115.9, 45.4; ESI-MS: m/z 277.1 (M+1)+.

![Figure 3.11 HPLC chromatogram of DNS-Az (67)](image)

Synthesis and characterization of DNS-NH₂ (68)

A solution of sodium sulfide (43 mg, 0.18 mmol) in 0.4 mL H₂O was added into a stirred solution of 67 in 17 mL acetonitrile. The reaction mixture was stirred for 2 hr at room temperature. Then solvents were evaporated under vacuum. The residue was purified by flash chromatography (DCM/MeOH 50:1) to give pure DNS-NH₂ (40 mg, 91%) as a yellow solid. ¹H NMR (400 MHz, DMSO-d₆): 8.44-8.42 (d, J = 8.4
Hz, 1H), 8.30-8.28 (d, J = 8.8 Hz, 1H), 8.13-8.11 (m, 1H), 7.63-7.56 (m, 2H), 7.59 (s, 2H), 7.27-7.25, (d, J = 8.4 Hz), 2.83 (s, 6H); $^{13}$C NMR (100 MHz, DMSO-$d_6$): 151.8, 140.2, 129.5, 129.4, 129.2, 128.0, 126.8, 124.0, 120.0, 115.5, 45.5; m.p. 215-217 °C; ESI-MS: m/z 251.1 (M+1)⁺.

**Figure 3.12** HPLC chromatogram of DNS-NH$_2$ (68)

### 3.3.2 Detection of hydrogen sulfide in aqueous solutions

#### 3.3.2.1 Detection of hydrogen sulfide using a fluorometer

A stock solution (30 mM in ethanol) of DNS-Az (10 µL, final concentration 300 µM) was added into 1.0 mL of sample solution containing 0-100 µM of hydrogen sulfide. Then it was mixed thoroughly and incubated for 1-5 min (depending on the reaction medium, for reaction time profile please see Figures S6-S8) and placed in a fluorometer for measurements with $\lambda_{ex}$= 340 nm. The reading was then compared to a standard curve to obtain the concentration of hydrogen sulfide.

#### 3.3.2.2 Detection of hydrogen sulfide using micro-plate reader

A stock solution (50 mM in ethanol) of DNS-Az (0.4 µL, final concentration 200 µM) was added into 100 µL of samples in a 96-well plate. Then the fluorescence intensity was measured on a micro-plate reader (excitation lamp filter 340 nm, emission filter 535 nm). The readings were then compared to a standard curve to obtain the concentration of hydrogen sulfide. For analysis of sulfide in serum, it is recommended that DNS-Az be added immediately after sample preparation to avoid fast catabolism of sulfide.
3.3.3 Detection of hydrogen sulfide in mouse blood

DNS-Az was dissolved in ethanol to make a stock solution of 50.0 mM. Male C57BL6/J mice 8 wk of age were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were anesthetized with ketamine/xylazine and blood was drawn with a syringe from the inferior vena cava of male C57BL6/J mice (n = 5). These procedures were approved by the Institute for Animal Care and Use Committee at Emory University School of Medicine and conformed to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996), and with federal and state regulations. Blood (100 µL × 4) was added into eppendorf tubes with DNS-Az (0.4 µL, final concentration 200 µM). Na$_2$S was spiked into each sample to a final concentration of 10, 50 and 100 µM. The samples were mixed thoroughly and centrifuged. Then 50 µL of serum was transferred from each sample into a 96-well plate. Zero point was obtained by trapping sulfide with ZnCl$_2$ (1 µL in 100 µL blood, final concentration 1 mM) followed by centrifugation and addition of DNS-Az into serum. The plate was read on a micro-plate reader (excitation lamp filter 360 nm, emission filter 528 nm).

3.4 Conclusions

In conclusion, a novel reduction-sensitive fluorescence chemoprobe was developed for hydrogen sulfide in aqueous solutions, including blood serum and whole blood. The probe was found to be very selective for sulfide among 18 anions tested and other common reducing species, with a detection limit of 1 µM in buffer/Tween and 5 µM in bovine serum with a signal-to-noise ratio of 3:1. The linear relationship obtained in bovine serum covers the reported endogenous concentration range of hydrogen sulfide. The probe was used in the detection of hydrogen sulfide in mouse blood using C57BL6/J mice model. The result (31.9 ± 9.4 µM) was very close to the previously reported serum concentration of hydrogen sulfide. In addition, the simplicity and ease in measurements and the compatibility of this probe to 96-well plates and a micro-plate reader, which are readily accessible in biology labs, has made this...
agent extremely easy to use. Hydrogen sulfide level in the biological system is tightly regulated and can experience rapid changes in concentration. The unprecedented fast response by DNS-Az (67) to hydrogen sulfide allows it to be used for the detection of transient changes in sulfide levels without sample pre-treatment. The probe, DNS-Az (67), is simple in structure, very easy to synthesize, stable and amenable to long-term storage. A new research field has emerged in the past decade because of the newly recognized significance of hydrogen sulfide as an endogenous gasotransmitter. The molecular mechanism of sulfide’s cellular actions remains to be understood, and novel H₂S releasing drugs need to be developed. We feel that this fast, selective, efficient and low-cost detection method for sulfide will be very useful in the booming research field of hydrogen sulfide. In addition, we are very pleased to see more fluorescent probes being developed based on this redox mechanism.¹⁵⁷, ¹⁵⁸, ¹⁶³-¹⁶⁷

3.5 Acknowledgment

I would like to thank Dr. Yunfeng Cheng for his HPLC work and our collaborators Dr. Adrienne L. King, Dr. Benjamin L. Predmore, and Dr. David J. Lefer for their help in detection of H₂S in mouse blood.
4. PUBLICATIONS AND MANUSCRIPTS IN PREPARATION


REFERENCES


69. Tornoe, C. W.; Christensen, C.; Meldal, M. Peptidotriazoles on solid phase: 1,2,3-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. J. Org. Chem 2002, 67, 3057-3064.


95. Meroni, G.; Ciana, P.; Maggi, A.; Santaniello, E. A new synthesis of 2-cyano-6-hydroxybenzoiazole, the key Intermediate of D-luciferin, starting from 1,4-benzoquinone. Synlett 2009, 2682-2684.

96. Meroni, G.; Rajabi, M.; Ciana, P.; Maggi, A.; Santaniello, E. Synthesis of 2-substituted-6-hydroxy and 6-methoxy benzoiazoles from 1,4-benzoquinone. Arkivoc 2010, 53-60.


110. Wang, R. Two's company, three's a crowd: can H$_2$S be the third endogenous gaseous transmitter? FASEB J. 2002, 16, 1792-1798.


APPENDICES

Appendix A. $^1$H- and $^{13}$C-NMR spectra of final compounds used in quorum sensing inhibition tests
Appendix B. $^1$H- and $^{13}$C-NMR spectra of intermediates in CBT-TTP synthesis
Appendix C. $^1$H- and $^{13}$C-NMR spectra of DNS-Az and DNS-NH$_2$