NEW CHEMISTRY OF S-NITROSOTHIOLS

By

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A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

WASHINGTON STATE UNIVERSITY
Department of Chemistry

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation/thesis of JIA PAN find it satisfactory and recommend that it be accepted.

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Dedicated to my parents and my wife Hongli Li
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New Chemistry of S-Nitrosothiols

Abstract

by Jia Pan, Ph.D.
Washington State University
May 2012

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Protein S-nitrosation is an important post-translational modification that not only transmits nitric oxide (NO) biological activity, but also regulates protein functions. However, development of reliable methods for the detection of protein S-nitrosation is still a challenge due to the lability of its products – S-nitrosothiols (SNOs).

The thesis will discuss three aspects in detail: first, the development of fluorescent probes for the detection of SNOs based on the reductive ligation; second, the study on the reactivity of sulfenamides which is the reductive ligation product from SNOs, towards different nucleophiles, and further development of a one-pot disulfide formation reaction; lastly, the synthetic application of S-nitrosation in amide/peptide synthesis.

The new chemistry of S-nitrosothiols not only provides a promising platform for the study of S-nitrosation in biological systems, but also reveals the potential usage of S-nitrosation in organic synthesis.
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-butyloxycarbonyl</td>
</tr>
<tr>
<td>Ca</td>
<td>calcium</td>
</tr>
<tr>
<td>Cbz</td>
<td>benzyloxycarbonyl</td>
</tr>
<tr>
<td>CDCl₃</td>
<td>deuterated chloroform</td>
</tr>
<tr>
<td>CH₃CN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>CK</td>
<td>creatine kinase</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>Cu</td>
<td>copper</td>
</tr>
<tr>
<td>CysNO</td>
<td>S-nitrosocysteine</td>
</tr>
<tr>
<td>DAF-2</td>
<td>4,5-diaminofluorescein</td>
</tr>
<tr>
<td>DAN</td>
<td>2,3-diaminonaphthalene</td>
</tr>
<tr>
<td>DBU</td>
<td>1,8-diazabicycloundec-7-ene</td>
</tr>
<tr>
<td>DCC</td>
<td>N,N'-dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DM</td>
<td>dialysis membrane</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-(N,N-dimethylamino)pyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethyl formamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>Et₂O</td>
<td>diethyl ether</td>
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</table>
EtOAc  ethyl acetate
fALS  familial amyotrophic lateral sclerosis
Fmoc  9-fluorenylmethyloxycarbonyl
GC  gas chromatography
GSH  glutathione
GSNO  S-nitrosoglutathione
GSSG  glutathione disulfide
HCl  hydrogenchloride
HgCl₂  mercury dichloride
H₂O  water
HPLC  high performance liquid chromatography
Hz  hertz
IHC  immunohistochemistry
IR  infrared
K₂CO₃  potassium carbonate
α-KGDH  α-ketoglutarate dehydrogenase
KO  knockout
LC  liquid chromatography
M  mole per liter
MeI  methyl iodide
MeOH  methanol
MMTS  methyl methanethiosulfonate
MS  mass spectrometry
N  mole per liter
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>NACB</td>
<td>S-nitroso N-acetylcysteine benzylamide</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>sodium bicarbonate</td>
</tr>
<tr>
<td>NaNO₂</td>
<td>sodium nitrite</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NAPDH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>sodium sulfate</td>
</tr>
<tr>
<td>NBS</td>
<td>N-bromo succinimide</td>
</tr>
<tr>
<td>NCL</td>
<td>native chemical ligation</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxyl succinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>nitrite</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>nitrate</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NTA</td>
<td>S-nitrosothioacid</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
</tr>
<tr>
<td>ONO</td>
<td>nitrite</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>phosphocreatine</td>
</tr>
<tr>
<td>POCl₃</td>
<td>phosphoryl chloride</td>
</tr>
<tr>
<td>PPh₃</td>
<td>triphenylphosphine</td>
</tr>
<tr>
<td>PTM</td>
<td>post-translational modification</td>
</tr>
<tr>
<td>PyBop</td>
<td>benzotriazol-1-yl-oxy-tris-pyrrolidino phosphonium hexafluorophosphate</td>
</tr>
<tr>
<td>rt</td>
<td>room temperature</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>SERCA2a</td>
<td>sarcoplasmic reticulum Ca2+ ATPase</td>
</tr>
<tr>
<td>SNAP</td>
<td>S-nitroso-N-acetylpenicillamine</td>
</tr>
<tr>
<td>SNO</td>
<td>S-nitrosothiol</td>
</tr>
<tr>
<td>SOCl₂</td>
<td>thionyl chloride</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TrSH</td>
<td>triphenylmethyl mercaptan</td>
</tr>
<tr>
<td>TrSNO</td>
<td>S-nitroso triphenylmethyl mercaptan</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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CHAPTER ONE

INTRODUCTION

1.1 NITRIC OXIDE

Nitric oxide (NO), a free radical gas, has long been known as a component of air polluting NOx complex. It can be oxidized by O2 to generate NOx or hydrolyzed to afford nitrite (NO2⁻) or nitrate (NO3⁻). Not until 1987 NO was recognized as a gaseous signaling molecule,⁠¹⁻¹ and it has so far been found to play an important role in many physiological and pathophysiological processes. In mammalian cells, NO can be synthesized from L-arginine in the presence of oxygen and the reduced form of nicotinamide adenine dinucleotide phosphate (NAPDH) under the catalysis of nitric oxide synthase (NOS) enzymes (Scheme 1.1).⁠¹⁻²

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{NH} & \quad \text{CH}_{2} & \quad \text{CH}_{2} & \quad \text{O} & \quad \text{H} \\
\text{L-arginine} & \quad \text{NH} & \quad \text{CH}_{2} & \quad \text{CH}_{2} & \quad \text{O} & \quad \text{H} \\
\text{O}_2, \text{NADPH} & \quad \text{Nitric oxide synthase} & \quad (\text{NOS}) & \quad \text{NH} & \quad \text{CH}_{2} & \quad \text{CH}_{2} & \quad \text{O} & \quad \text{H} \\
\text{H}_2\text{N} & \quad \text{N} & \quad \text{CH}_{2} & \quad \text{CH}_{2} & \quad \text{O} & \quad \text{H} & \quad \cdot \text{NO} \\
\text{L-citrulline} & \quad \text{NH} & \quad \text{CH}_{2} & \quad \text{CH}_{2} & \quad \text{O} & \quad \text{H} & \quad \cdot \text{NO} \\
\end{align*}
\]

Scheme 1.1 Biological synthesis of nitric oxide.

There are at least 3 different classes of NOS: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). In general, nNOS produces NO in both the central and peripheral nervous system to help cell communication. In the immune system iNOS produces NO for immune defense against pathogens. In addition, NO generation by eNOS in blood vessels leads to vasodilation.⁠¹⁻³ All these NOS activities regulate a large number of protein functions in biological systems.
1.2 PROTEIN S-NITROSATION

The cellular response to NO is transduced via multiple chemical reactions, including direct reactions with heme centers, metalloproteins, and indirect biochemical reactions after oxidation or other metabolism to various reactive nitrogen species (RNS). For example, NO can nitrosate many protein nucleophilic sites such as amines, alcohols, thiols and aromatic rings. Among all these nitrosation reactions, protein S-nitrosation that protein cysteine residues (−SH) bond to NO to form S-nitrosothiol (SNO) (Scheme 1.2) has received much attention.

![Scheme 1.2 S-Nitrosation and S-nitrosothiol](image)

Scheme 1.2 S-Nitrosation and S-nitrosothiol

Mechanism of S-nitrosation is highly dependent on the context of specific physiological environments.\textsuperscript{[1,4,15]} Endogenous S-nitrosation is strongly related to NOS signaling since a range of S-nitrosated proteins were identified in endothelial cells (eNOS), epithelial cells (nNOS) and lymphocyte cell lines (iNOS), in cytokine-activated macrophages (iNOS), in skeletal and cardiac muscle and developing neurons (nNOS), and throughout the wall of blood vessels stimulated by calcium or acetylcholine (eNOS).\textsuperscript{[1,6,1,10]} S-Nitrosation can also be catalyzed by heme in blood flow or catalyzed by copper in plasma.\textsuperscript{[1,6]} Other chemical mechanisms such as NO or thiol oxidation, transnitrosation, and nitrite-mediated S-nitrosation are also involved.\textsuperscript{[1,6]}

Protein S-nitrosation is an important post-translational modification (PTM) which regulates a large number of protein functions.\textsuperscript{[1,11]} It has been reported that S-nitrosation
of the mitochondrial complex IV (cytochrome c oxidase) via binding of NO• to its binuclear Cuβ/haem-a3 active site inhibits its activities.[1.12-1.15] Some other research revealed that the activity of the F1F0ATPase (ATP synthase) can also be inhibited by S-nitrosation.[1.16] S-Nitrosation of creatine kinase (CK) can inhibit its catalytic conversion of creatine with consumption of adenosine triphosphate (ATP) to create phosphocreatine (PCr) and adenosine diphosphate (ADP).[1.17] S-Nitrosation has also been reported to increase the activity of some enzymes. For example, α-ketoglutarate dehydrogenase (α-KGDH) can be S-nitrosated and increases its activity in the conversion of α-ketoglutarate into succinyl CoA in the citric acid cycle.[1.16] S-nitrosation of the L-type Ca2+ channel has been reported to reduce the channel activity in the heart under adrenergic stimulation,[1.18] under oxidative stress,[1.19] and with atrial fibrillation.[1.20] S-nitrosation of the cardiac sarcoplasmic reticulum (SR) calcium release channel/ryanodine receptor (RyR2) has been shown to increase its open channel probability. The decreased S-nitrosation of RyR2 in nNOS-KO hearts has also been suggested to cause cysteine residues of RyR2 to be more oxidized under oxidative stress, leading to SR Ca2+ leakage and arrhythmogenesis.[1.21, 1.22] Cardiac SR Ca2+-ATPase (SERCA2a) has also been shown to be activated by an NO-dependent modification. Adachi et al showed that NO-dependent S-glutathionylation (occurring via peroxynitrite and therefore dependent on superoxide generation as well as NO and GSH) of SERCA2a resulted in an increase in SERCA2a activity.[1.21, 1.23]

Protein S-nitrosation also plays an important cardioprotective role[1.11] by modulating the activity of proteins involved in apoptosis and oxidative stress such as caspase 3,[1.24] cyclooxygenase-2,[1.25] hypoxia inducible factor 1α,[1.26] NADPH oxidase,[1.27] and
Furthermore, recent studies suggest that NOS generates NO locally and regulates compartmentalized S-nitrosation and protein trafficking in the cardiovascular system.\textsuperscript{[1.29,1.30]} Several important proteins that regulate protein trafficking, such as dynamin,\textsuperscript{[1.31,1.32]} N-ethylmaleimide–sensitive factor,\textsuperscript{[1.33]} G protein–coupled receptor kinase 2,\textsuperscript{[1.34]} and β-arrestin 2,\textsuperscript{[1.35]} have been identified to be S-nitrosylated and S-nitrosation of these proteins play an important role in regulating protein trafficking and signaling transduction.

In addition, S-nitrosation has been considered as an important factor in diseases.\textsuperscript{[1.6]} Elevation of S-nitrosothiols (nitrosative stress) has been found in diseases such as tuberculosis,\textsuperscript{[1.36]} pneumonia,\textsuperscript{[1.37]} diabetics,\textsuperscript{[1.38]} pre-eclampsia\textsuperscript{[1.39]} and inflammatory lung diseases\textsuperscript{[1.40]}. In another aspect, depletion of S-nitrosothiols is also importantly related to diseases such as asthma,\textsuperscript{[1.41]} familial amyotrophic lateral sclerosis (fALS),\textsuperscript{[1.42]} and Alzheimer’s disease.\textsuperscript{[1.43]}

### 1.3 Detection of S-Nitrosation

Protein S-nitrosation is so important that it requires efficient analysis tools for its study. However, identification of S-nitrosated proteins is still technically limited. It is primarily due to the fact that SNO is very unstable functional group since the S-NO bond is labile and sensitive towards light or heat and will decompose to NO and thyl radical (RS•).\textsuperscript{[1.44-1.46]} Therefore the processing, isolation and analysis of SNOs are still difficult. To date, there are six major methods for SNO detection\textsuperscript{[1.47-1.51]}:

1) Chemiluminescence assays\textsuperscript{[1.52,1.53]}. In this method, SNO is first decomposed by
laser light or the reductants such as Cu(I) and cysteine to NO radical. And NO is then reacted with ozone to generate NO$_2^*$, which decays to NO$_2$ and emits light. It will then be measured by chemiluminescence spectroscopy. The reaction occurs in the gas phase and is dependent on the rate of mixing of NO and ozone.

2) Colorimetry-based assays (also known as Saville assay)$^{[1.54]}$: SNOs are converted to nitrite (NO$_2^-$) by HgCl$_2$. The concentration of NO$_2^-$ can be determined by using the Griess reagents (sulfanilamide and N-(1-naphthyl)-ethylenediamine). This method is not suited for the detection of biological levels of SNOs because its detection limit is close to the biological concentration of SNOs.

3) Fluorescence assays: SNOs undergo the same procedure as in Saville assay to generate NO$_2^-$ or through photolysis to generate NO, which will then react with fluorescent probes 2,3-diaminonaphthalene (DAN)$^{[1.55]}$ or 4,5-diaminofluorescein (DAF-2)$^{[1.56]}$ to afford fluorescent triazole compounds for fluorescence detection.

4) Mass spectrometric assays: Gas chromatography-mass spectrometry (GC-MS)$^{[1.57]}$ and liquid chromatography-mass spectrometry (LC-MS)$^{[1.58]}$ both utilize isotope labeled analogues of the endogenous SNOs as internal standards for the measurement. The method is selective and sensitive, however, it is also limited in the detection of complex mixture of S-nitrosated proteins.

5) Electrochemical/amperometric SNO sensors$^{[1.59-1.63]}$: A indirect method utilizing a thin layer immobilized with catalysts (Cu(I/II), glutathione and organoselenium, e.g.) between the tip of an underlying amperometric NO sensor and protective outer
dialysis membrane (DM). The catalyst promote the decomposition of SNOs to generate NO, which is then detected by the NO sensor. This method is limited for the detection of low molecular weight SNOs due to the molecular-weight cut-off of the applied DM.

6) Anti-S-nitroso-cysteine antibody based assay[1.64,1.65]: Polyclonal and monoclonal antibodies are isolated and react with SNOs by immunohistochemistry (IHC). The preparation of the samples including proper tissue collection, fixation and sectioning is critical for this method, therefore it is not very reliable towards SNO antigens due to their lability in the procedure.

These methods have been successfully applied in some cases but still limited in analyzing cell extracts and tissue samples. Chemiluminescence, colorimetry, fluorescence and electrochemical assays require decomposition of SNO to NO or NO$\text{}_2^-$. These indirect methods can only provide the overall concentration of SNO moieties without the ability to identify the specific site of S-nitrosated proteins, and they may also be interfered with other NO metabolites such as nitrite in biological systems. Anti-S-nitroso-cysteine antibody and MS assays directly target SNO, however, they can only determine or quantify SNO in low molecular weight or relatively pure protein samples.

**Biotin switch method**

To achieve the detection of S-nitrosated proteins in complex biological systems such as cell extracts, Jaffrey et al developed a biotin switch method in 2001.[1.66,1.67] It
ultilizes 3 steps to selectively convert unstable SNO to stable and detectable biotin conjugate (Scheme 1.3): The first step is to use methyl methanethiosulfonate (MMTS)\textsuperscript{[1,68]} to block and convert all the free thiols to methyl disulfides, and then in the second step ascorbate is used to reduce only SNO to free thiols, \textsuperscript{[1,69-1,71]} which in the third step are reacted with biotin-HPDP (N-[6-(Biotinamido)hexyl]-3′-(2′-pyridyldithio)-propionamide) to form detectable biotin conjugates. These products can then be detected by mass spectrometry or Western blot analysis.

\begin{center}
\includegraphics[width=\textwidth]{Scheme1_3.png}
\end{center}

\textbf{Scheme 1.3} Biotin switch method

The biotin switch method has been applied successfully in various protein systems for SNO research.\textsuperscript{[1,72-1,74]} However, it still suffers some drawbacks: 1) the blocking of free thiols in the first step is difficult to achieve completion, which will lead to considerable interference with the low SNO concentration; 2) in the second step ascorbate reduction is not efficient towards some SNO moieties\textsuperscript{[1,49, 1,75,1,76]}, and it’s also found to reduce disulfides in some circumstances to generate wrong signals\textsuperscript{[1,77-1,79]}. Although it has been reported that the false-positive signal (i.e. the reduction of disulfides) might be caused by indirect sunlight\textsuperscript{[1,76,1,80]}, it is still suggested that additional control experiments be performed to ensure positive SNO signaling in the biotin switch method.\textsuperscript{[1,81]}
With all the analytical methods mentioned above, there are still huge differences in the detection values of S-nitrosated proteins in the same tissue or biological fluid by different research groups.\cite{1.8,1.81,1.82} Therefore, it is extremely important to develop reliable and accurate method for measuring or detecting SNO moities in biological environments, to facilitate the understanding of protein S-nitrosation.

### 1.4 CHEMISTRY OF S-NITROSOThIOLS

Although serving as an important product from protein S-nitrosation in biological systems, S-nitrosothiol as a unique chemical or functional group has not yet received much attention. The chemistry of SNO was poorly explored during the last two decades, probably due to the lability of the group which prevents convenient preservation and handling. In fact, decomposition of S-NO bonds has been found to be accelerated by many factors such as light, heat, certain metal ions, superoxide, seleno compounds, ascorbate and thiols.\cite{1.83-1.85}

So far, most alkyl or aryl SNOs have been found to react with nucleophiles, such as free thiols\cite{1.84} or carbanions\cite{1.86} to replace NO and form sulfur conjugates such as disulfides or thioethers (Scheme 1.4A). Triphenylmethyl SNO (TrSNO) as one of the most stable small molecule SNOs, however, it has its unique reactivities. Since the S-NO bond of TrSNO is labile towards light or heat, its relatively stable radical products from photolysis can undergo a 1,2-addition of alkene or α,β-unsaturated conjugates (Scheme 1.4B).\cite{1.87} TrSNO can also be converted to thio-iminophosphine by triphenylphosphine (Scheme 1.4C).\cite{1.88}
Based on the unique reactivity of SNO towards triphenylphosphine, our laboratory first developed a reductive ligation of SNOs.\textsuperscript{[1,89]} As shown in Scheme 1.4, the reaction utilized a triphenylphosphine 1.1 which has an ester group serving as an electrophile. It reacted with SNO and the iminophosphine intermediate 1.3 generated during the reaction will then be trapped intramolecularly by the ester to form a sulfenamide product 1.5 (Scheme 1.5). The reaction occurred in very mild conditions within short time and proved to be selective towards SNOs.

\textbf{Scheme 1.4} Chemical reactions of SNOs.
When replacing the ester group with other electrophiles such as thioester groups, our lab has developed a series of ligation reactions which selectively convert SNOs to other sulfur-conjugates. For example, when tertiary SNOs were treated with phosphine 1.6, the product thioimidates 1.7 were formed (Scheme 1.6A).\[^{[1,90]}\]

However, if similar phosphine 1.8 was used to react with primary SNOs, disulfides as shown in Scheme 1.6B were produced.\[^{[1,91]}\] A similar reaction was also developed when the ester bond of the phosphine 1.10 was inverted, and the disulfide products 1.11 were obtained without phosphine tags (Scheme 1.6C).\[^{[1,92]}\]

All these reactions completed in minutes under mild conditions, and showed good selectivity towards SNOs. The bis-ligation and one-pot disulfide formation are the most promising methods for detecting protein S-nitrosation as they convert primary SNOs to disulfides which are stable in biological systems.

![Scheme 1.6 New ligation reactions of SNOs](image-url)
A synthetically useful reaction of SNOs was also developed in our group, which is the one-pot synthesis of 3-substituted benzisothiazoles from o-mercaptoacylphenones via S-nitrosation.\(^1\) As shown in Scheme 1.7, the aromatic SNO 1.13 generated via S-nitrosation was quickly converted to thio-iminophosphine 1.15 by phosphine 1.14, which was then reacted intramolecularly with the ketones to generate new aromatic products 1.16. The reaction provides an facile route for the synthesis of 3-substituted benzisothiazoles which are important molecules in medicinal chemistry and drug discovery.\(^1\)

![Scheme 1.7](image)

**Scheme 1.7** Synthesis of 3-substituted benzisothiazoles from o-mercaptoacylphenones via S-nitrosation.

### 1.5 REFERENCES


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1.17 Arstall, M. A.; Bailey, C.; Gross, W. L.; Bak, M.; Balligand, J. L.; Kelly, R. A.


CHAPTER TWO

FLUORESCENT DETECTION OF S-NITROSOTHIOLS

2.1 ABSTRACT

Due to their high sensitivity and convenience of operation, fluorescence based assays are widely used in the detection of biological molecules. Currently there is no fluorescence assay that directly targets SNO moieties. We hypothesized that the development of a fluorescence based assay which directly targets the SNO functional group would be more efficient for the detection of S-nitrosation. To prove this hypothesis, we designed and synthesized two series of fluorescent probes targeting SNOs based on different mechanisms. The specificity and efficacy towards the detection of SNOs were investigated. The methods developed are promising for the study of SNOs in complex biological systems.

2.2 FLUORESCENT DETECTION OF S-NITROSOTHIOLS VIA OXIDATION OF PHOSPHINES\(^{(2.1)}\)

2.2.1 RESEARCH DESIGN AND METHODOLOGY

The stoichiometry in the reductive ligation\(^{(2.1)}\) of SNOs is notable. In the reaction, two equivalents of phosphine substrates 2.1 were reacted with one equivalent of the SNO
and generated one equivalent of phosphine oxide 2.2 and one equivalent of thiaoazaylide intermediate 2.3 (Scheme 2.1).

![Scheme 2.1](image)

**Scheme 2.1** Stoichiometry in the reductive ligation of SNOs.

The formation of the corresponding phosphine oxide and azaylide intermediate is general for the reaction between SNOs and triaryl substituted phosphines.\[^{2,3}\] If there is no electrophilic group (i.e. an ester) in the phosphine substrate to trap the nucleophilic nitrogen, the hydrolysis of azaylide will predominate the reaction and lead to another molecule of phosphine oxide (Scheme 2.2). We envisioned that this reaction directly targets the SNO moiety and can be developed for a fluorescence based assay of SNOs by exploiting the fluorescence properties of the phosphine starting material and phosphine oxide product.

![Scheme 2.2](image)

**Scheme 2.2** Reaction between triarylphosphine and SNO

To test this hypothesis, we proposed the coumarin-phosphine compound 2.5 as a fluorogenic dye which when oxidized can be used to detect SNOs (Scheme 2.3). Compound 2.5 is a tertiary phosphine of which an aryl group is 7-amino coumarin attached via the 3-position. It is known that substituents at the 3-position strongly
influence the fluorescence properties of 7-amino coumarin dyes.\[^{[2,4-2,6]}\] A similar compound has been used as the fluorogenic probe for detecting azides based on the Staudinger ligation.\[^{[2,7]}\] The lone pair of electrons on the phosphorus of compound 2.5 quenches the excited state of the fluorophore, rendering the molecule nonfluorescent. Upon reaction with SNOs, the oxidation of the phosphine eliminates quenching and activates the fluorescence.

![Scheme 2.3](image)

**Scheme 2.3** A coumarin-phosphine fluorogenic dye (2.5) activated by SNOs.

**Synthesis of probe 2.5**

The synthesis of compound 2.5 is shown in Scheme 2.4. The commercially available 8-hydroxyjulolidine 2.7 was converted to coumarin 2.8 via formylation and the Wittig reaction. Iodination of coumarin 2.8 was then achieved with NIS/TsOH providing 3-iodocoumarin 2.9. Finally, the palladium-catalyzed coupling of 2.9 with diphenylphosphine furnished the final product 2.5.\[^{[2,7]}\]

![Scheme 2.4](image)

**Scheme 2.4** Synthesis of the probe 2.5
2.2.2 Fluorescence analysis

As expected, the reaction under aqueous conditions between compound 2.5 and S-nitrosothiols such as S-nitrosoglutathione (GSNO), S-nitrosocysteine (CysNO), and S-nitroso-N-acetylpenicillamine (SNAP) afforded the phosphine oxide 2.6 exclusively (Scheme 5). It resulted in the increase in fluorescence, which was immediately evident. However there was no fluorescence detected in control reactions lacking SNO (Scheme 2.5).

![Scheme 2.5 Reaction between compound 2.5 and GSNO](image)

In addition, another control experiment was performed to elucidate the reaction in the presence of a disulfide. Glutathione disulfide (GSSG), instead of GSNO, was mixed with probe 2.5 and did not lead to any fluorescence increase (Figure 2.1), confirming the previous observation that triaryl substituted phosphines are nonreactive towards disulfide bonds.\[2.2\]

![Figure 2.1 Fluorescence intensity image of compound 2.5 and its reactions.](image)
As shown in Table 2.1, phosphine 2.5 and phosphine oxide 2.6 have quite different photophysical parameters. When compared to phosphine 2.5, the oxidized product shows slightly red-shifted maximum absorbance. In addition, the molar absorptivity of the phosphine oxide is greater than the phosphine compound. We measured the excitation spectra of phosphine 2.5 and phosphine oxide 2.6 in PBS buffer (pure DMSO was used to dissolve these compounds, and was then diluted in PBS at pH 7.4). The phosphine 2.5 has maximum excitation wavelength at 437 nm while compound 2.6 at 448 nm. The most significant difference between phosphine and phosphine oxide is their fluorescence quantum efficiency. Phosphine 2.5 exhibited a weak fluorescence with a quantum yield of 0.033 ± 0.002 (relative to quinine sulfate) and an emission maximum at 486 nm. However, the phosphine oxide 2.6 shows an intense emission with a maximum at 489 nm and a quantum yield of 0.79 ± 0.04. Therefore, the reaction between the phosphine 2.5 and the SNOs should produce a phosphine oxide with an easily distinguishable fluorescence signal. The normalized excitation and emission spectra of the phosphine 2.5 and the phosphine oxide 2.6 is shown in Figure 2.2.

<table>
<thead>
<tr>
<th></th>
<th>ε(M⁻¹cm⁻¹)</th>
<th>λabs (nm)</th>
<th>λex (nm)</th>
<th>λem (nm)</th>
<th>Quantum yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>21200 ± 1100</td>
<td>433</td>
<td>437</td>
<td>486</td>
<td>0.033 ± 0.002</td>
</tr>
<tr>
<td>2.6</td>
<td>29500 ± 1600</td>
<td>442</td>
<td>448</td>
<td>489</td>
<td>0.79 ± 0.04</td>
</tr>
</tbody>
</table>

a All measured in phosphate-buffered saline (PBS).
Figure 2.2 The excitation and emission spectra of compounds 2.5 and 2.6. Excitation spectra of 2.5 (green) and 2.6 (red). Emission spectra of 2.5 (yellow) and 2.6 (blue).

To demonstrate the efficiency of the phosphine 2.5 in the measurement of SNO concentration, we reacted the phosphine 2.5 with GSNO under a series of different concentrations, in order to obtain a standard curve of fluorescence versus SNO concentration. In all samples (in PBS pH 7.4 solutions) the concentration of the phosphine 2.5 was maintained at 1.2 mM, while the concentrations of GSNO were 0.033 mM, 0.066 mM, 0.133 mM, 0.265 mM and 0.530 mM, respectively. The fluorescence intensity of each sample was recorded and plotted vs. the concentration of GSNO. As shown in Figure 2.3, the fluorescent signal was linearly related to the concentration of GSNO in this concentration range. In addition, we observed that the fluorescence signal generated from the reaction between phosphine 2.5 and other SNO compounds also fitted into this curve (examples of SNAP are also shown in Figure 2.3). Under the conditions described above, the detection limit of SNO is around 0.01 mM, which is comparable to some colorimetry/fluorescence-based assays for SNOs.
2.3 FLUORESCENT DETECTION OF S-NITROSOTHIOLS VIA THE REDUCTIVE LIGATION

2.3.1 RESEARCH DESIGN AND METHODOLOGY

In our preliminary study of the fluorescent probe via oxidative-activation\textsuperscript{[2,1]}, we were able to obtain good result for small molecule SNO models. However, we will have to consider the reactive oxygen species (ROS) broadly present in biological systems which could also oxidize the phosphine in some circumstances to generate false signals. Thus, the method should require multiple control experiments as well as complex sample processing to obviate the background from ROS for its application in real biological systems. In our opinion, a fluorescent turn-on method which cannot be interfered by the oxidation of phosphine should be more efficient and selective.

**Figure 2.3** Effect of SNO concentration on the fluorescence intensity. (●) GSNO; (■) SNAP.
We investigated the mechanism of the reductive ligation\cite{2,2} and envisioned that fluorescent probes for SNOs could be designed by replacing the phenoxy group of the phosphine compound 1.1 with a fluorophore. It is known that the fluorescence of some fluorophores can be quenched by converting its hydroxyl groups to other functionalities such as ethers or esters.\cite{2.8-2.10} The probes we designed, i.e. compound 2.11 (Scheme 2.6), should be nonfluorescent. Upon treatment with SNOs, the reductive ligation should proceed and release the fluorescent molecule 2.12. Meanwhile, the phosphine oxide of the probes will not emit fluorescence.

**Scheme 2.6** Proposed fluorescent probes based on reductive ligation.

**Synthesis of fluorescent probes 2.13 – 2.16.**

Synthesis of the probe 2.13 and 2.14 is shown in Scheme 2.7A. The commercially available fluorescein was treated in basic condition with tert-butyl 2-bromoacetate to afford 2.18 in 35% of yield. 2.18 was then coupled with 2.17 using similar conditions described above to afford probe 2.13 in 76% yield. Probe 2.14 was obtained in 96% yield using TFA.\cite{2.11}
Synthesis of the probe 2.15 is described in Scheme 2.7B. Fluorescein was treated with MeI in basic condition to form dimethylated product 2.19, which was then hydrolyzed in basic condition to afford 2.20. EDC coupling reaction between 2.20 and 2.17 gave probe 2.15 in 66% yield.\textsuperscript{[2,12]}

Synthesis of the probe 2.16 is shown in Scheme 2.7C. It was easily obtained via the coupling reaction between phosphine acid 2.17 and the commercially available 7-hydroxylcoumarin using EDC and DMAP in 90% yield.

Scheme 2.7 Synthesis of fluorescent probes based on reductive ligation.
2.3.2 Fluorescence analysis

We first used the probe 2.13 to study the efficiency of the reaction with CysNO methyl ester 2.21 in a mixed solution of PBS (pH 7.4)/CH₃CN (3:1), and isolated fluorescent product 2.18 in very good yield (89%) (Scheme 2.8).

Scheme 2.8 Reaction between probe 2.13 and SNO 2.21.

A linear relationship between the emission intensity and the concentration of 2.21 was also obtained by adding various amount of 8.08 μM 2.21 in CH₃CN to buffer solution of 2.13 (12.05 μM in PBS (pH 7.4)/ CH₃CN 3:1), and measuring the fluorescent emission of each reaction mixture (15 times diluted) via fluorescent spectroscopy after 2 hours (Figure 2.4).
Figure 2.4 A linear correlation between emission intensity and the concentration of 2.21.

To investigate whether the ester bond is stable in the buffer solution during the reaction, we also dissolved probe 2.13 in buffer solutions with different pH and tested them in time-based fluorescence and UV spectroscopy (Scheme 2.9). As shown in Figure 2.5, we were delighted to see the probe was quite stable in neutral or acidic conditions even after 2 hours. And it was not surprising that the fluorescence of the probe in basic condition significantly increased possibly due to the basic hydrolysis of the ester bond which release the fluorescence.

Scheme 2.9 Hydrolysis of the ester bond of probe 2.13 at different pHs.
To investigate the specificity of the reaction towards SNOs, we also reacted probe 2.13 with cysteine thiol 2.24 and disulfide 2.25 (Scheme 2.10) which represent two other sulfur species commonly existing in biological systems.

**Scheme 2.10** Cysteine 2.24 and cysteine disulfide 2.25.

Figure 2.6 indicates that in the same concentration level (1 μM), the reaction of 2.13
(after mixed for 2 hours) with 2.21 gave more than 7 fold increase of fluorescence intensity, while the reactions with 2.24 and 2.25 led to an increase less than 1 fold. Therefore, 2.13 appears to be a highly selective fluorescent probe for the detection of SNOs. Images of phosphine 2.13, phosphine oxide 2.23, the mixture of 2.13 with 2.21, 2.24 and 2.25 (after mixed for 2 hours) were also taken and shown in Figure 2.7. As we can see, the strong fluorescence were only generated by mixing probe 2.13 with SNO 2.21.

Figure 2.6 Fluorescence increase of the reactions between 2.13 and 2.21, 2.24, 2.25.

Figure 2.7 Images of phosphine 2.13, phosphine oxide 2.23, the mixture of 2.13 with 2.21, 2.24 and 2.25.
To test the efficiency of the probe $2.13$ in complex biological systems, we applied it in the RAW267.4 cell study. The cells were cultured with 250 µM CysNO for 50 min, and washed twice to scavenge small molecules. Then 25 µM probe $2.13$ was added to the cells, and the sample were taken to fluorescence spectroscopy at 5, 10 and 15 minutes. As shown in Figure 2.8, the fluorescence increase was visible during the time and compared to the control sample, however, it also showed moderate fluorescent background in the control sample.

![Enlarged image](image.png)

**Figure 2.8** Efficiency of probe $2.13$ towards SNOs in living cells.

To look for a probe with lower background and higher response towards SNO, we tested probe $2.15$ and $2.16$ in the model study. Two SNO models – S-nitroso N-acetylcysteine benzylamide (NACB) (Scheme 2.11) and GSNO (Scheme 2.12) were chosen and reacted with both coumarin and fluorescein probes. As shown in Figure 2.9, both probes showed low response towards NACB in relatively low concentration range (0.5–5 µM). The high fluorescence response towards NACB was only obtained using fluorescein probe $2.15$.
and it had more than 10-fold fluorescence increase than background at 50 µM concentration level.

Scheme 2.11 Reactions between probe 2.15, 2.16 and NACB.

Figure 2.9. Fluorescence response of the probes 2.15, 2.16 towards different NACB concentration over background.

Interestingly, when we tested the probes with GSNO, it had different result compared to that with NACB. It showed that coumarin probe 2.16 worked better towards GSNO and gave higher fluorescence response. When GSNO was in the concentration range of
10~100 µM, coumarin probe had 3- to 9-fold fluorescence increase over background (Figure 2.10). The efficiency of fluorescein probe dropped possibly due to the fact that the GSNO has a reactive site with bigger steric effect, which may not react as efficiently as NACB with the fluorescein probe 2.15, a larger molecule with more steric effect compared to the coumarin probe 2.16.

Scheme 2.12 Reactions between probe 2.15, 2.16 and GSNO

Figure 2.10. Fluorescence response of the probes 2.15, 2.16 towards different GSNO concentration over background.
In an organic solution with trace buffer (CH$_3$CN/buff 100:1), we were able to obtain a linear plot between fluorescence response of probe 2.16 and the NACB concentration (Figure 2.11). However, attempt to obtain good linear correlation between the fluorescence and the GSNO concentration failed. This is possibly due to the fact that the free amine and carboxylic groups of the GSNO may lead to the intramolecular protonation of the aza-ylide intermediate which is then not a reactive intermediate, thus decrease the efficiency of the reaction.

![Figure 2.11](image)

**Figure 2.11** Linear correlation between the fluorescence response of probe 2.16 and the NACB concentration.

**2.4 CONCLUSION**

We have developed two fluorescent detection methods of S-nitrosothiols based on turn-on different mechanisms. The method based on the oxidation of the phosphine has been successfully used in measuring the concentration of small molecule SNOs. The fluorescent probes based on the reductive ligation are better candidates for fluorescent
study of SNOs than the probes based on oxidation of the phosphines. Those probes showed good fluorescent response towards small SNO models in certain concentration range, although with limitation in the quantification of the SNO concentration. Future study should be focused on improving the efficiency of the reaction in aqueous media with larger SNO molecules such as protein SNOs, and careful control experiments to ensure the specificity of the reaction in biological systems should also be performed. Refinement of these preliminary studies aiming at the application of this tool in the detection of SNOs in vitro and in vivo is of significant future interest.

2.5 EXPERIMENTAL

Materials and Methods: Reactions were carried out in oven or flame-dried glassware under an argon atmosphere, unless otherwise noted. All solvents were reagent grade. Diethyl ether (Et$_2$O) and tetrahydrofuran (THF) were freshly distilled from sodium / benzophenone under argon. Reactions were magnetically stirred and monitored by thin layer chromatography (TLC) with 0.25 mm pre-coated silica gel plates. Flash chromatography was performed with silica gel 60 (particle size 0.040 – 0.062mm). Yields refer to chromatographically and spectroscopically pure compounds, unless otherwise stated. Proton and carbon-13 NMR spectra were recorded on a 300 MHz spectrometer. Chemical shifts are reported relative to chloroform (δ 7.26) for $^1$H NMR and chloroform (δ 77.0) $^{13}$C NMR. Absorption spectra were recorded on a Lambda 20 UV/Vis spectrophotometer using 1 cm quartz cells. Fluorescence excitation and emission spectra were measured on QuantaMaster QM4 from Photon Technologies, Inc.
2.5.1 Synthesis

*Oxidation activated fluorescent probe:*

![Chemical Structure](image)

**Salicaldehyde 2.7a.** A solution of 8-hydroxyjulolidine 2.7 (5.0 g, 26.4 mmol) in 5 mL DMF was added dropwise to a cold solution of POCl$_3$ (4.46g, 29.1 mmol, 2.66 mL) in 10 mL DMF which had been stirring for 15 min. After 30 min at rt, the solution was heated to 100 °C for 30 min and was then cooled to rt. 25 mL water was added with stirring and a blue-green solid formed slowly. The precipitate was filtered, washed with water, and dried in a vacuum. The solid was dissolved in benzene/5% EtOAc and filtered through silica gel. A yellowish oil (4.92g, 88%) was obtained after the solvent was removed: $^1$H NMR (300 MHz, CDCl$_3$) δ 11.81 (s, 1H), 9.37 (s, 1H), 6.84 (s, 1H), 3.3 (dd, J=11.6, 4.1 Hz, 4H), 2.68 (t, J=6.3 Hz, 4H), 1.97-1.91 (m, 4H). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 191.7, 159.5, 149.7, 131.3, 113.8, 110.8, 105.4, 50.5, 27.4, 21.8, 20.7, 19.8; MS (MSI+) m/z 218.1 (M+H$^+$).

![Chemical Structure](image)

**Coumarin 2.8.** A solution of salicaldehyde 2.7a (2.2 g, 10 mmol), (2-methoxy-2-oxoethyl)-tri-phenylphosphonium bromide (5.0 g, 12 mmol), and DBU (1.8 mL, 1.8 g,
12 mmol) in DMSO (40 mL) was heated at reflux for 20 min. The solution was then cooled to rt, and poured into a mixture of crushed ice and water in a separatory funnel, then extracted four times with DCM. The combined DCM extracts were washed twice with water and dried over Na$_2$SO$_4$. The product was purified by silica gel chromatography (toluene/EtOAc, 10:1). A brilliant yellow solid was obtained (2.25 g, 93%): $^1$H NMR (300 MHz, CDCl$_3$) δ 7.46 (d, J=9.0 Hz, 1H), 6.84 (s, 1H), 5.99 (d, J=9.3 Hz, 1H), 3.1 (dd, J=11.1, 4.8 Hz, 4H), 2.88 (t, J=6.5 Hz, 2H), 2.75 (t, J=6.5 Hz, 2H), 2.01-1.92 (m, 4H). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 163.0, 151.9, 146.2, 144.3, 125.2, 118.5, 108.4, 108.3, 106.8, 50.2, 49.8, 27.7, 21.7, 20.8, 20.5; MS (MSI+) m/z 242.5 (M+H$^+$).

3-Iodocoumarin 2.9. A solution of NIS (2.52 g, 11.2 mmol) in THF (25 mL) was added dropwise to a vigorously stirred mixture of coumarin 2.8 (2.25 g, 9.3 mmol) and TsOH (0.18 g, 9.3 mmol) in 100 mL THF at rt. After stirring for 30 min following the addition the reaction was diluted with DCM, washed twice with 5% Na$_2$S$_2$O$_3$, once each with sat. NaHCO$_3$ and brine, then dried over Na$_2$SO$_4$. A yellow-orange solid (3.0 g, 87%) was obtained following purification over a silica column eluting with DCM: $^1$H NMR (300 MHz, CDCl$_3$) δ 8.00 (s, 1H), 6.76 (s, 1H), 3.4-3.24 (m, 4H), 2.86 (t, J=6.6 Hz, 2H), 2.74 (t, J=6.2 Hz, 2H), 1.99-1.91 (m, 4H). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 159.2, 152.6, 152.1, 146.6, 124.3, 118.9, 110.2, 106.5, 74.5, 50.2, 49.8, 27.7, 21.5, 20.6, 20.4; MS (MSI+) m/z 367.1 (M+H$^+$).
**Phosphine 2.5.** A solution of 3-iodocoumarin 2.9 (1 g, 2.7 mmol), Pd(OAc)$_2$ (1.7 mg, 7.6 μmol), triethylamine (0.51 mL) in acetonitrile (50 mL) was cooled to -70 °C, degassed and filled with Ar. Diphenylphosphine (0.46 mL, 2.7 mmol) was added after the system warmed to rt. Then the solution was heated to reflux for 5 h. When cooled to rt, a solution of 300 mL water and 400mL ethyl acetate was used for partition followed by washing twice with brine and drying with Na$_2$SO$_4$. A brilliant yellow solid (1.04g, 90%) was obtained following purification over silica gel (hexane/EtOAc, 5:1 to 2:1): $^1$H NMR (300 MHz, CDCl$_3$) δ 7.42-7.33 (m, 10H), 6.97 (d, J=3.6 Hz, 1H), 6.67 (s, 1H), 3.1 (t, J=5.4 Hz, 4H), 2.88 (t, J=6.5 Hz, 2H), 2.68 (t, J=6.2 Hz, 2H), 1.94 (m, 4H). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 163.1, 162.8, 152.1, 148.5, 148.4, 146.4, 136.0, 135.8, 134.2, 134.1, 133.9, 129.3, 129.2, 128.9, 128.8, 125.4, 118.5, 118.4, 117.4, 117.3, 109.1, 106.5, 50.3, 49.9, 27.6, 21.6, 20.7, 20.5; $^{31}$P NMR (121 MHz, CDCl$_3$) δ -14.4; MS (MSI+) m/z 426.1 (M+H$^+$).

**Compound 2.6.** A solution of S-nitrosoglutathione (0.677 g, 2 mmol) in water (7 mL)
was added dropwise to a solution of phosphine 5 (0.0830 g, 0.2 mmol) in THF (21 mL), stirred at rt. for 2 hours. After extracted with dichloromethene (30 mL) for 3 times, the organic layer was washed twice with brine and dried with Na₂SO₄. A yellow-orange solid (0.0792 g, 92%) was obtained following purification over silica gel (hexane/ EtOAc, 2:1 to 1:1): ¹H NMR (300 MHz, CDCl₃) δ 8.50 (d, J=13.5 Hz, 1H), 7.89-7.82 (m, 4H), 7.52-7.38 (m, 6H), 6.95 (s, 1H), 3.2 (q, J=5.7 Hz, 4H), 2.79 (t, J=6.5 Hz, 2H), 2.72 (t, J=6.3 Hz, 2H), 1.91 (m, 4H). ¹³C NMR (75 MHz, CDCl₃) δ 161.1, 160.9, 153.6, 153.5, 153.4, 148.3, 132.9, 132.2, 132.1, 132.0, 131.4, 128.5, 128.4, 126.9, 119.2, 109.3, 108.5, 108.4, 107.8, 106.0, 50.4, 50.0, 27.7, 21.4, 20.4, 20.2; ³¹P NMR (121 MHz, CDCl₃) δ 26.9; MS (MSI+) m/z 442.2 (M+H⁺).

**Reductive ligation activated fluorescent probes:**

![Reductive ligation activated fluorescent probes](image)

**Compound 2.18.** To a stirred solution of fluorescein (1.33 g, 4 mmol) in 20 mL DMF was added sodium hydride (60%, 0.32 g, 8 mmol), and the solution was stirred for an additional 30 mins. Then t-butyl bromoacetate (2.26 mL, 11.6 mmol) and sodium iodide (0.06 g, 0.4 mmol) were added, and the solution was then heated to reflux and stirred for 12 h. After cooled to room temperature, the solution was diluted with 100 mL DCM and washed with water (50 mL 3 times) and the organic layer was dried over Na₂SO₄. Flash chromatography (DCM/MeOH, 10:1) gave pure product 2.18 as red solid (0.57 g, 35%):
\(^1\)H NMR (300 MHz, DMSO-d\(_6\)) \(\delta\) 8.26 (1H, dd, \(J_1 = 7.8\) Hz, \(J_2 = 1.5\) Hz), 7.89 (1H, dt, \(J_1 = 7.5\) Hz, \(J_2 = 1.5\) Hz), 7.80 (1H, dt, \(J_1 = 7.5\) Hz, \(J_2 = 1.5\) Hz), 7.50 (1H, dd, \(J_1 = 7.5\) Hz, \(J_2 = 1.5\) Hz), 6.75 (2H, d, \(J = 9.9\) Hz), 6.54-6.51 (6H, m), 4.53 (2H, s), 1.27 (9H, s).

![Chemical structure of Compound 2.13](image)

**Compound 2.13.** A solution of compound 2.18 (0.115 g, 0.26 mmol), \(o\)-diphenylphosphinobenzoic acid 2.17 (0.087 g, 0.28 mmol), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (0.066 g, 0.34 mmol) and 4-(dimethylamino)-pyridine (0.004 g, 0.03 mmol) in 10 mL DCM was stirred at room temperature for 6 h. After the solvent was removed, the pure product 2.13 was obtained via flash chromatography (hexane/aceton, 1:1) as a light yellow solid (0.145g, 76%). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 8.36 (dd, \(J_1 = 7.5\) Hz, \(J_2 = 1.5\), 1H), 8.26-8.22 (m, 1H), 7.79-7.29 (m, 16H), 7.08 (d, \(J = 2.1\) Hz, 1H), 7.03-6.98 (m, 1H), 6.90-6.88 (m, 1H), 6.79 (dd, \(J_1 = 9.0\) Hz, \(J_2 = 2.4\), 1H), 6.54 (dd, \(J_1 = 9.6\) Hz, \(J_2 = 2.1\), 1H), 6.42 (d, \(J = 1.8\) Hz, 1H), 4.48 (d, \(J = 6.0\) Hz, 2H); \(^31\)P NMR (121 MHz, CDCl\(_3\)) \(\delta\) -2.98.

![Chemical structure of Compound 2.14](image)
**Compound 2.14.** To a stirred solution of **2.13** (0.145 g, 0.198 mmol) in 2 ml DCM was added 2 ml of trifluoroacetic acid (TFA) dropwise at 0 °C. The reaction was monitored by TLC and was complete in 2 h. The solvent was evaporated in vacuo and coevaporated 5 times with acetonitrile to yield **2.14** as a light yellow solid (0.129 g, 96%): $^1$H NMR (300 MHz, DMSO-d$_6$) δ 8.26-8.24 (1H, m), 8.02 (1H, d, $J = 6.9$ Hz), 7.67-7.59 (4H, m), 7.47 (3H, t, $J = 4.2$ Hz), 7.34-7.28 (8H, m), 7.14 (1H, d, $J = 7.2$ Hz), 7.00-6.97 (1H, m), 6.95 (1H, d, $J = 2.1$ Hz), 6.77-6.60 (4H, m), 4.70 (2H, s); $^{31}$P NMR (121 MHz, CDCl$_3$) δ -2.98.

**Compound 2.19.** To a stirred solution of fluorescein (1 g, 3.01 mmol) and K$_2$CO$_3$ (0.83 g, 6.01 mmol) in 3 ml N,N-dimethylformamide was added dropwise MeI (0.86 g, 6.06 mmol), and the solution was stirred for 12 hours. The solution was then diluted with 30 mL methylene chloride and washed with saturated ammonium chloride solution. The organic layer was separated and dried upon anhydrous Na$_2$SO$_4$. After the solvent was removed, the pure product **2.19** was obtained via flash chromatography (methylene chloride/methanol, 50:1) as a yellow solid (0.867 g, 76%): $^1$H NMR (300 MHz, DMSO-d$_6$) δ 8.26-8.23 (1H, m), 7.77-7.64 (2H, m), 7.33-7.30 (1H, m), 6.97 (1H, d, $J = 2.4$ Hz), 6.90-6.84 (2H, m), 6.74 (1H, dd, $J_1 = 9.0$ Hz, $J_2 = 2.7$ Hz), 6.55 (1H, dd, $J_1 = 9.6$ Hz, $J_2 = 2.1$ Hz), 6.47 (1H, d, $J = 1.8$ Hz), 3.92 (3H, s), 3.64 (3H, s).
**Compound 2.20.** To a stirred solution of 2.19 (0.573 g, 1.59 mmol) in 10 ml methanol was added dropwise 1.6 mL 10% NaOH, and the solution was stirred for 4 hours at room temperature. Methanol was then removed under vaccum, and concentrated HCl was added to adjust pH to 5. Then the solution was diluted with 20 mL DCM and the organic layer was separated and dried upon anhydrous Na$_2$SO$_4$. After the solvent was removed, the pure product 2.20 was obtained via flash chromatography (methylene chloride/methanol, 50:1) as a light yellow solid (0.419 g, 76%): $^1$H NMR (300 MHz, DMSO-d$_6$) δ 8.04-8.00 (1H, m), 7.69-7.59 (2H, m), 7.17-7.15 (1H, m), 7.00 (1H, d, $J = 9.0$ Hz), 6.75 (1H, dd, $J_1 = 10.8$ Hz, $J_2 = 2.7$ Hz), 6.69-6.59 (2H, m), 6.54-6.50 (1H, m), 6.31 (1H, dd, $J_1 = 9.0$ Hz, $J_2 = 2.4$ Hz), 3.84 (3H, s).

**Compound 2.15.** A solution of 2.20 (0.406 g, 1.17 mmol), acid 2.17 (0.395 g, 1.29 mmol), EDC (0.248 g, 1.29 mmol) and DMAP (0.016 g, 0.13 mmol) in 20 ml methylene chloride was stirred at room temperature for 4 hours. The solution was then diluted with 20 mL methylene chloride and washed with brine. The organic layer was separated and dried upon anhydrous Na$_2$SO$_4$. After the solvent was removed, the pure product 2.15 was
obtained via flash chromatography (methylene chloride/methanol, 50:1) as a yellow solid (0.487g, 66%): $^1$H NMR (300 MHz, CDCl$_3$) δ 8.06-7.99 (2H, m), 7.68-7.49 (11H, m), 7.45-7.39 (m, 4H), 7.15-7.13 (1H, m), 7.05 (1H, d, $J = 2.1$ Hz), 6.75 (1H, d, $J = 2.4$ Hz), 6.70-6.58 (4H, m), 3.82 (3H, s); $^{31}$P NMR (121 MHz, CDCl$_3$) δ -3.0.

**Compound 2.16.** A solution of 2.20 (0.406 g, 1.17 mmol), acid 2.17 (0.395 g, 1.29 mmol), EDC (0.248 g, 1.29 mmol) and DMAP (0.016 g, 0.13 mmol) in 20 ml methylene chloride was stirred at room temperature for 4 hours. The solution was then diluted with 20 mL methylene chloride and washed with brine. The organic layer was separated and dried upon anhydrous Na$_2$SO$_4$. After the solvent was removed, the pure product 2.15 was obtained via flash chromatography (methylene chloride/methanol, 50:1) as a yellow solid (0.487g, 66%): $^1$H NMR (300 MHz, CDCl$_3$) δ 8.28-8.24 (1H, m), 7.65 (1H, d, $J = 9.6$ Hz), 7.50-7.46 (2H, m), 7.43-7.27 (12H, m), 7.04-6.99 (1H, m), 6.93-6.89 (2H, m), 6.36 (1H, d, $J = 9.6$ Hz); $^{31}$P NMR (121 MHz, CDCl$_3$) δ -2.67.

*Ligation reaction between probe 2.13 and N-acyl cysteine SNO 2.21.*
To a stirred solution of SNO 2.21 (0.008 g, 0.038 mmol) in 6 mL CH<sub>3</sub>CN/PBS (pH 7.4) (2:1) was added phosphine 2.13 (0.0500 g, 0.068 mmol), the solution was then stirred for 5 minutes at room temperature. After that 2 mL of H<sub>2</sub>O<sub>2</sub> (5%) was added. The solution was then extracted with ethyl acetate (20mL, 3 times) and the organic layer was seperated and dried over Na<sub>2</sub>SO<sub>4</sub>. After the solvent was removed, flash chromatography (DCM/MeOH, 40:1) gave the ligation product 2.22 (0.009 g, 60%), fluorophore 2.18 (0.014 g, 89%) and phosphine oxide 2.23 (0.024 g, 95%).

2.5.2 Fluorescence analysis

**Determination of fluorescence quantum yields.** The fluorescence quantum yield (Φ) for the same compound has a relationship with several factors described in equation 1:

\[
I = 2.3 \Phi I_o \varepsilon c l
\]

In equation 1, \(I\) and \(I_o\) are fluorescent exitation and emission intensities in emission spectrum, \(\varepsilon\) is the molar absorption coefficient of the compound, \(c\) is the concentration of the compound and \(l\) is the width of the cuvette. For the determination
of fluorescence quantum yields, it is convenient to use the same excitation wavelength for the emission detection of the samples for we can eliminate and avoid the detection of excitation intensity. Then do the same detection of a standard compound with known quantum yield ($\Phi_0$), we can determine the unknown’s ($\Phi$) by using equation 2:

$$\Phi = \Phi_0 \times [(\varepsilon c_0) / (\varepsilon c)] \times (E / E_0)$$  \hspace{1cm} (2)

Here, the molar absorption coefficient of the unknown ($\varepsilon$) was determined by plotting a series of absorption versus different concentrations (Figure 2.11).

![Figure 2.11](image-url) Determination of the molar absorption coefficients of compound 2.5 (♦) and 2.6 (■).

The area under the entire emission spectrum for both unknown (E) and the standard ($E_0$) which represent the emission intensity of each was determined by numeric integration using the computer program Origin 8.0. Quinine bisulfate ($\Phi = 0.55$ in 1M H$_2$SO$_4$, $\varepsilon_0 = 1205$ cm$^{-1}$ M$^{-1}$ at 380 nm) was used as a standard to determine the quantum yields of 2.5 and 2.6 in phosphate-buffered saline (PBS). Optically dilute solutions of the quinine bisulfate, 2.5 or 2.6 were exited at 380 nm and the areas
under the emission spectra were determined by numerical integration (quinine bisulfate: 385-650 nm, 5,6: 450-600 nm).

**Linear relationship between fluorescent emission intensity and the concentrations of S-nitrosoglutathione (GSNO) and S-nitroso-N-acetylpenicillamine (SNAP):** A series of 0, 20, 40, 80, 160, 320 μL stock solution of GSNO in water (1.412 mM) were each mixed with a stock solution of phosphine 2.5 in DMSO (1.104 mM) and diluted to a 4240 μL PBS solution (pH 7.4). After 1 h, 20 μL of each were taken to 4000 μL PBS solution (pH 7.4), and detected by fluorescent emission spectrum ($\lambda_{ex} = 437$ nm, $\lambda_{em} = 487$ nm). The curve of the emission intensity at any concentration of GSNO was determined. To test its applicability for other SNOs, a series of 0, 20, 40, 60, 80, 100 μL stock solution of SNAP in THF (2.664 mM) were each mixed with a stock solution of phosphine 2.5 in DMSO (1.104 mM). After 5 h, 20 μL of each were taken to 4000 μL PBS solution (pH 7.4), and detected by fluorescent emission spectrum ($\lambda_{ex} = 437$ nm, $\lambda_{em} = 487$ nm). All the data are combined and displayed in Figure 2.12.
**Figure 2.12** Determination of concentration of SNAP (■) using the linear relationship between the emission intensity and the concentration of GSNO (●) after the reaction with compound 2.5.

The linear equation of the plot is \( y = 9.737 \times 10^{11} x + 1.512 \times 10^{4} \), is good in linearity \( (R^2=0.9944) \), where \( 1.512 \times 10^{4} \) is the average background of compound 2.5.

**Linear correlation between fluorescent emission intensity and the concentrations of S-nitroso-N-acetyl cysteine methylester 2.21:** A series of 0, 20, 40, 80, 160, 320 μL stock solution of SNO 2.21 in CH₃CN (1.22 mM) were each mixed with 200 μL stock solution of phosphine 2.13 in CH₃CN (1.82 mM) and diluted to a 3200 μL CH₃CN/PBS (pH 7.4) (2:1) solution. After 2 h, 20 μL of each were taken to 45 mL PBS solution (pH 7.4), and detected by fluorescent emission spectrum (\( \lambda_{\text{ex}} = 467 \text{ nm}, \lambda_{\text{em}} = 514 \text{ nm} \)). The curve of the emission intensity at any concentration of GSNO was determined (Figure 2.3). The equation of the plot: \( y = 1.381 \times 10^{7} x \), is good in linearity \( (R^2=0.98) \).

**Figure 2.4** A linear correlation between emission intensity and the concentration of 2.21.
**Stability test of the ester bond in different pH.** As shown in Figure 2.5A, each of 30 μL stock solution of phosphine 2.13 in CH$_3$CN (4.05 μM) was diluted in 3000 μL CH$_3$CN/PBS (pH 3.9) (2:1), CH$_3$CN/PBS (pH 7.4) (2:1), CH$_3$CN/PBS (pH 9.6) (2:1) solution, and immediately taken to the detection by the timed-based fluorescent spectrum ($\lambda_{ex} = 467$ nm, $\lambda_{em} = 514$ nm) for 2.5 h. Time-based UV analysis was shown in Figure 2.5B, each of 60 μL stock solution of phosphine 2.13 in CH$_3$CN (0.409 mM) was diluted in 3000 μL CH$_3$CN/PBS (pH 3.9) (2:1), CH$_3$CN/PBS (pH 7.4) (2:1), CH$_3$CN/PBS (pH 9.6) (2:1) solution, and immediately taken to the detection by the timed based UV spectrum ($\lambda_{max} = 454$ nm) for 2.5 h.

![Scheme 2.10](image)

**Scheme 2.10** Hydrolysis test of the ester bond of probe 2.13 in different pH
Figure 2.5 A Time-based fluorescence spectroscopy of the probe 2.13 in different pH (4 7.4 10); 4B Time-based UV-vis spectroscopy of the probe 2.13 in different pH (4 7.4 10).

Reactivity of probe 2.13 towards different cysteine species. 20 μL stock of SNO 2.21, cysteine 2.24 and cysteine disulfide 2.25 (1.00 mM) were each mixed with a 60 μL stock solution of phosphine 2.13 in CH₃CN (1.49 mM) and diluted in 2000 μL CH₃CN/PBS (pH 7.4) (2:1) solution. After 2 hours the solution were detected by the fluorescent spectrum (λₑₓ = 467 nm, λₑₘ = 514 nm). The emission spectrum and bar plot of each case was expressed in Figure 2.5 below.
**Figure 2.6** Fluorescence response of the probe 2.13 towards cysteine species 2.21, 2.24, 2.25.

**Fluorescent response of probes towards GSNO and NACB.** 200 mL stock solution of probe 2.15 and 2.16 were each diluted in 2000 µL mixed solution of PBS (pH 7.4, 0.1 M) and CH$_3$CN (v/v 3:1). 100 µL stock solution of 0, 10 µM, 100 µM, 1 mM, 10 mM CysNO was then added respectively. The mixed solutions were stirred at room temperature for 2 hours, and then taken to the fluorescence spectroscopy (2.15: $\lambda_{ex}$ 465 nm, $\lambda_{em}$ 510 nm; 2.16: $\lambda_{ex}$ 375 nm, $\lambda_{em}$ 450 nm). The bar plot of signal over background ratio at different CysNO concentration was presented in Figure 2.9.
Figure 2.9. Fluorescence response towards different CysNO concentration over background

20 mL stock solution of probe 2.15 and 2.16 were each diluted in 2000 µL mixed solution of PBS (pH 7.4, 0.1 M) and CH₃CN (v/v 1:1). 100 µL stock solution of 0, 0.2 mM, 0.4 mM, 0.8 mM, 1.2 mM, 1.6 mM, 2 mM CysNO was then added respectively. The mixed solutions were stirred at room temperature for 2 hours, and then taken to the fluorescence spectroscopy (2.15: λex 465 nm, λem 510 nm; 2.16: λex 375 nm, λem 450 nm). The bar plot of signal over background ratio at different GSNO concentration was presented in Figure 2.9.

![Bar plot of signal over background ratio at different GSNO concentration](image)

Figure 2.10. Fluorescence response towards different CysNO concentration over background

Linear correlation between fluorescence response of probe 2.16 versus the concentration of CysNO: A series of 0, 10 µL, 15 µL, 30 µL, 60 µL and 200 µL stock solution of CysNO (146 µM) were added respectively to 2000 µL stock solution of probe 2.16 (0.62 mM) in mixed solution of CH₃CN and PBS (pH 7.4) 100:1, and mixed at room
temperature for 1 hour. The solution were then taken to fluorescence spectroscopy at $\lambda_{ex}$ 375 nm, $\lambda_{em}$ 450 nm. A linear correlation is shown in Figure 2.11 with equation $y = 26384 x + 32142$ ($R^2 = 0.996$).

**Figure 2.11** Linear correlation of fluorescence response of probe 2.16 versus CysNO concentration

### 2.6 REFERENCES


CHAPTER THREE

REACTIVITY OF SULFENAMIDES TOWARDS NUCLEOPHILES

3.1 ABSTRACT

The reductive ligation is a specific reaction towards SNOs and converts SNOs to sulfenamides. We hypothesized that those sulfenamides can react with certain nucleophiles to generate more stable sulfur conjugates. To prove our hypothesis, we investigated a series of sulfur and carbon nucleophiles and studied their reactivity and selectivity towards SNOs. Based on that, we found a one-pot disulfide formation reaction from SNOs. The study revealed the potential usage of the one-pot reaction in the detection of SNOs.

3.2 INTRODUCTION

The reductive ligation of SNOs selectively converts unstable SNOs to relatively stable sulfenamide products via a Staudinger ligation-like mechanism. However, we also noticed that sulfenamides generated from cysteine derivatives could be over-reduced by the excess of phosphine ligation reagents. This might be a problem for directly applying reductive ligation in SNO detection.

To solve this problem, we developed a bis-ligation of SNO in 2009 (Scheme 3.1). This reaction can convert SNOs to stable disulfide-iminophosphorane conjugates in
one-step. Previous studies have revealed that disulfides are stable towards excess triarylphosphines such as the phosphine reagents used in our reaction.\(^{[3.1,3.4]}\)

\[
\begin{align*}
\text{R-SNO} & \quad \xrightarrow{3.1} \quad \text{Scheme 3.1 Bis-ligation of SNOs}
\end{align*}
\]

Given the high reactivity of the sulfenamide intermediates towards the thiolate observed in bis-ligation, we proposed another one-step reaction to convert unstable SNOs to stable products (Scheme 3.2). As such, SNO will be treated with regular reductive ligation reagent 3.5. As the reductive ligation is a very fast reaction, sulfenamide products should be formed in minutes. Then, if a nucleophile is added into the reaction mixture, it may convert sulfenamide 3.6 to a stable final product 3.7 and liberate the phosphine oxide 3.8. The formation of simple adducts, without the bulky triarylpiposphene-oxide, would be attractive for applications in protein systems. A critical concern of the one-pot reaction being used in the detection of protein SNO, is that the nucleophile should only react with sulfenamides, not with protein disulfides.

\[
\begin{align*}
\text{R-SNO} & \quad \xrightarrow{3.5} \quad \text{Scheme 3.2 Proposed one-pot reaction to convert SNO to stable products}
\end{align*}
\]
3.3 RESEARCH DESIGN AND METHODOLOGY\textsuperscript{[3,5]}

To test our hypothesis, sulfenamide 3.9 was prepared via the reductive ligation\textsuperscript{[3,1]} of cysteine SNO 3.10A (Scheme 3.3A) and used as a model compound to test the reactivity of a series of nucleophiles. To examine the selectivity of the nucleophiles under the same condition, disulfide 3.11 was also synthesized from the oxidation of cysteine 3.10 (Scheme 3.3B).

Scheme 3.3 A Synthesis of sulfenamide 3.9; B synthesis of disulfide 3.11.

The reaction of 3.9 and 3.11 with nucleophiles 3.12a-j were both carried out in a mixture of THF and PBS buffer (pH 7.4) solution at room temperature, and the products were isolated for comparison (Table 3.1).
As shown in Table 3.1, primary thiol 3.13a and thiobenzoic acid 3.13c showed good reactivity towards both sulfenamide and disulfide. Tertiary thiol 3.13b showed no reactivity possibly due to its steric effect. Carbon nucleophiles 3.13d and 3.13e were also tested in our reactions. 3.13d was previously reported to react with cyclic sulfenamides.⁹ However, both 3.13d and dimedone 3.13e failed to show any reactivity towards sulfenamide 3.9. Interestingly, most of the aromatic thiol nucleophiles (3.13g-j) showed some selectivity for the sulfenamide compared to the
disulfide. Among them 2-mercapto benzothiazole \textbf{3.13h} showed best selectivity and efficiency.

\textit{One-pot disulfide formation via SNOs}

With these results in hand, we turned to the one-step disulfide formation from S-nitrosothiols using \textbf{3.13h}, the best substrate found in Table 3.1. A series of SNO substrates \textbf{3.14a-g} were tested (Table 3.2).

Table 3.2 One-pot disulfide formation from SNOs.

<table>
<thead>
<tr>
<th>RSNO (3.14)</th>
<th>Yield % (3.16)</th>
<th>RSNO (3.14)</th>
<th>Yield % (3.16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeO₂C\text{NHAc}\text{SNO} a</td>
<td>85</td>
<td>Boc\text{HN}\text{O}\text{CO₃Me}\text{SNO} e</td>
<td>65</td>
</tr>
<tr>
<td>BnHN\text{NHAc}\text{SNO} b</td>
<td>86</td>
<td>Cbz\text{HN}\text{O}\text{CO₃Me}\text{SNO} f</td>
<td>82</td>
</tr>
<tr>
<td>MeO₂C\text{NHAc}\text{SNO} c</td>
<td>43</td>
<td>Ac\text{HN}\text{O}\text{CO₃Me}\text{SNO} g</td>
<td>75</td>
</tr>
<tr>
<td>MeO₂C\text{NHAc}\text{SNO} d</td>
<td>55</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The reaction was carried out as follows: freshly prepared SNO substrates were treated with phosphine \textbf{3.15} in a mixture of 3:1 THF/PBS buffer (pH 7.4) at room temperature. After 10 minutes, \textbf{3.13h} was added and the reaction mixture was stirred for an additional 30 minutes to afford the desired product \textbf{3.16a-g}. As shown in Table
3.2, all of the SNO substrates exhibited good reactivity in the reaction and the desired disulfide products were obtained in good yields.

**Crossover control experiment**

In order to confirm that the reaction is specific only towards SNOs, while not affecting disulfides, we performed a crossover experiment. As shown in Scheme 3.4, the one-step disulfide formation using SNO 3.17 was carried out in the presence of disulfide 3.11. In this reaction, 3.18 was the only product and no crossover product 3.12h was observed. Disulfide 3.11 was completely recovered.

![Scheme 3.4 Crossover reactivity test in the presence of cysteine disulfide.](image)

**3.4 CONCLUSION**

In conclusion, the study revealed the reactivity of the sulfenamide towards various nucleophiles and provided a one-pot disulfide formation from S-nitrosothiols under very mild conditions. Importantly, this reaction does not affect disulfide bonds. Further modification towards the structure of 2-mercaptobenzothiazole with reporters such as biotin or fluorophores will lead to compounds useful in the detection of SNOs.
3.5 EXPERIMENTAL

Materials and Methods: All solvents were reagent grade. Tetrahydrofuran (THF) was freshly distilled from sodium / benzophenone under argon. Reactions were magnetically stirred and monitored by thin layer chromatography (TLC) with 0.25 mm pre-coated silica gel plates. Flash chromatography was performed with silica gel 60 (particle size 0.040-0.062mm). Yields refer to chromatographically and spectroscopically pure compounds, unless otherwise stated. Proton and carbon-13 NMR spectra were recorded on a 300 MHz spectrometer. Chemical shifts are reported relative to chloroform (δ 7.26) for $^1$H NMR and chloroform (δ 77.0) for $^{13}$C NMR.

Preparation of sulfonamide 3.9 and disulfide 3.11.

\[ \text{ON} \quad \text{NHAc} \quad \text{CO}_2\text{Me} \quad \text{PhPh}_2 \quad \text{CO}_2\text{Ph} \quad \text{H}_2\text{O} \quad \text{H}_2\text{O} \quad \text{DCM} \quad \text{MeOH} \quad \text{5 : 1} \quad \text{86%} \]

To a stirred solution of 3.10A (301 mg, 1.46 mmol) in 27 mL mixed solution of CH$_3$CN, THF and H$_2$O (1.5 : 1.5 : 1), was added 1.1 (1.117g, 2.92 mmol). The solution was then stirred for 10 minutes at room temperature in darkness and later a aqueous solution of H$_2$O$_2$ (5%, 5 mL) was added to quench the reaction. The solution was extracted with DCM (20 mL, 3 times) and the organic layer was washed with brine (20 mL), separated and dried over anhydrous Na$_2$SO$_4$. The solvent was then removed under vaccum and the product 3.9 was isolated as white solid (626 mg, 86%) via flash chromatography (DCM/MeOH 50 : 1).
To a stirred solution of 3.10 (1.77 g, 10 mmol) in 5 mL DCM, was added NBS (0.89 g, 5 mmol), and the solution was stirred at room temperature for 1 hour. The solution was then diluted with 30 mL DCM and washed with saturated NaHCO$_3$ (20 mL) and brine (20 mL). The organic layer was separated and dried over anhydrous Na$_2$SO$_4$, and then concentrated under the vacuum. The product 3.11 was isolated as white solid (1.708 g, 97%) via flash chromatography (DCM/MeOH 50 : 1).

**General procedure for the nucleophilic substitution reactions of sulfenamide 3.9 or disulfide 3.11 with nucleophiles.**

To a stirred solution of sulfenamide 3.9 or disulfide 3.11 (0.1 mmol) in 4 mL THF/PBS (pH 7.4) (3:1), was added nucleophile 3.13 (0.11 mmol), the reaction mixture was stirred at room temperature for 0.5 to 2 hours. After the reaction was done (by TLC), the mixture was diluted with dichloromethane (25 mL) and washed with brine (25 mL). The organic solvent was then dried over anhydrous Na$_2$SO$_4$. The product was isolated by flash column chromatography (dichloromethane/methanol 100:1).
Compound 3.12a

![Compound 3.12a structure](image)

White solid; m.p. 89-90°C; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.36-7.28 (5H, m), 7.30 (1H, d, $J = 6.9$ Hz), 4.80-4.74 (1H, m), 3.89 (2H, s), 3.73 (3H, s), 2.89-2.75 (2H, m), 2.02 (3H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 171.2, 170.1, 137.0, 129.6, 128.9, 127.9, 52.9, 51.8, 43.7, 40.0, 23.4; IR (thin film) cm$^{-1}$ 3281, 3030, 1747, 1656, 1541, 1215, 700; MS m/z 300.0 [M+H$^+$].

Compound 3.12c

![Compound 3.12c structure](image)

White solid; m.p. 104-105°C; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.96-7.93 (2H, m), 7.62 (1H, dt, $J_1 = 7.5$ Hz, $J_2 = 1.5$ Hz), 7.49-7.44 (2H, m), 7.16 (1H, d, $J = 7.5$ Hz), 4.87-4.81 (1H, m), 3.68 (3H, s), 3.44 (1H, dd, $J_1 = 14.4$ Hz, $J_2 = 4.8$ Hz), 3.13 (1H, dd, $J_1 = 14.4$ Hz, $J_2 = 4.8$ Hz), 2.07 (3H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 171.1, 170.1, 167.5, 136.8, 129.5, 128.5, 127.7, 53.0, 51.9, 40.8, 23.2; IR (thin film) cm$^{-1}$ 3287, 3062, 2953, 1747, 1686, 1540, 1448, 1206, 1176, 887, 679; MS m/z 314.0 [M+H$^+$].

Compound 3.12f

![Compound 3.12f structure](image)
Colorless thick oil; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.70-7.55 (1H, m), 7.54-7.47 (2H, m), 7.37-7.31 (2H, m), 7.28-7.22 (1H, m), 6.35 (1H, d, $J$ = 7.2 Hz), 4.91-4.82 (1H, m), 3.73 (3H, s), 3.23 (1H, q, $J$ = 2.4 Hz), 1.93 (3H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 171.1, 170.1, 167.5, 136.8, 129.5, 128.5, 127.7, 53.0, 51.9, 40.8, 23.2; IR (thin film) cm$^{-1}$ 3275, 3060, 1747, 1660, 1539, 1438, 1217, 1024, 744; MS m/z 285.9 [M+H$^+$].

**Compound 3.12g**

White solid; m.p. 151-152°C; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.69-7.66 (1H, m), 7.59 (1H, d, $J$ = 7.8 Hz), 7.52-7.49 (1H, m), 7.36-7.32 (2H, m), 4.99-4.94 (1H, m), 3.72 (3H, s), 3.65 (1H, dd, $J_1$ = 14.6 Hz, $J_2$ = 5.3 Hz), 3.41 (1H, dd, $J_1$ = 14.6, $J_2$ = 4.8 Hz), 2.07 (3H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 170.8, 170.4, 164.2, 152.8, 141.5, 125.4, 125.2, 119.3, 110.8, 53.1, 51.6, 42.1, 23.3; IR (thin film) cm$^{-1}$ 3280, 3063, 1747, 1660, 1538, 1504, 1452, 1237, 1216, 1127, 1094, 745; MS m/z 327.4 [M+H$^+$].

**Compound 3.12h**

White solid; m.p. 153°C; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.89 (1H, dd, $J_1$ = 8.1 Hz, $J_2$ = 0.6 Hz), 7.81 (1H, dd, $J_1$ = 8.0 Hz, $J_2$ = 0.6 Hz), 7.46 (1H, dt, $J_1$ = 7.8 Hz, $J_2$ = 1.2 Hz), 7.35 (1H, dt, $J_1$ = 7.8 Hz, $J_2$ = 1.2 Hz), 6.90 (1H, d, $J$ = 7.2 Hz), 4.99-4.94 (1H, m), 3.75 (3H, s), 3.50 (2H, d, $J$ = 4.8 Hz), 2.04 (3H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 170.7,
170.7, 170.2, 154.7, 136.1, 126.7, 125.2, 122.4, 121.5, 53.2, 52.0, 41.7, 23.4; IR (thin film) cm\(^{-1}\) 3275, 3061, 1747, 1660, 1540, 1459, 1426, 1236, 1216, 1006, 758; HRMS m/z 343.0247 [M+H\(^{+}\)], calcd for C\(_{13}\)H\(_{15}\)N\(_{2}\)O\(_{3}\)S\(_{3}\) 343.0239.

Compound 3.12i

Colorless thick oil; \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 8.65 (2H, d, \(J = 4.8\) Hz), 7.23 (1H, s), 7.16 (1H, t, \(J = 4.8\) Hz), 4.95-4.89 (1H, m), 3.71 (3H, s), 3.53 (1H, dd, \(J_1 = 14.4\) Hz, \(J_2 = 4.8\) Hz), 3.4 (1H, dd, \(J_1 = 14.4\) Hz, \(J_2 = 4.8\) Hz), 2.06 (3H, s); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) 171.6, 171.0, 170.0, 158.2, 118.4, 52.9, 51.5, 41.2, 23.4; IR (thin film) cm\(^{-1}\) 3267, 3049, 2953, 1746, 1659, 1574, 1561, 1447, 1418, 1216, 1118, 763; MS m/z 287.8 [M+H\(^{+}\)].

Compound 3.12j

Colorless thick oil; \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 8.55-8.52 (1H, m), 7.65 (1H, dt, \(J_1 = 8.1\) Hz, \(J_2 = 1.8\) Hz), 7.52 (1H, d, \(J = 8.1\) Hz), 7.43 (1H, d, \(J = 7.2\) Hz), 7.16 (1H, ddd, \(J_1 = 7.2\) Hz, \(J_2 = 4.8\) Hz, \(J_3 = 1.2\) Hz), 4.94-4.89 (1H, m), 3.72 (3H, s), 3.44 (1H, dd, \(J_1 = 14.4\) Hz, \(J_2 = 4.8\) Hz), 3.4 (1H, dd, \(J_1 = 14.4\) Hz, \(J_2 = 4.8\) Hz), 2.02 (3H, s); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) 171.0, 170.2, 159.2, 150.1, 137.4, 121.6, 121.3, 52.9, 51.9, 41.6, 23.3; IR (thin film) cm\(^{-1}\) 3267, 3049, 2953, 1746, 1659, 1574, 1561, 1447, 1418, 1216, 1118, 763; MS m/z 286.9 [M+H\(^{+}\)].

To a stirred solution of RSNO 3.14 (3 mmol) in 4 mL THF/PBS (pH 7.4) 3:1, was added phosphine 3.15 (6 mmol) and stirred for 10 minutes, which was followed by adding thiol 3.13h (3.3 mmol). The reaction mixture was stirred at room temperature for 30 minutes, and then diluted with dichloromethane (50 mL) and washed with brine (25 mL). The organic layer was dried over anhydrous Na₂SO₄. The product was isolated by flash column chromatography (dichloromethane/methanol 100:1).

Compound 3.16a

White solid; m.p. 153°C; $^1$H NMR (300 MHz, CDCl₃): $\delta$ 7.89 (1H, dd, $J_1 = 8.1$ Hz, $J_2 = 0.6$ Hz), 7.81 (1H, dd, $J_1 = 8.0$ Hz, $J_2 = 0.6$ Hz), 7.46 (1H, dt, $J_1 = 7.8$ Hz, $J_2 = 1.2$ Hz), 7.35 (1H, dt, $J_1 = 7.8$ Hz, $J_2 = 1.2$ Hz), 6.90 (1H, d, $J = 7.2$ Hz), 4.99-4.94 (1H, m), 3.75 (3H, s), 3.50 (2H, d, $J = 4.8$ Hz), 2.04 (3H, s); $^{13}$C NMR (75 MHz, CDCl₃): $\delta$ 170.7, 170.7, 170.2, 154.7, 136.1, 126.7, 125.2, 122.4, 121.5, 53.2, 52.0, 41.7, 23.4; IR (thin film) cm⁻¹ 3275, 3061, 1747, 1660, 1540, 1459, 1426, 1236, 1216, 1006, 758; HRMS m/z 343.0247 [M+H⁺], calcd for C₁₃H₁₅N₂O₃S₃ 343.0239.
Compound 3.16b

White solid; m.p. 223-224°C; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 8.69 (1H, t, $J = 6.0$ Hz), 8.39 (1H, dd, $J = 7.8$ Hz), 8.04 (1H, d, $J = 7.8$ Hz), 7.84 (1H, d, $J = 8.1$ Hz), 7.48 (1H, t, $J = 7.2$ Hz), 7.48 (1H, t, $J = 7.2$ Hz), 7.30-7.21 (5H, m), 4.70-4.63 (1H, m), 4.27 (2H, d, $J = 5.7$ Hz), 3.39 (1H, dd, $J_1 = 13.5$ Hz, $J_2 = 5.7$ Hz), 3.18 (1H, dd, $J_1 = 13.5$ Hz, $J_2 = 5.7$ Hz), 1.87 (3H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 170.4, 169.7, 137.8, 129.1, 129.0, 128.1, 127.8, 126.8, 126.8, 125.3, 121.7, 121.6, 52.8, 44.0, 42.8, 23.4; IR (thin film) cm$^{-1}$ 3276, 1647, 1541, 1426, 1006, 698; MS(MSI+) m/z 418.0739 [M+H$^+$], calcd for C$_{19}$H$_{20}$N$_3$O$_2$S$_3$ 418.0712.

Compound 3.16c

Colorless thick oil; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 8.09 (1H, t, $J = 5.6$ Hz), 7.86 (1H, d, $J = 8.1$ Hz), 7.78 (1H, dd, $J_1 = 8.1$ Hz, $J_2 = 0.8$ Hz), 7.44 (1H, dt, $J_1 = 7.8$ Hz, $J_2 = 1.2$ Hz), 7.33 (1H, dt, $J_1 = 7.7$ Hz, $J_2 = 1.2$ Hz), 7.19 (1H, d, $J = 7.8$ Hz), 4.95 (1H, q, $J = 6.8$ Hz), 4.07 (2H, dd, $J_1 = 5.7$ Hz, $J_2 = 0.9$ Hz), 3.72 (3H, s), 3.45 (1H, dd, $J_1 = 14.3$ Hz, $J_2 = 5.9$ Hz), 3.22 (1H, dd, $J_1 = 14.1$ Hz, $J_2 = 6.9$ Hz), 1.99 (3H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 170.8, 170.4, 170.0, 153.9, 136.2, 126.7, 125.2, 122.0, 121.6, 52.6, 52.6, 42.4, 41.6, 23.3;
IR (thin film) cm	extsuperscript{-1} 3289, 3061, 2952, 1750, 1652, 1538, 1426, 1122, 1006, 758, 729; MS m/z 399.9 [M+H]+.

**Compound 3.16d**

![Chemical structure image](image)

White solid; m.p. 193-194°C; \(^1\)H NMR (300 MHz, CDCl\textsubscript{3}): \(\delta\) 7.75 (1H, dd, \(J_1 = 7.8\) Hz, \(J_2 = 0.9\) Hz), 7.70 (1H, dd, \(J_1 = 7.8\) Hz, \(J_2 = 0.9\) Hz), 7.64 (1H, d, \(J = 7.5\) Hz), 7.38 (1H, dt, \(J_1 = 7.5\) Hz, \(J_2 = 1.2\) Hz), 7.30 (1H, dt, \(J_1 = 7.5\) Hz, \(J_2 = 1.2\) Hz), 7.26-7.08 (6H, m), 4.92 (1H, dd, \(J_1 = 14.1\) Hz, \(J_2 = 6.0\) Hz), 4.83 (1H, dd, \(J_1 = 13.2\) Hz, \(J_2 = 6.9\) Hz), 3.67 (3H, s), 3.41 (1H, dd, \(J_1 = 14.1\) Hz, \(J_2 = 6.0\) Hz), 3.20 (2H, dt, \(J_1 = 13.8\) Hz, \(J_2 = 6.0\) Hz), 3.06 (1H, dd, \(J_1 = 13.8\) Hz, \(J_2 = 6.9\) Hz), 1.92 (3H, s); \(^{13}\)C NMR (75 MHz, CDCl\textsubscript{3}): \(\delta\) 171.6, 170.8, 170.7, 169.9, 154.2, 136.2, 136.0, 129.5, 128.8, 127.3, 126.7, 125.1, 122.2, 121.5, 54.1, 52.7, 52.6, 42.7, 37.8, 23.3; IR (thin film) cm	extsuperscript{-1} 3285, 3061, 1743, 1648, 1541, 1426, 1215, 1006, 757; MS m/z 489.9 [M+H]+.

**Compound 3.16e**

![Chemical structure image](image)

Colorless thick oil; \(^1\)H NMR (300 MHz, CDCl\textsubscript{3}): \(\delta\) 7.90 (1H, dd, \(J_1 = 8.4\) Hz, \(J_2 = 0.6\) Hz), 7.83 (1H, dd, \(J_1 = 8.1\) Hz, \(J_2 = 0.6\) Hz), 7.46 (1H, dt, \(J_1 = 7.2\) Hz, \(J_2 = 1.2\) Hz), 7.36 (1H, dt, \(J_1 = 7.5\) Hz, \(J_2 = 1.2\) Hz), 7.33 (1H, s), 5.16-5.15 (1H, m), 4.98-4.92 (1H, m), 3.86 (2H,
d, \( J = 5.7 \) Hz), 3.75 (3H, s), 3.50 (2H, ddd, \( J_1 = 21.3 \) Hz, \( J_2 = 14.4 \) Hz, \( J_3 = 1.5 \) Hz), 1.46 (9H, s); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)); \( \delta \) 170.3, 169.8, 154.8, 136.1, 126.7, 125.2, 122.5, 121.5, 53.2, 51.9, 41.3, 28.5; IR (thin film) cm\(^{-1}\) 3314, 3062, 2978, 1745, 1678, 1518, 1427, 1367, 1250, 1169, 1006, 758, 728; MS m/z 457.8 [M+H\(^+\)].

Compound 3.16f

![Chemical structure](image)

Colorless thick oil; \(^1\)H NMR (300 MHz, CDCl\(_3\)); \( \delta \) 7.90 (1H, d, \( J = 8.4 \) Hz), 7.80 (1H, d, \( J = 7.8 \) Hz), 7.44 (1H, dt, \( J_1 = 7.8 \) Hz, \( J_2 = 1.2 \) Hz), 7.38-7.30 (7H, m), 5.38 (1H, d, \( J = 7.2 \) Hz), 5.12 (2H, s), 4.91 (1H, d, \( J = 7.2 \) Hz), 4.36-4.31 (1H, m), 3.74 (3H, s), 3.54-3.39 (2H, m), 1.41 (3H, d, \( J = 6.9 \) Hz); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)); \( \delta \) 202.6, 172.4, 170.3, 156.2, 154.7, 136.3, 136.2, 128.7, 128.5, 128.4, 126.7, 125.2, 122.5, 121.5, 67.4, 53.2, 52.1, 50.7, 41.2; IR (thin film) cm\(^{-1}\) 3310, 3063, 1718, 1670, 1522, 1006, 728; MS m/z 506.0 [M+H\(^+\)].

Compound 3.16g

![Chemical structure](image)

White solid; m.p. 214-215°C; \(^1\)H NMR (300 MHz, CDCl\(_3\)); \( \delta \) 7.79 (1H, d, \( J = 8.1 \) Hz), 7.72 (1H, d, \( J = 7.8 \) Hz), 7.39-7.10 (8H, m), 6.23 (1H, d, \( J = 7.5 \) Hz), 4.81-4.71 (2H, m), 3.65 (3H, s), 3.39 (1H, dd, \( J_1 = 14.4 \) Hz, \( J_2 = 4.8 \) Hz), 3.2 (1H, dd, \( J_1 = 14.1 \) Hz, \( J_2 = 5.7 \) Hz).
Hz), 3.08-2.94 (2H, m), 1.88 (3H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): δ 171.3, 171.1, 170.5, 170.0, 154.9, 136.5, 136.1, 129.5, 128.9, 127.3, 126.7, 125.2, 122.5, 121.5, 54.5, 53.1, 52.1, 41.1, 38.4, 23.4; IR (thin film) cm$^{-1}$ 3276, 3062, 1747, 1648, 1542, 1426, 1236, 1006, 728; MS m/z 490.0 [M+H$^+$].

**Crossover experiment**

![Chemical structure diagram]

To a stirred solution of SNO 3.17 (0.055 g, 0.196 mmol) and disulfide 3.11 (0.069 g, 0.196 mmol) in 3:1 THF/PBS (pH 7.4) (8 mL), was added phosphine 3.15 (0.150 g, 0.392 mmol) and the reaction mixture was stirred for 10 minutes at room temperature. Then 3.13h (0.065 g, 0.392 mmol) was added and the reaction mixture was stirred at room temperature for an additional 30 minutes. After the reaction was done, the mixture was diluted with dichloromethane (25 mL) and washed with brine (25 mL). The organic solvent was then dried over anhydrous Na$_2$SO$_4$. The disulfide product 3.18 was isolated (white solid, 0.050 g, 72%) by flash column chromatography (dichloromethane/methanol 100:1), and starting material 3.11 was fully recovered (white solid, 0.067 g, quant.).
3.6 REFERENCES


CHAPTER FOUR

FACILE AMIDE FORMATION VIA S-NITROSATION

4.1 ABSTRACT

Due to the importance in biology and drug discovery, amide or peptide bond formation is an active research area in organic chemistry. In the past decade, a number of new strategies for the construction of amide bonds have been discovered. In particular, thioacid or thioester derivatives are attractive starting materials. Recent studies have revealed some unique reactivity of these sulfur-based compounds and demonstrated some advantages of them compared to carboxylic acid derivatives in amide and peptide bond forming sequences. In our recent efforts to study new chemistry of thiol S-nitrosation, we hypothesized that if thioacids were subjected to nitrosation, the corresponding S-nitrosothioacids (NTA) could be formed. Such a S-nitrosation process may activate thioacids and lead to a facile acylation with certain nucleophiles. To prove the hypothesis, we synthesized a series of NTAs and reacted them with amines in situ to generate amides. The scope and efficacy of the reaction have been investigated. And it is found to be promising in peptide synthesis.

4.2 Introduction

Amide/peptide
Amide, a carbonyl group attaching to a nitrogen atom, is an important structure that broadly exists in nature and also plays an important role in biology and drug discovery. In nature, amides are mostly found in proteins, where amino acids are assembled by amide or peptide bonds. Proteins play a crucial role in virtually all biological processes such as enzymatic catalysis (nearly all known enzymes are proteins), transport/storage (haemoglobin), immune protection (antibodies) and mechanical support (collagen).[4.1] There are also a large number of drugs which contains amide structure as they are neutral, stable and can both donate and accept hydrogen bonds.

*Amide/peptide synthesis*

Chemical synthesis of amide/peptide has long been considered an important area and it is still growing with the increasing interest in pharmaceutical chemistry and drug discovery.

Synthesis of amides can be achieved by the direct condensation of a carboxylic acid and an amine. The reaction occurs in relatively high temperature (160-180 °C)[4.2,4.3], which is not compatible with certain functionalities or structures. Thus, introducing a leaving group to the carboxylic acid to activate it becomes a common way to solve the problem. This is usually achieved by adding some activating reagents or coupling reagents (Scheme 4.1).
So far, there have been many synthetic routes utilizing different activating or coupling reagents towards amide synthesis. For example, thionyl chloride (SOCl₂)\[^{4.4-4.6}\] is commonly used to convert carboxylic acid to acyl chloride, which then reacts with an amine to form an amide. The method is efficient but it also suffers drawbacks such as possible hydrolysis and racemization, which is common in simple activation of carboxylic acids with leaving groups.\[^{4.1}\] The mechanism shown in Scheme 4.2 demonstrates the racemization of the peptides in basic condition during the coupling reaction. An oxazolone intermediate A can be formed through the intramolecular substitution, which will then epimerize in basic condition to its epimer B to achieve the racemization.

**Scheme 4.1** Synthesis of amide from carboxylic acid and amine.

**Scheme 4.2** Racemization in peptide synthesis
To address this problem, efforts were made towards the development of activating reagents which disfavor the oxazolone formation. So far the most commonly used reagents are carbodiimides and phosphonium salts which have been successfully commercialized.\[^{[4.1]}\] Phosphonium salts, e.g. benzotriazol-1-yl-oxo-tris-pyrrolidino phosphonium hexafluorophosphate (PyBop), showed very good ability to suppress the racemization during the peptide formation. However, they have low efficiency towards the coupling of certain substrates, such as N-methyl-α-amino acids.\[^{[4.1]}\] In addition, the expense of these reagents limited their usage in large scale or multi-step synthesis.

**Thioacid derivatives in peptide synthesis**

In recent years, thioacid or thioacid derivatives as building blocks for amide/peptide synthesis has received considerable attention. Compared to carboxylic acid derivatives, these sulfur-based compounds have shown advantages for efficient amidation in certain cases.

Thioesters, the most common thioacid derivatives, were not considered as important building blocks for peptide synthesis until 1994. In that year, Dawson *et al* discovered a “native chemical ligation” (NCL) which linked peptide C-terminal thioester with another peptide N-terminal free cysteine to form a new peptide bond (Scheme 4.3).\[^{[4.7]}\] In the first step of the reaction, a reversible thiol-thioester exchange occurred in the solution to form a new thioester between the two peptide segments. That thioester was then attacked intramolecularly by the amine group from the previous N-terminal cysteine to form an amide bond. The reaction is chemoselective only between the
thioester and cysteine N-terminus, therefore it has been widely used in polypeptide or protein synthesis without protecting peptide side chains.

**Scheme 4.3** Native chemical ligation.

Thioacids have not been considered a good acylating agents in amide synthesis according to the early studies.\(^4.8\) However, activation of thioacid by different mechanisms has been found to largely improve the efficiency of the acylation reaction during last decades. Generally, thioacids can conjugate with different reactants to form good leaving groups on the carbonyl group, thus activates the amide formation.\(^4.9,4.10\) So far, there are many reagents found to be effectively activating this process, such as isonitriles\(^4.11-4.13\), Sanger reagent\(^4.14-4.18\) and Mukaiyama reagent\(^4.18\) (Scheme 4.4). These methods have shown very low epimerization rates during the reactions and are efficient towards some cases where carboxylic acid derivatives do not work well.\(^4.11,4.12,4.18\)
Since both thioesters and thioacids can be conveniently and largely prepared from carboxylic acids on the solid phase\(^{[4.19-4.21]}\), they have been applied more and more in amide or peptide synthesis as alternative or better building blocks compared to carboxylic acids.

### 4.3 RESEARCH DESIGN AND METHODOLOGY\(^{[4.22]}\)

It is known that S-nitrosothiols are unstable moieties. Their chemistry, especially in synthetically useful reactions, has not been well studied.\(^{[4.23-4.26]}\) NTA type molecules have never been clearly identified, although such compounds may be involved in some thiy radical formation processes.\(^{[4.27]}\) In our study, we first tested the preparation of NTA. One example using thiobenzoic acid 4.1 is shown in Scheme 4.2. Compound 4.1 was treated with organonitrite (RONO) or HCl/NaNO\(_2\) in organic solutions at room temperature and 0 °C. The resulting species, presumably NTA 4.2, showed a deep green color, which is the characteristic color of tertiary S-nitrosothiols. The UV spectra of 4.2
also showed specific absorption band at \( \sim 300 - 400 \) nm, representing the unique absorption band of SNOs.

**Figure 4.1** Color and UV spectrum of thiobenzoic acid 4.1 and S-nitroso thiobenzoic acid 4.2

The NTA 4.2 appeared to be unstable as the green color readily faded when we attempted to isolate compound 4.2. Based on the TLC analysis, there were complicated decomposition products such as thiols, diacyldisulfides, SNOs and other minor compounds. As shown in Scheme 4.5, although NTA 4.2 was unstable, it was reacted in situ with some nucleophiles. It showed that NTA, unlike other activated carboxylic acid derivatives, did not show any reactivity towards the hydroxyl nucleophiles (such as benzyl alcohol, phenol, and N-hydroxysuccinimide). However, amines proved to be excellent substrates, and the formation of amide bonds was effectively achieved (see Table 4.2 below).
Scheme 4.5 Synthesis and reactivity of NTA 4.1.

We then optimized the conditions for this NTA mediated coupling between thioacids and amines. As shown in Table 4.1, the best procedure was to mix the thioacid (1.0 equiv) and the amine (1.1 equiv) at 0 °C. the commercially available amyl nitrite (2.0 equiv) was then added dropwise into the solution. By this method, no additional base was needed. The formation of the desired amide product was immediately observed and isolated in high yields. As shown in entries 1-4 (Table 4.1), this reaction worked nicely in a number of common organic solvents including THF, DCM, DMF, and CH$_3$CN. Water seemed to have little effect on the coupling as the reaction gave similar results in buffer-containing systems (entries 5 and 6). The reaction was performed at 0 °C and completed within minutes.
Table 4.1 Optimization of the NTA reaction conditions.

<table>
<thead>
<tr>
<th>entry</th>
<th>solvent</th>
<th>equiv.(ONO)</th>
<th>reaction time</th>
<th>yield of 64a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>THF</td>
<td>2</td>
<td>5 min</td>
<td>96%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>&gt; 1 hr</td>
<td>93%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>&gt; 1 hr</td>
<td>49%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>&gt; 1 hr</td>
<td>14%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>DCM</td>
<td>2</td>
<td>10 min</td>
<td>82%</td>
</tr>
<tr>
<td>3</td>
<td>DMF</td>
<td>2</td>
<td>10 min</td>
<td>75%</td>
</tr>
<tr>
<td>4</td>
<td>CH₃CN</td>
<td>2</td>
<td>5 min</td>
<td>94%b</td>
</tr>
<tr>
<td>5</td>
<td>THF/PBS buffer (3/1)</td>
<td>2</td>
<td>10 min</td>
<td>95%</td>
</tr>
<tr>
<td>6</td>
<td>DMF/PBS buffer (1/1)</td>
<td>2</td>
<td>20 min</td>
<td>71%</td>
</tr>
</tbody>
</table>

a. isolated yield  
b. precipitate formed from the reaction solution

To prove the reaction was involved NTA, we carried out several control experiments. As shown in Scheme 4.6, the reaction between thioacid 4.1 and α-methyl-benzylamine 4.4 in the air only led to the formation of amide 4.5 in trace amounts at room temperature, even when the amine was used in large excess (10 equiv). A previous report by Orgel et al. also suggested that thioacids should not directly react with amines to form amides.⁴¹⁰

Scheme 4.6 Reaction between thiobenzoic acid 4.1 and amine 4.4 without nitrosation reagent.
As NTAs are unstable species and could decompose to various sulfur containing compounds such as diacyldisulfides, we then tested whether the reaction underwent a mechanism to generate disulfide as a reactive intermediate. As shown in Scheme 4.7, the more stable substrate N-benzylxycarbonyl thioglycine 4.6 and non-nucleophilic triethylamine (TEA) were reacted under the above reaction conditions. After the reaction, only less than 10% of thioacid 4.6 was converted to disulfide 4.7 and 87% of hydrolysis product benzoic acid 4.8 was isolated as the major component at the end (Scheme 4.8A). Only when there was no amine added in the reaction, the disulfide 4.7 was isolated with a 36% yield, and a nonpolar compound with no UV activity was left behind for mass recovery (Scheme 4.8B). This result indicated that the disulfide is not the essential reactive intermediate involved in the reaction.

![Mechanistic study of disulfide formation in SNO coupling reaction](image)

**Scheme 4.7** Mechanistic study of disulfide formation in SNO coupling reaction A with TEA, B without any amine.

Meanwhile, we were able to capture the unstable NTA intermediates using the reductive ligation which is specific towards SNOs. The desired ligation product 4.12 was obtained from the reductive ligation of substrate 4.9 (Scheme 4.8). Although the yield of 4.12 was
only 23%, the formation of this sulfenamide product strongly supported the presence of an NTA intermediate in the reaction.

![Scheme 4.8 Capture of NTA via the reductive ligation.](image)

The results shown above suggested that S-nitrosation acts as an effective activating route to facilitate amide formation. To test the generality of this reaction, a series of thioacids and amines were employed under the optimized conditions (Table 4.2). The reaction proved to be very efficient with not only primary amines (4.15a, c, d, g) but also sterically hindered secondary amines (4.15b, e, f, h). In these cases, simple amino acid substrates were also tested. As expected, the corresponding products were obtained in good yields in all substrates (4.15c, d, g, h). In all the cases, the reaction was able to be complete within 10 min at 0 °C without addition of base.
Another encouraging result was obtained when we applied the NTA coupling reaction to the peptide synthesis. As shown in Table 4.3, dipeptides and tripeptides with different amino acid sequences were synthesized. The reaction showed good compatibility towards different amine protecting groups such as tert-butyloxycarbonyl (Boc) (4.18a), benzyloxycarbonyl (Cbz) (4.18b-f) and 9-fluorenlymethylxycarbonyl (Fmoc) (4.18g-i). In addition, the aminoacid sidechain functionalities like hydroxyl from tyrosine (4.18c) and serine, (4.18e), amide from asparagine (4.18b), carboxylic acid from aspartic acid (4.18f), thioether from methionine (4.18d) and aromatic amine from tryptophan (4.18h) did not interfere with the NTA reaction. A sterically hindered peptide bond in 4.18i was also successfully generated with good efficacy. Finally two tripeptides (4.18j, k) were employed to investigate the level of epimerization during the reaction. We were able to isolate the tripeptide products 4.18j and 4.18k, while no significant epimerization was observed from the HPLC data. More control experiments including the synthesis of corresponding epimeric tripeptides for comparison are ongoing. Overall, the yields of all cases turned out to be excellent.
Table 4.3 NTA coupling reaction in peptide synthesis.

Finally, we looked back into the mechanism of the reaction, where we proposed an interesting HSNO intermediate \(4.23\) generated during the nucleophilic substitution step (Scheme 4.9).

Scheme 4.9 Proposed mechanism of the NTA coupling reaction.
This reminded us of the trans-nitrosation reaction, which delivers the NO group between free thiols. In that case, the intermediate HSNO 4.23 can also serve as a nitrosation reagent to nitrosate thioacid 4.19 to S-nitrosothioacid 4.22. And then a catalytic cycle should occur when HSNO 4.23 is regenerated during the nucleophilic substitution reaction (Scheme 4.10). If the hypothesis is true for the reaction, we could modify the reaction condition by decreasing the amount of nitrosation reagent to achieve a catalytic reaction, which is unusual and more efficient in amide formation.

Scheme 4.10 Proposed catalytic cycle in NTA coupling reaction.

To test our hypothesis, we employed Cbz glycine thioacid 4.24, which is a more stable substrate compared to thiobenzoic acid, as our model substrate in the NTA coupling reaction. The reactions were all performed in THF at 0 °C for 30 minutes, and the isolated yield of the product 4.15d was determined. By using different amounts of isopentyl nitrite (less than 1 equivalent), we summarized the results in Scheme 4.11. When using 0.10 equivalent of isopentyl nitrite, 18% yield of 4.15d was obtained which is not significantly higher than the equivalent of the nitrosation reagent. However, when 0.20
equivalent of the isopentyl nitrite was added, the yield of 4.15d increased dramatically to 51%. An even higher equivalent 0.30 of the isopentyl nitrite led to 63% yield of 4.15d. As the data shows, yields were all higher than expected and not entirely based on the equivalent of isopentyl nitrite in all cases. Although the reaction mechanism is still under investigation, these preliminary studies revealed the potential of the reaction to be catalytically driven.

Scheme 4.11 Catalytic NTA reactions between Cbz glycine thioacid and benzylamine.

4.4 Conclusion

In summary, we have developed a novel amide bond formation strategy from simple thioacids and amines. This process was mediated by reactive NTA intermediates. It revealed S-nitrosation as a novel strategy for thioacid activation. Compared to common amide formation methods, this reaction only utilized readily available organonitrite as the activation reagent. It took place under very mild reaction conditions, and the reaction was extremely fast. In addition the chemistry was easily executed. It also showed excellent selectivity towards amines over other functionalities which are present in aminoacid side chains. In our opinion, this method should be promising for peptide synthesis and the
selective N-acylation, with the potential usage in a catalytic manner. Further studies will be focused on the detailed reaction mechanism, the study of epimerization, the application of this method in solid phase peptide and protein synthesis as well as the exploration of new chemistry.

4.5 Experimental

Materials and Methods: All solvents were reagent grade. Tetrahydrofuran (THF) was freshly distilled from sodium / benzophenone under argon. Reactions were magnetically stirred and monitored by thin layer chromatography (TLC) with 0.25 mm pre-coated silica gel plates. Flash chromatography was performed with silica gel 60 (particle size 0.040-0.062mm). Yields refer to chromatographically and spectroscopically pure compounds, unless otherwise stated. High performance liquid chromatography (HPLC) analysis was carried out using Hypersil GOLD C18 column on Finnigan Surveyor system from Thermo Scientific®. HPLC eluent B was a solution of 0.05% TFA in CH$_3$CN and eluent A was a 0.05% TFA aqueous solution. HPLC analysis was monitored on a dual channel UV detector at 215 nm and 254 nm. Optical rotation were recorded at room temperature at the sodium D line (589 nm). Proton and carbon-13 NMR spectra were recorded on a 300 MHz spectrometer. Chemical shifts are reported relative to chloroform (δ 7.26) for $^1$H NMR and chloroform (δ 77.0) for $^{13}$C NMR. Absorption spectra were recorded on a Lambda 20 UV/Vis spectrophotometer using 1 cm quartz cells. Low resolution mass spectroscopy (LRMS) was performed using ESI-MS.

Experimental Procedures and Compound Characterization Data
UV spectra of S-nitroso thiobenzoic acid 4.2 (in CH$_3$CN):

These three UV-vis absorption bands of compound 4.2 are very similar to the characteristic UV-vis bands of S-nitrosothiols.$^{[4.28-4.30]}$ A) 213-268 nm region (39 μM, $\varepsilon = 1.14 \times 10^4$ M$^{-1}$cm$^{-1}$), attributed to the $\pi \rightarrow \pi^*$ transition$^{[4.28]}$; B) 315-385 nm region (460 μM, $\varepsilon = 9.67 \times 10^2$ M$^{-1}$cm$^{-1}$), attributed to the $n_0 \rightarrow \pi^*$ transition$^{[4.29]}$; C) 531-633 nm region (27 mM, $\varepsilon = 13.2$ M$^{-1}$cm$^{-1}$), attributed to the forbidden $n_N \rightarrow \pi^*$ transition (this band determines the compound’s color)$^{[4.30]}$.

Color of thiobenzoic acid 4.1 and S-nitroso thiobenzoic acid 4.2:
Left (yellow) – thiobenzoic acid (83 mM). Right (green) – thiobenzoic acid (83 mM) + iso-amyl nitrite (167 mM).

**Preparation of thioacids**

Thiobenzoic acid 1 was purchased from Aldrich. Preparation of other thioacids were described below via modified routes based on the known procedures.[4,31,432]

**Method A:** A solution of carboxylic acid (1.0 mmol), N-hydroxysuccinimide (NHS, 126 mg, 1.1 mmol), EDC (211 mg, 1.1 mmol) and DMAP (13 mg, 0.05 mmol) in 10 mL dichloromethane was stirred at room temperature for 3 hours. The organic layer was washed with saturated ammonium chloride and then dried upon anhydrous sodium sulfate. The solvent was removed under vaccum and the product NHS ester was obtained through flash chromatography.

A solution of NHS ester (1.0 mmol) in 10 mL methanol was added a solution of NaHS (mg, 3.0 mmol) in 1 mL water. The reaction mixture was stirred at room temperature for 3 hours, and then washed with 10 mL dichloromethane for 3 times. The solution was then neutralized with 1 N HCl to adjust the solution to pH 4. The solution was then extracted with 15 mL dichloromethane for 3 times. The organic layer was separated and dried upon
anhydrous sodium sulfate. The solvent was removed under vaccum and the final product thioacid was obtained as pure product through flash chromatography.

**Method B**: A solution of carboxylic acid (1.0 mmol), triphenylmethyl mercaptan (TrSH, 304 mg, 1.1 mmol), EDC (211 mg, 1.1 mmol) and DMAP (13 mg, 0.05 mmol) in 10 mL dichloromethane was stirred at room temperature for 3 hours. The organic layer was washed with saturated ammonium chloride and then dried upon anhydrous sodium sulfate. The solvent was removed under vaccum and the product Tr-thioester was obtained through flash chromatography.

A solution of Tr-thioester (1.0 mmol) in 10 mL dichloromethane was added dropwise trifluoroacetic acid (TFA, mL, 3.0 mmol) and triethylsilane ( mL, mmol). The reaction mixture was stirred at room temperature for 30 minutes, and then neutralized with saturated sodium bicarbonate to adjust the solution to pH 10. The solution was then washed with 10 mL dichloromethane for 3 times and neutralized with 1N HCl back to pH 4. The solution was then extracted with 15 mL dichloromethane for 3 times and the organic layer was separated and dried upon anhydrous sodium sulfate. The solvent was removed under vaccum and the final product thioacid was obtained as pure product through flash chromatography.

**General procedure of NTA mediated amide formation**

\[
\begin{align*}
\text{O} &\quad \text{H}_2\text{N} \\
\text{S} &\quad \text{ONO} \\
\text{1} &\quad \text{THF} \\
&\quad 0 \degree \text{C}
\end{align*}
\]

\[
\begin{align*}
\text{N} &\quad \text{4a}
\end{align*}
\]
To a stirred solution of thioacid 1 (69 mg, 0.5 mmol) and α-methyl benzylamine (67 mg, 0.55 mmol) in THF (5 mL) at 0 °C in darkness was added amyl nitrite (117 mg, 1 mmol) slowly (dropwise). The reaction was stirred at 0 °C for 5 minutes. The reaction mixture was then diluted with DCM (20 mL) and washed with water and brine. The organic layer was dried (with anhydrous Na₂SO₄). The solvent was then removed under reduced pressure. The crude product was purified by flash column chromatography (hexane/ethyl acetate, 10/1) to afford the desired product 4a (108 mg, 96%).

Compound 4.5

Yield 96%; white solid, m.p. 120-121 °C; ¹H NMR (300 MHz, CDCl₃): δ 7.66 (2H, d, J = 7.8 Hz), 7.37-7.12 (8H, m), 6.70 (1H, d, J = 7.2 Hz), 5.21 (1H, dt, J = 7.2 Hz), 1.46 (3H, d, J = 6.9 Hz); ¹³C NMR (75 MHz, CDCl₃): δ 167.0, 143.5, 134.8, 131.7, 128.9, 128.7, 127.6, 127.3, 126.5, 49.5, 22.0; IR (thin film) cm⁻¹ 3341, 3067, 2974, 1633, 1579, 1530, 1491, 1449, 1322, 1276, 1210, 1148, 1029, 872, 802, 762, 698; MS m/z 248.1 [M+Na⁺].

Compound 4.15a

4.15a
Yield 100%; white solid, m.p. 101-102 °C; \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta 7.80\) (2H, d, \(J = 6.9\) Hz), 7.46 (1H, tt, \(J_1 = 7.5\) Hz, \(J_2 = 1.5\) Hz), 7.37-7.23 (8H, m), 4.55 (2H, d, \(J = 6.0\) Hz); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)): \(\delta 167.9\), 138.7, 134.6, 131.7, 128.9, 128.7, 128.0, 127.6, 127.4, 44.2; IR (thin film) cm\(^{-1}\) 3323, 3060, 2930, 2856, 1643, 1604, 1578, 1547, 1490, 1453, 1419, 1362, 1313, 1260, 1158, 1080, 1058, 1029, 990, 928, 805, 794, 728, 696, 666; MS m/z 212.1 [M+Na\(^+\)].

Compound 4.15b

![4.15b](image)

Yield 88%; white solid, m.p. 96-97 °C; \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta 7.55\) (2H, m), 7.41-7.32 (11H, m), 7.18-7.16 (2H, m), 4.74 (2H, s), 4.43 (2H, s); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)): \(\delta 172.5\), 137.2, 136.7, 136.7, 136.4, 129.9, 129.1, 129.0, 128.8, 128.7, 127.9, 127.8, 127.3, 127.0, 51.8, 47.1; IR (thin film) cm\(^{-1}\) 3028, 2924, 1632, 1602, 1495, 1450, 1422, 1365, 1307, 1259, 1204, 1142, 1077, 1028, 992, 731, 698; MS m/z 302.2 [M+H\(^+\)].

Compound 4.15c

![4.15c](image)
Yield 89%; colorless oil; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.80 (2H, dt, $J_1 = 6.6$ Hz, $J_2 = 1.5$ Hz), 7.49 (1H, tt, $J_1 = 7.5$ Hz, $J_2 = 1.5$ Hz), 7.42-7.37 (2H, m), 6.95 (1H, s), 4.21 (2H, d, $J = 5.1$ Hz), 3.76 (3H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 170.8, 167.9, 133.8, 132.0, 128.8, 127.3, 52.7, 41.9; IR (thin film) cm$^{-1}$ 3331, 3063, 2953, 1754, 1650, 1536, 1491, 1439, 1408, 1371, 1314, 1211, 1183, 1080, 1006, 976, 719, 693; HRMS m/z 194.0811 [M+H]$^+$; calcd for C$_{10}$H$_{12}$NO$_3$: 194.0812.

Compound 4.15d

![4.15d](image)

Yield 86%; white solid, m.p. 107-108 °C; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.35-7.21 (10H, m), 6.78 (1H, s), 5.75 (1H, s), 5.03 (2H, s), 4.38 (2H, d, $J = 5.7$ Hz), 3.84 (2H, d, $J = 5.4$ Hz); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 169.3, 156.9, 138.0, 136.3, 128.9, 128.8, 128.7, 128.5, 128.3, 127.9, 127.8, 67.4, 44.8, 43.7; IR (thin film) cm$^{-1}$ 3321, 3062, 2925, 1693, 1666, 1548, 1537, 1454, 1427, 1360, 1287, 1246, 1164, 1068, 1006, 736, 696; HRMS m/z 299.1384 [M+H]$^+$; calcd for C$_{17}$H$_{19}$N$_2$O$_3$: 299.1390.

Compound 4.15e

![4.15e](image)
Yield 97%; white solid, m.p. 131-132 °C; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.68 (2H, d, $J = 7.5$ Hz), 7.41-7.29 (3H, m), 6.14 (1H, s), 1.42 (9H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 167.2, 136.1, 131.2, 128.6, 127.0, 51.8, 29.0; IR (thin film) cm$^{-1}$ 3317, 2965, 1632, 1578, 1534, 1492, 1450, 1364, 1312, 1218, 1078, 936, 877, 720, 694; MS m/z 178.0 [M+H$^+$].

Compound 4.15f

Yield 80%; colorless oil; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.48-7.44 (2H, m), 7.36-7.31 (3H, m), 3.59 (2H, t, $J = 6.9$ Hz), 3.36 (2H, t, $J = 6.6$ Hz), 1.95-1.76 (4H, m); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 169.9, 137.4, 129.9, 128.4, 127.3, 49.8, 46.3, 26.6, 24.7; IR (thin film) cm$^{-1}$ 2971, 2877, 1626, 1575, 1447, 1422, 719, 700, 658; MS m/z 198.1 [M+Na$^+$].

Compound 4.15g

Yield 77%; colorless oil $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.72 (2H, dt, $J_1 = 6.6$ Hz, $J_2 = 1.5$ Hz), 7.50 (1H, tt, $J_1 = 7.5$ Hz, $J_2 = 1.5$ Hz), 7.44-7.38 (2H, m), 7.33-7.25 (3H, m),
7.15-7.12 (2H, m), 6.65 (1H, d, J = 7.2 Hz), 5.12-5.06 (1H, m), 3.76 (3H, s), 3.33-3.19 (2H, m); $^{13}$C NMR (75 MHz, CDCl$_3$): δ 172.2, 167.1, 136.1, 134.1, 132.0, 129.6, 128.9, 127.4, 127.2, 53.8, 52.7, 38.1; IR (thin film) cm$^{-1}$ 3319, 3030, 2952, 1745, 1643, 1603, 1580, 1537, 1490, 1437, 1360, 1217, 1098, 1028, 912, 701; HRMS m/z 284.1294 [M+H]$^+$; calcd for C$_{17}$H$_{18}$NO$_3$: 284.1281. $[\alpha]_{D}^{25} +74.4$ (c 0.58, DCM), lit. +70.5.$^{[4,33]}

Compound 4.15h

$\text{CbzHN} \text{N} \text{O}$

4.15h

Yield 87%; white solid, m.p. 62-63 °C; $^1$H NMR (300 MHz, CDCl$_3$): δ 7.37-7.30 (5H, m), 5.79-5.78 (1H, m), 5.12 (2H, s), 3.94 (2H, d, J = 4.2 Hz), 3.49 (2H, t, J = 6.6 Hz), 3.37 (2H, t, J = 6.6 Hz), 2.03-1.94 (2H, m), 1.92-1.82 (2H, m); $^{13}$C NMR (75 MHz, CDCl$_3$): δ 166.5, 156.5, 136.7, 128.7, 128.3, 67.1, 46.2, 45.6, 43.7, 26.2, 24.4; IR (thin film) cm$^{-1}$ 3539, 3487, 3279, 2957, 1696, 1628, 1549, 1452, 1412, 1333, 1266, 1173, 1052, 981, 763, 704; HRMS m/z 263.1379 [M+H]$^+$; calcd for C$_{14}$H$_{19}$N$_2$O$_3$: 263.1390.

*General procedure of NTA mediated peptide synthesis*

\[
\text{CbzHN} \text{N} \text{O} \text{SH} + \text{HCl HN} \text{MeCO}_2 \xrightarrow{\text{Et}_3\text{N, THF, 0 °C}} \text{CbzHN} \text{N} \text{CO}_2\text{Me} \text{NHPh}
\]
To a stirred solution of amino thioacid 1 (69 mg, 0.5 mmol), phenylalanine methylester hydrochloride (67 mg, 0.55 mmol) and triethylamine (ml, mmol) in THF (5 mL) at 0 °C in darkness was added amyl nitrite (117 mg, 1 mmol) slowly (dropwise). The reaction was stirred at 0 °C for 5 minutes. The reaction mixture was then diluted with DCM (20 mL) and washed with water and brine. The organic layer was dried (with anhydrous Na₂SO₄). The solvent was then removed under reduced pressure. The crude product was purified by flash column chromatography (hexane/ethyl acetate, 10/1) to afford the desired product 4a (108 mg, 96%).

**Compound 4.18a**

![Chemical Structure](image)

**4.18a**

Yield 87%; colorless oil; ¹H NMR (300 MHz, CDCl₃): δ 7.29-7.18 (5H, m), 6.77 (1H, t, J = 4.8 Hz), 5.23 (1H, d, J = 5.1 Hz), 4.45 (1H, d, J = 6.0 Hz), 4.05-3.88 (2H, m), 3.70 (3H, s), 3.12 (1H, dd, J₁ = 13.8 Hz, J₂ = 4.8 Hz), 3.03-2.96 (1H, m), 1.36 (9H, s); ¹³C NMR (75 MHz, CDCl₃): δ 172.0, 170.2, 155.7, 136.9, 129.5, 128.8, 127.1, 80.3, 55.8, 53.7, 52.5, 41.4, 38.6, 28.4; IR (thin film) cm⁻¹ 3314, 2978, 1754, 1665, 1530, 1501, 1440, 1367, 1250, 1211, 1170, 1022, 856, 700; HRMS m/z 337.1756 [M+H]^+; calcd for C₁₇H₂₅N₂O₅: 337.1758.

**Compound 4.18b**
Yield 88%; colorless oil; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.70 (1H, d, $J = 7.8$ Hz), 7.29-7.20 (10H, m), 6.10 (2H, d, $J = 16.2$ Hz), 5.91 (1H, s), 5.12 (2H, s), 5.05 (2H, s), 4.80 (1H, s), 3.85 (2H, s), 2.86 (1H, d, $J = 15.0$ Hz), 2.71 (1H, d, $J = 15.0$ Hz); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 172.8, 171.2, 169.8, 169.8, 156.9, 156.5, 135.5, 128.8, 128.6, 128.4, 128.3, 67.7, 67.3, 49.3, 44.3, 37.0; MS m/z 414.2 [M+H]$^+$; calcd for C$_{21}$H$_{24}$N$_2$O$_6$: 414.1.

Compound 4.18c

Yield 80%; colorless oil; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.70 (1H, d, $J = 7.8$ Hz), 7.29-7.20 (10H, m), 6.10 (2H, d, $J = 16.2$ Hz), 5.91 (1H, s), 5.12 (2H, s), 5.05 (2H, s), 4.80 (1H, s), 3.85 (2H, s), 2.86 (1H, d, $J = 15.0$ Hz), 2.71 (1H, d, $J = 15.0$ Hz); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 172.2, 172.0, 170.8, 170.7, 157.8, 156.3, 144.1, 141.4, 130.1, 127.6, 127.2, 127.0, 125.1, 119.8, 115.1, 67.0, 54.3, 54.2, 51.5, 43.5, 36.5, 36.5; MS m/z 475.2 [M+H]$^+$; calcd for C$_{27}$H$_{27}$N$_2$O$_6$: 475.2.
Compound 4.18d

\[ \text{CbzHN} \quad \text{O} \quad \text{SMe} \]
\[ \text{H} \quad \text{CO}_2\text{Bn} \]

4.18d

Yield 80%; colorless oil; 3°C; \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 7.70 (1H, d, \(J = 7.8\) Hz), 7.29-7.20 (10H, m), 6.10 (2H, d, \(J = 16.2\) Hz), 5.91 (1H, s), 5.12 (2H, s), 5.05 (2H, s), 4.80 (1H, s), 3.85 (2H, s), 2.86 (1H, d, \(J = 15.0\) Hz), 2.71 (1H, d, \(J = 15.0\) Hz); \(^1^3\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) 172.8, 171.2, 169.8, 169.8, 156.9, 136.5, 135.5, 128.8, 128.6, 128.4, 128.3, 67.7, 67.3, 49.3, 44.3, 37.0; MS m/z 475.2 [M+H]+; calcd for C\(_{27}\)H\(_{27}\)N\(_2\)O\(_6\): 475.2.

Compound 4.18e

\[ \text{CbzHN} \quad \text{O} \quad \text{OH} \]
\[ \text{H} \quad \text{CO}_2\text{Me} \]

4.18e

Yield 81%; white solid, m.p. 90-91°C; \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 7.37-7.31 (5H, m), 7.29-7.28 (1H, m), 5.86 (1H, t, \(J = 5.4\) Hz), 5.09 (2H, s), 4.65-4.61 (1H, m), 3.97-3.82 (4H, m), 3.73 (3H, s), 3.62 (1H, t, \(J = 4.8\) Hz); \(^1^3\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) 171.2, 169.9, 157.2, 136.3, 128.8, 128.5, 128.3, 67.5, 62.8, 54.9, 53.0, 44.5; IR (thin film) cm\(^{-1}\) 3331, 2954, 1726, 1710, 1666, 1547, 1530, 1441, 1345, 1236, 1134, 1052, 738, 698;
HRMS m/z 311.1233 \([\text{M}+\text{H}]^+\); calcd for \(\text{C}_{14}\text{H}_{19}\text{N}_2\text{O}_6\): 311.1238. \([\alpha]\)^{25}_D +22.3 (c 1.05, CHCl$_3$).

Compound **4.18f**

![4.18f](image)

Yield 79%; colorless oil; \(^1\)H NMR (300 MHz, CDCl$_3$): \(\delta\) 7.34-7.30 (10H, m), 5.72 (1H, s), 5.16 (2H, s), 5.08 (2H, s), 4.89 (1H, t, \(J = 1.5\) Hz), 3.99-3.92 (1H, m), 3.85 (1H, dd, \(J_1 = 8.4\) Hz, \(J_2 = 2.4\) Hz), 3.05 (1H, dd, \(J_1 = 8.7\) Hz, \(J_2 = 2.1\) Hz), 2.87 (1H, dd, \(J_1 = 8.7\) Hz, \(J_2 = 1.8\) Hz); \(^{13}\)C NMR (75 MHz, CDCl$_3$): \(\delta\) 174.0, 170.6, 169.7, 157.1, 136.2, 135.2, 128.8, 128.8, 128.7, 128.5, 128.4, 128.3, 68.0, 67.6, 67.6, 48.9, 44.3, 36.1; MS m/z 415.1 \([\text{M}+\text{H}]^+\); calcd for \(\text{C}_{21}\text{H}_{23}\text{N}_2\text{O}_7\): 415.2.

Compound **4.18g**

![4.18g](image)

Yield 86%; white solid, m.p. 157-158 °C; \(^1\)H NMR (300 MHz, CDCl$_3$): \(\delta\) 7.77 (2H, d, \(J = 7.5\) Hz), 7.59 (2H, d, \(J = 7.2\) Hz), 7.41 (2H, t, \(J = 7.2\) Hz), 7.32 (2H, dt, \(J_1 = 7.2\) Hz, \(J_2 = 1.8\) Hz).
1.2 Hz), 7.21 (3H, t, J = 7.5 Hz), 7.07 (2H, t, J = 3.6 Hz), 6.38 (1H, d, J = 7.5 Hz), 5.28 (1H, d, J = 6.9 Hz), 4.89-4.83 (1H, m), 4.44-4.31 (2H, m), 4.25-4.19 (2H, m), 3.72 (3H, s), 3.20-3.04 (2H, m), 1.36 (3H, d, J = 6.9 Hz); $^{13}$C NMR (75 MHz, CDCl$_3$): δ 171.9, 171.8, 144.0, 141.5, 135.8, 129.4, 128.2, 128.0, 127.4, 127.3, 125.3, 125.3, 120.2, 67.4, 53.4, 52.7, 50.6, 47.3, 38.0; IR (thin film) cm$^{-1}$ 3303, 3063, 2951, 1742, 1711, 1661, 1530, 1450, 1252, 1215, 1118, 1080, 1046, 759, 740, 701; HRMS m/z 473.2071 [M+H]$^+$; calcd for C$_{28}$H$_{29}$N$_2$O$_5$: 473.2071. [$\alpha$]$^\circ_{25}$D +26.2 (c 0.95, CHCl$_3$), lit. +22.7.$^{[4,34]}

Compound 4.18h

\[
\begin{align*}
\text{FmocHN} & \quad \text{N} \\
\text{C} & \quad \text{O} \\
\text{Me} & \quad \text{CO}_2
\end{align*}
\]

Yield 86%; white solid, m.p. 170-171 °C; $^1$H NMR (300 MHz, CDCl$_3$): δ 8.07(1H, s), 7.78 (2H, d, J = 7.5 Hz), 7.56 (2H, t, J = 7.2 Hz), 7.52 (1H, d, J = 9.9 Hz), 7.41 (2H, t, J = 7.5 Hz), 7.15-7.04 (4H, m), 6.90 (2H, d, J = 2.1 Hz), 6.67 (1H, d, J = 7.2 Hz), 5.39 (1H, d, J = 7.8 Hz), 4.92 (1H, m), 4.37-4.13 (4H, m), 3.65 (3H, s), 3.5 (2H, d, J = 5.4 Hz), 1.32 (3H, d, J = 6.9 Hz); $^{13}$C NMR (75 MHz, CDCl$_3$): δ 171.3, 156.1, 144.0, 141.5, 136.2, 128.0, 127.7, 127.3, 125.4, 125.3, 122.4, 120.3, 119.8, 118.6, 111.6, 109.8, 67.3, 53.1, 52.7, 50.6, 47.2, 31.8, 27.7, 22.9, 18.9, 14.4 ; IR (thin film) cm$^{-1}$ 3303, 3063, 2951, 1742, 1711, 1661, 1530, 1450, 1252, 1215, 1118, 1080, 1046, 759, 740, 701; MS m/z 534.3 [M+Na]$^+$; calcd for C$_{30}$H$_{29}$N$_3$NaO$_5$: 534.2.
Compound **4.18i**

![Structure of Compound 4.18i](image)

Yield 80%; white solid, m.p. 49-50 °C; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.76 (2H, d, $J$ = 7.5 Hz), 7.59 (2H, d, $J$ = 7.2 Hz), 7.42-7.28 (9H, m), 5.72 (1H, d, $J$ = 8.1 Hz), 5.22, 5.12 (2H, AB, $J$ = 12.3 Hz), 4.63-4.58 (1H, m), 4.56-4.48 (1H, m), 4.34 (2H, d, $J$ = 7.2 Hz), 4.21 (1H, t, $J$ = 7.2 Hz), 3.77-3.57 (2H, m), 2.27-2.19 (1H, m), 2.11-1.94 (3H, m), 1.36 (3H, d, $J$ = 6.9 Hz); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 171.8, 171.5, 155.8, 144.1, 144.0, 141.5, 135.7, 128.8, 128.6, 128.4, 127.9, 127.3, 125.4, 120.2, 67.2, 59.1, 48.5, 47.3, 47.1, 29.2, 25.2, 18.6; IR (thin film) cm$^{-1}$ 3286, 3064, 2978, 2881, 1742, 1721, 1530, 1501, 1451, 1379, 1248, 1172, 1044, 910, 759, 739, 699; HRMS m/z 499.2230 [M+H]$^+$; calcd for C$_{30}$H$_{31}$N$_2$O$_5$: 499.2227.

Compound **4.18j**

![Structure of Compound 4.18j](image)

Yield 75%; white solid, m.p. 206-208 °C; HPLC: isocratic 20% B in A for 4 min, then gradient 20% to 80% B in A over 42 min, 0.5 mL/min $t_R$: 29.5 min; $^1$H NMR (300 MHz,
DMSO-d$_6$): δ 8.46 (1H, d, $J = 6.9$ Hz), 8.00 (1H, d, $J = 8.4$ Hz), 7.89 (2H, d, $J = 7.2$ Hz), 7.20 (2H, q, $J = 3.6$ Hz), 7.41 (2H, t, $J = 7.2$ Hz), 7.32 (3H, t, $J = 7.5$ Hz), 7.26-7.21 (4H, m), 7.14 (1H, d, $J = 5.4$ Hz), 4.63-4.56 (1H, m), 4.33-4.21 (4H, m), 3.79 (1H, t, $J = 8.1$ Hz), 3.60 (3H, s), 3.02 (1H, dd, $J_{1} = 13.8$ Hz, $J_{2} = 4.2$ Hz), 2.77 (1H, dd, $J_{1} = 13.8$ Hz, $J_{2} = 9.6$ Hz), 1.86 (1H, m), 1.27 (3H, d, $J = 7.2$ Hz), 0.72 (6H, dd, $J_{1} = 12.0$ Hz, $J_{2} = 6.9$ Hz);

$^{13}$C NMR (75 MHz, CDCl$_3$): δ 172.8, 171.0, 170.9, 155.9, 143.9, 143.7, 140.7, 137.5, 129.2, 127.9, 127.6, 127.0, 126.2, 125.3, 120.1, 65.6, 60.4, 53.2, 51.8, 47.6, 46.7, 37.5, 30.4, 19.0, 18.2, 16.8; MS m/z 572.2 [M+H]$^+$; calcd for C$_{33}$H$_{38}$N$_3$O$_6$: 572.3. $[^{23}]$D$_{23} = -12.7$ (c 0.5, CHCl$_3$).

Compound 4.18k

![Diagram of 4.18k]

Yield 76%; white solid, m.p. 217-219 °C; HPLC: isocratic 20% B in A for 4 min, then gradient 20% to 80% B in A over 42 min, 80% 5 min, 0.5 mL/min $t_R$: 13.7 min; $^1$H NMR (300 MHz, DMSO-d$_6$): δ 8.44 (1H, d, $J = 6.3$ Hz), 7.66 (1H, d, $J = 9.0$ Hz), 7.52 (1H, d, $J = 7.8$ Hz), 7.34 (5H, q, $J = 3.6$ Hz), 5.01 (2H, s), 4.27-4.18 (2H, m), 4.11 (1H, t, $J = 7.5$ Hz), 3.60 (3H, s), 1.98-1.89 (1H, m), 1.27 (3H, d, $J = 7.2$ Hz), 1.18 (3H, d, $J = 7.2$ Hz), 0.85 (6H, dd, $J_1 = 12.9$ Hz, $J_2 = 6.6$ Hz); $^{13}$C NMR (75 MHz, CDCl$_3$): δ 172.9, 172.3, 170.8, 155.6, 137.0, 128.3, 127.8, 127.7, 65.3, 56.8, 51.7, 50.1, 47.6, 31.1, 19.0, 18.1,
17.9, 16.7; MS m/z 408.1 [M+H]^+; calcd for C\textsubscript{20}H\textsubscript{30}N\textsubscript{3}O\textsubscript{6}: 408.2. [\alpha]\textsuperscript{23}\textsubscript{D} -33.0 (c 1.0, CHCl\textsubscript{3}).

The reductive ligation for capturing NTA

![Chemical reaction diagram]

To a solution of 4.9 (36 mg, 0.16 mmol) in THF/CH\textsubscript{3}CN/H\textsubscript{2}O (1.5 mL/1.5 mL/1.0 mL) was added amyl nitrite (64 \(\mu\)L, 0.47 mmol) at 0°C. The resulting green solution was stirred for ~5 min at 0°C. Then, phosphine 4.11 (114 mg, 0.30 mmol, in 1.0 mL THF) was added into the solution. The reaction was stirred for additional 5 min. The reaction mixture was diluted with ethyl acetate (15 mL) and washed with an aqueous solution of 5% H\textsubscript{2}O\textsubscript{2} (3 mL), saturated NaHSO\textsubscript{3} (3 mL) and brine. The organic layer was dried over Na\textsubscript{2}SO\textsubscript{4} and concentrated. The crude product was purified by flash column chromatography (MeOH:DCM/1:200) to give 4.12 in 23% yield. \(^1\)H NMR (600 MHz, CDCl\textsubscript{3}) \(\delta\) 9.92 (s, 1H), 8.07-8.06 (m, 1H), 7.66-7.61 (m, 5H), 7.57-7.55 (m, 2H), 7.47-7.44 (m, 5H), 7.30-7.26 (m, 10H), 7.16-7.13 (m, 1H); \(^{13}\)C NMR (150 MHz, CDCl\textsubscript{3}) 196.9, 168.2, 139.1(d), 137.2, 133.4(d), 132.7(d), 132.6(d), 132.5(d), 132.4(d), 131.9(d), 131.8, 130.7, 130.0, 129.0, 128.9, 128.8(d), 128.7(d), 128.6, 127.6, 61.3; \(^{31}\)P NMR (121 Hz, CDCl\textsubscript{3}) 36.6. IR (thin film) cm\(^{-1}\) 3061, 2923, 2855, 1715, 1682, 1589, 1495, 1437, 1252, 1168, 1120, 725, 695; HRMS m/z, 548.1449 [M+H]; calcd for C\textsubscript{33}H\textsubscript{27}NO\textsubscript{3}PS, 548.1449.

4.6 REFERENCES


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**JIA PAN**

DEPARTMENT OF CHEMISTRY

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(509) 230-2481

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**Education**


**Awards and Honors**

- WSU GPSA travel grant, 2011.
- WSU GCS travel grant, 2010.
- Frank Fowler Scholarship, 2008.
- Guanghua Scholarship, 2005.

**Research Experience**

*Doctoral Research:* Department of Chemistry, Washington State University, 2007-present (research adviser: Dr. Ming Xian)

- Development of phosphine-based ligation reactions of S-nitrosothiols.
- Design, synthesis and application of phosphine-based fluorescent probes for the detection of S-nitrosothiols.
- Exploration of S-nitrosation in peptide synthesis.
- Investigation of new strategies in the detection of hydrogen sulfide.


- Synthesis of Schiff-base type dendrimers as organic nonlinear optical materials.
- Practical study of Sonogashira reaction in dendrimer synthesis.

*Undergraduate Research:* Department of Chemistry, Nanjing University, 2000-2004 (research adviser: Dr. Shaozhong Wang)

- Investigation of Iron(III) catalyzed annulation reactions in heterocycle synthesis.
Teaching Experience


Presentations


Publications


Appendix

119
$^{13}$C NMR (300 MHz, CDCl$_3$) spectrum of 2.8
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 2.9
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 2.5
$^{13}$C NMR (300 MHz, CDCl$_3$) spectrum of 2.5
$^{31}$P NMR (300 MHz, CDCl$_3$) spectrum of 2.6
\(^1\)H NMR (300 MHz, CDCl\(_3\)) spectrum of 2.13
$^{31}$P NMR (300 MHz, CDCl$_3$) spectrum of 2.13
$^{31}$P NMR (300 MHz, CDCl$_3$) spectrum of 2.15
$^{31}P$ NMR (300 MHz, CDCl$_3$) spectrum of 2.16
$^{13}$C NMR (300 MHz, CDCl$_3$) spectrum of 3.12a

3.12a
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 3.12c
$^{13}$C NMR (300 MHz, CDCl$_3$) spectrum of 3.12f
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 3.12g
$^{13}$C NMR (300 MHz, CDCl$_3$) spectrum of 3.12g
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 3.12h
$^{13}$C NMR (300 MHz, CDCl$_3$) spectrum of 3.12h

3.12h
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 3.12i
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 3.16b
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 3.16c
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 3.16d
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 3.16e
$^{13}$C NMR (300 MHz, CDCl$_3$) spectrum of 3.16e
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 3.16f

3.16f
$^{13}$C NMR (300 MHz, CDCl$_3$) spectrum of $3.16f$
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 3.16g
$^{13}$C NMR (300 MHz, CDCl$_3$) spectrum of 3.16g
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 4.5
$^{13}$C NMR (300 MHz, CDCl$_3$) spectrum of 4.5
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 4.15a
$^{13}$C NMR (300 MHz, CDCl$_3$) spectrum of 4.15a
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 4.15b
$^{1}H$ NMR (300 MHz, CDCl$_3$) spectrum of 4.15e
$^{13}\text{C} \text{ NMR (300 MHz, CDCl}_3\text{) spectrum of 4.15e}$
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 4.15f
$^{13}$C NMR (300 MHz, CDCl$_3$) spectrum of 4.15f
$^{13}$C NMR (300 MHz, CDCl$_3$) spectrum of 4b
$^1$H NMR (300 MHz, CDCl₃) spectrum of 4.15h
$^{13}$C NMR (300 MHz, CDCl$_3$) spectrum of 4.15h

![Chemical Structure]

\[ \text{CbzHNCO} \]

4.15h
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 4.18a

4.18a
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 4.18b
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 4.18c
$^{13}$C NMR (300 MHz, CDCl$_3$) spectrum of 4.18c
$^{1}H$ NMR (300 MHz, CDCl$_3$) spectrum of 4.18d

C2zHN

$\text{CO}_2\text{Bn}$
$^{13}$C NMR (300 MHz, CDCl$_3$) spectrum of 4.18d
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 4.18e

![Diagram of 4.18e](image)
$^{13}$C NMR (300 MHz, CDCl$_3$) spectrum of 4.18e

![Chemical Structure Image]

4.18e
$\text{^{1}H NMR (300 MHz, CDCl}_3\text{) spectrum of 4.18f}$

![NMR spectrum of 4.18f](image)
"{}\textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) spectrum of 4.18g"
$^{1}H$ NMR (300 MHz, CDCl$_3$) spectrum of 4.18i
$^{13}$C NMR (300 MHz, CDCl$_3$) spectrum of 4.18i
$^1$H NMR (300 MHz, DMSO-d$_6$) spectrum of 4.18j
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Method: DH 80% ACN
$^1$H NMR (300 MHz, DMSO-$d_6$) spectrum of 4.18k

![Chemical structure of 4.18k](image)
>![](image)

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**Method:** DH 80% ACN
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 4.12

4.12
$^{31}$P NMR (300 MHz, CDCl$_3$) spectrum of 4.12

4.12