

NEW METHODS FOR DETECTING BIOMARKERS OF OXIDATIVE STRESS
AND REDOX SIGNALING ON PROTEIN CYSTEINE RESIDUES

BY

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ABBREVIATIONS USED

AhpC	Alkyl Hydroperoxide Reductase Component C
biotin-IAA	biotin polyethylene oxide iodoacetamide
C165S	Mutant with cysteine at position 165 converted to serine
CAN	cerium ammonium nitrate
Cys-SNO	S-nitrosocysteine
DABCYL	4-(4-dimethylaminophenylazo)benzoic acid
DCC	N,N'-Dicyclohexylcarbodiimide
DTNB	5,5'-dithio- <i>bis</i> -(2-nitrobenzoic acid) or Ellman's Reagent
ESI-MS	Electrospray ionization mass spectrometry
FRET	Fluorescence resonance energy transfer
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Glutathione (or reduced glutathione)
GSSG	Glutathione disulfide (or oxidized glutathione)
HASMC	Human aortic smooth muscle cell
IAA	Iodoacetamide
LC-MS-MS	Liquid chromatography-tandem mass spectrometry
LPO	Lipid peroxidation
MMTS	Methyl methanethiosulfonate
MS	Mass spectrometry
Msr	Methionine sulfoxide reductase
NBD-Cl	7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole

NEM	N-Ethyl maleimide
NF- κ B	Nuclear factor kappa B
NMR	Nuclear magnetic resonance
NO	Nitric oxide
PDGF	Platelet-derived growth factor
Prx	Peroxiredoxin(s)
PTP	Protein tyrosine phosphatase
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RSNO	S-Nitrosothiol
RSOH	Sulfenic Acid
RSO ₂ H	Sulfinic Acid
RSO ₃ H	Sulfonic Acid
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
TBAF	Tetrabutyl ammonium fluoride
TCEP	Tris carboxyethyl phosphine
TNB	2-nitro-5-thiobenzoic acid
TNF- α	Tumor necrosis factor- α
Trx	Thioredoxin
TXPTS	Tris(4,6-dimethyl-3-sulfonatophenyl) phosphine trisodium salt hydrate
VEGF	Vascular endothelial growth factor

ABSTRACT

Both nitric oxide and hydrogen peroxide, as well as their respective metabolites (reactive nitrogen or oxygen species), participate in a variety of cellular redox processes and have become well recognized as messengers in cellular signal transduction. One important mechanism by which cellular redox-based signaling occurs is reversible oxidation of cysteine residues in the presence of low concentrations of these oxidants. S-Nitrosothiols (RSNO) and sulfenic acids (RSOH) are thought to be two of the most common cysteine modifications, and formation of these species reversibly alters protein function. Protein oxidation is studied to a lesser extent than lipid and DNA oxidation in part because of a lack of sensitive, stable, readily detectable markers for tracking these unstable intermediates.

We addressed the specificity of current RSNO detection methods, including the Biotin Switch Technique, using a mutated C165S alkyl hydroperoxide reductase (AhpC) enzyme from *Salmonella typhimurium*. Through both SDS PAGE and MS-based methods, we showed that the thiol alkylating agents used during this assay had an unexpected cross-reactivity with our model sulfenic acid, and in all cases formed covalent sulfoxide products. This cross-reactivity suggests that the Biotin Switch Technique may quantify both -SNO and -SOH versions of our enzyme, questioning the reliability of this assay.

We also report the development of new chemical probes for the detection of S-nitrosothiols and sulfenic acids. Using the water-soluble phosphine, tris(4,6-dimethyl-3-sulfonatophenyl)phosphine trisodium salt hydrate (TXPTS), we covalently labeled S-

nitrosated cysteine, glutathione, and C165S AhpC. A combination of NMR and MS techniques reveals that these reactions produce covalent S-alkylphosphonium ion adducts (with S-P⁺ connectivity), TXPTS oxide, and a TXPTS-derived aza-ylide. Mechanistically, this reaction may proceed through an S-substituted aza-ylide or the direct displacement of nitroxyl from the RSNO group. This work provides a new means for detecting and quantifying S-nitrosated species in solution and highlights the role of triaryl phosphines as a new tools to elucidate the role of S-nitrosothiols in nitric oxide metabolism.

The synthesis of novel dimedone-based chemical probes for the detection of protein sulfenic acids are also presented. These probes all contain a dimedone-based 1,3-cyclohexadione core, which covalently binds RSOHs, and reporter tags such as biotin for affinity capture and fluorescent labels for visual detection of cellular sulfenic acids. The application of these new chemical probes has improved our understanding of the role of sulfenic acid formation in a variety of cellular processes. These new probes have been used to monitor protein oxidation events in models of the immune response, angiogenesis, and cancer signaling pathways and have enhanced our understanding of the biological roles of hydrogen peroxide and other cellular ROS in signal transduction.

CHAPTER 1

AN INTRODUCTION TO PROTEIN SULFENIC ACID AND S-NITROSOTHIOL REDOX BIOLOGY

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Biological signaling associated with reactive oxygen and nitrogen species (ROS and RNS respectively) is a subject of intense research interest. ROS and RNS are produced endogenously and have a variety of effects within the cell. At low concentrations, these oxidants are believed to be involved in a diverse range of signaling events, but higher concentrations can lead to cytotoxicity¹⁻³. Accumulating evidence suggests that many proteins actually require oxidation by ROS and RNS for their normal catalytic function⁴⁻⁶. However, the cellular balance between oxidative signaling and damage is still unclear. Herein we will discuss research which supports the view that protein oxidation, once believed to be toxic in nature, is indeed a carefully regulated event required for cell growth and signaling. The work comprised in this dissertation will focus on our efforts to develop chemical probes to detect certain types of cysteine-based oxidation. We hope to better understand the mechanism by which protein oxidation functions as an important post-translational signaling event.

Elevated levels of ROS/RNS result in oxidative stress, leading to oxidative damage of cellular tissue which is implicated in aging and in the development of heart disease, diabetes, chronic inflammatory disease, cancer, and several neurodegenerative diseases⁷⁻¹¹. Common oxidative damage includes lipid peroxidation, DNA oxidation, and several types of protein oxidation (Figure 1). ROS encompass a variety of oxygen-based oxidants including: hydrogen peroxide (H_2O_2), peroxynitrite (ONOO^-), hydroxide radical ($\cdot\text{OH}$), and superoxide (O_2^-). RNS typically include nitric oxide ($\cdot\text{NO}$), nitrogen dioxide (NO_2), and dinitrogen trioxide (N_2O_3). Peroxynitrite is also considered a potent RNS.

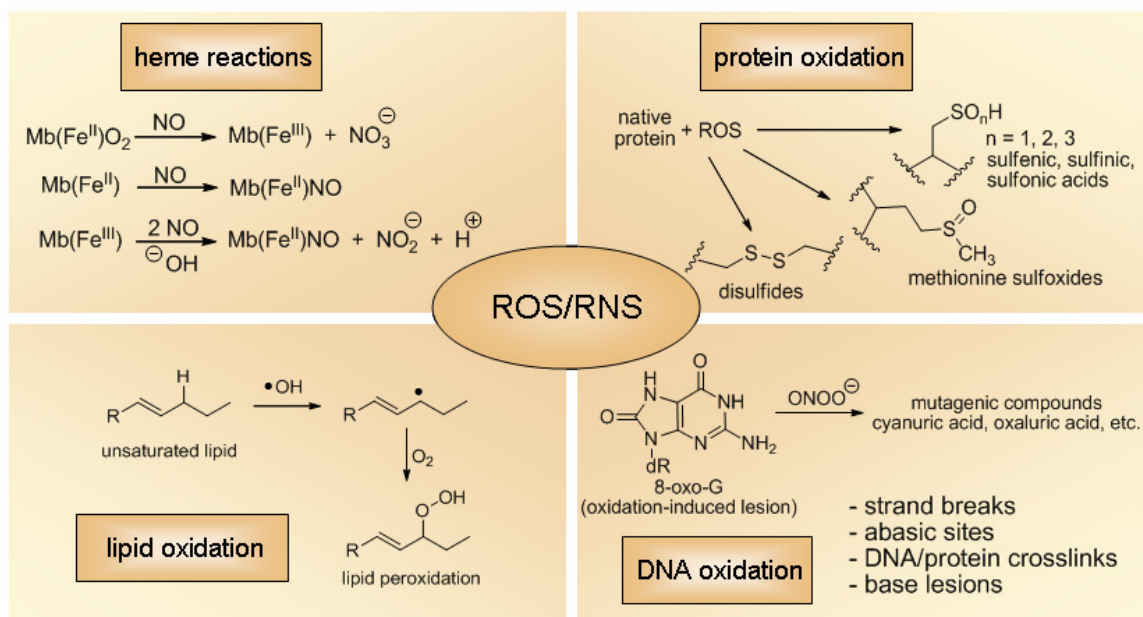
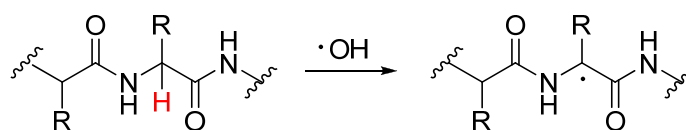


Figure 1. Biological targets of oxidative stress. Several ROS/RNS oxidize proteins at heme centers as well as individual amino acid residues. Lipid oxidation leads to the formation of lipid peroxides. DNA oxidation results in several types of oxidative induced lesions.

•NO, the endogenously produced signaling agent which covalently modifies or oxidizes proteins in a variety of ways by generating several RNS. The interaction of •NO and other RNS at the heme center of proteins has been extensively investigated and reviewed by us and others and leads to a variety of oxidized and reduced products (Figure 1)¹²⁻¹⁴. Following DNA oxidation, RNS can cleave oxidative lesions, resulting in the formation of a variety of toxic and mutagenic compounds (Figure 1)¹⁵. Additionally, RNS can produce nitrotyrosine adducts, which are a marker of oxidative damage¹⁶⁻¹⁸.

ROS also elicit a variety of cellular effects. Elevated levels of ROS, specifically hydroxyl radical, can initiate lipid peroxidation (LPO) and LPO-derived DNA-damage,

which is implicated in disease pathogenesis¹⁹⁻²¹. Hydroxyl radical can also oxidize proteins in a variety of ways, including hydrogen abstraction of the peptide backbone to induce peptide cross-linking or fragmentation (Scheme 1)²². The ability of metal ions in oxygenated environments to catalyze the endogenous production of ROS is well established. For instance, Fe(II) reacts with H₂O₂ to produce a ferryl ion complex, Fe(IV)O, capable of oxidizing methionine residues to methionine sulfoxide (MetO)²².



Scheme 1. Hydrogen abstraction from a peptide backbone

Lower levels of RNS are capable of forming S-nitrosothiols (RSNOs) on cysteine residues, which reversibly alter protein function but do not necessarily indicate toxicity. RSNOs are suggested to act as endogenous transporters of NO and may have other beneficial effects on the cell independent of NO-related signaling^{23, 24}. We will discuss these aspects of NO in greater detail later in this chapter. Low concentrations of ROS form cysteine sulfenic acids (RSOH) in several protein systems^{2, 25}. Analogous to S-nitrosothiols, an increasing number of functions are being ascribed to RSOHs, including their role in cancer cell proliferation and many other redox signaling pathways²⁶⁻²⁸. As ROS levels increase, RSOH will oxidize to sulfinic (RSO₂H) and sulfonic (RSO₃H) acids, which are irreversible cysteine-based modifications that are markers of oxidative damage (Figure 1).

Reversible protein oxidation tends to be complex and varies in the degree of toxicity it causes. The formation of disulfide bonds (the simplest type of cysteine oxidation) is readily reversible by endogenous cellular reductants and typically not associated with toxic events. However, under conditions of oxidative stress, perturbation in the disulfide proteome can overwhelm the natural antioxidant mechanisms of the cell and lead to the development of disease. This fine balance is further complicated by the fact that cellular reductants (for example: GSH, Grx, Prx or Trx) are also susceptible to oxidation when ROS/RNS concentrations are high, leading to their inactivation^{29, 30}. While cysteine is the most common, other amino acids are also susceptible to oxidation. Methionine oxidation (Figure 1), a result of intracellular ROS, is also reversible through the action of free methionine-R-sulfoxide reductases (fRMsR)^{31, 32}. Cysteine and methionine are the only amino acids known to have repair mechanisms in place following oxidation.

1.1 Cysteine Oxidation in Cellular Signal Transduction

The sulfur on cysteine has the ability to exist in numerous oxidation states *in vivo* leading to a range of oxidative modifications that include thiolates, thiyl radicals, disulfides, sulfenic (SOH), sulfinic (SO₂H), and sulfonic acids (SO₃H), disulfide-S-oxides, selenodisulfides, and S-nitrosothiols (RSNO). Most of these higher-oxidized products have the potential to reversibly alter protein function, increasing the redox diversity of the proteome. Figure 2 summarizes the formation of a variety of these

cysteine-based modifications. Two of these oxidative thiol species, S-nitrosothiols and sulfenic acids, are reversible modifications that can participate in several biochemical

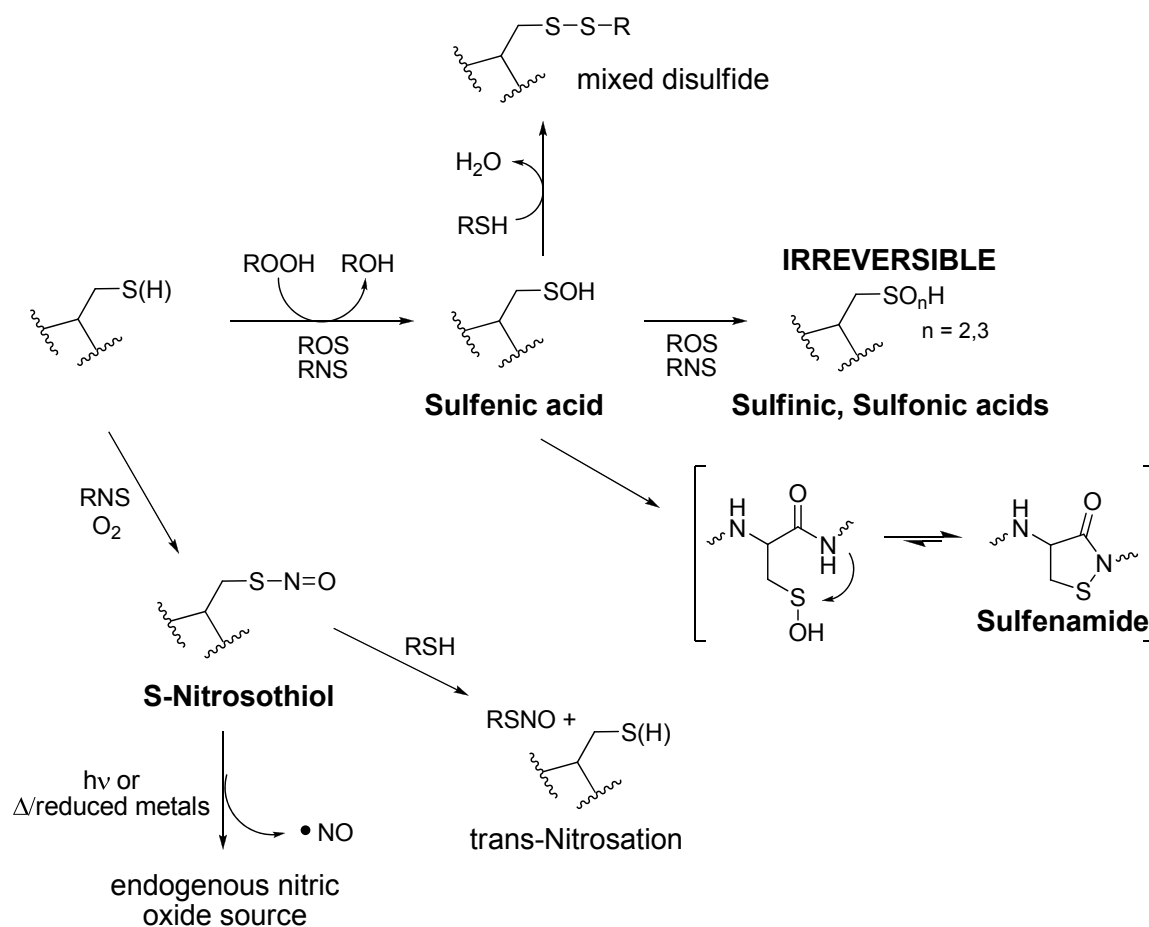


Figure 2. A schematic view of protein cysteine oxidation. RSNOs are generated in the presence of RNS and O₂. Sulfenic acids form upon exposure to low levels of ROS or peroxide; higher concentrations will result in the formation of sulfinic and sulfonic acids, which are irreversible species that represent oxidative damage.

pathways such as redox sensing, catalysis, transcriptional regulation, and redox signaling³³. Various families of proteins, such as glyceraldehyde-3-phosphate dehydrogenase³⁴⁻³⁷, tyrosine phosphatases^{5, 38, 39}, and proteases⁴⁰⁻⁴², contain active-site

cysteine residues that are essential for function and thus, are inhibited by the oxidation of these thiols to RSNO/RSOH.

S-Nitrosothiols

S-Nitrosothiols have the general formula R-S-N=O. Cysteine is oxidized to an RSNO in the presence of certain RNS with O₂, and this process elicits a variety of biological responses. Figure 3 shows the proposed *in vivo* formation of S-nitrosothiols. Interestingly, the reaction of reduced glutathione (GSH) with ·NO does not produce S-nitrosoglutathione (GSNO) directly, but instead generates glutathione disulfide (GSSG)

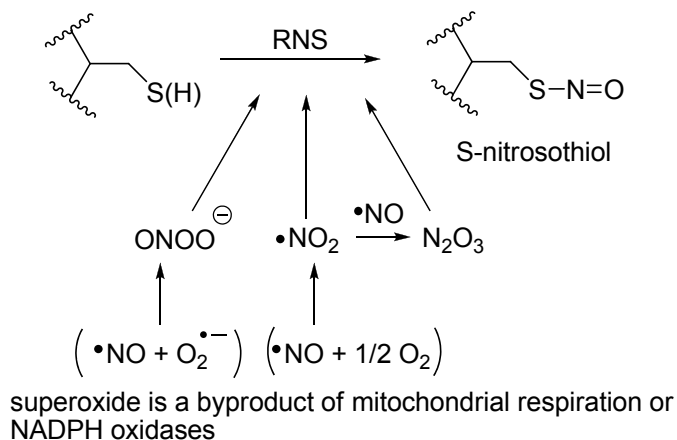


Figure 3. The formation of protein S-nitrosothiols. Several types of RNS can form RSNOs *in vivo*, including peroxynitrite (ONOO⁻), nitrogen dioxide (NO₂) or dinitrogen trioxide (N₂O₃).

and nitroxyl anion (HNO/NO⁻)⁴³. The formation of GSNO, or any RSNO, from ·NO is an oxygen-dependent process and only occurs after NO is oxidized by O₂ to form ·NO₂ or N₂O₃ (Figure 3)⁴⁴. The reaction of ·NO with superoxide anion (O₂⁻) generates

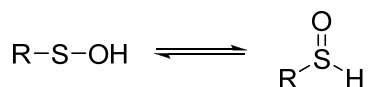
peroxynitrite (ONOO^-), a potent oxidant capable of activating guanylyl cyclase, inhibiting platelet aggregation and inducing vasodilation; an S-nitrosated intermediate accounts for this activity^{45, 46}. Thiol nitrosation can also occur *via* the transfer of $[\text{NO}^+]$ from iron nitrosyl species, which are generated from the reaction of NO with both ferrous and ferric heme centers (Figure 1) or through the trans-nitrosation of a neighbouring RSNO^{12, 14, 47}. However, the most likely RNS nitrosating species in biological systems is N_2O_3 (as its formation is kinetically facilitated in hydrophobic environments such as biological membranes)⁴⁸. Generally RSNOs decompose *via* homolytic cleavage of the S- $\cdot\text{NO}$ bond, generating $\cdot\text{NO}$ and a thiyl radical ($\text{RS}\cdot$)^{49, 50} and this suggests a biological role for RSNOs as $\cdot\text{NO}$ -storage and transport devices. However, the decomposition of RSNOs by copper ions in solution is also well established. Cu(I) ions at concentrations as low as 10^{-6} M were shown to decompose RSNOs and generate $\cdot\text{NO}$ ²³. Protein-bound copper sources are also capable of generating $\cdot\text{NO}$ from RSNOs⁵¹.

While the mechanism by which low molecular weight RSNOs form and decompose is well understood, S-nitrosoprotein formation remains controversial. The propensity for protein thiolates to become S-nitrosothiols depends on a variety of factors including the protein microenvironment, metal ion availability, ROS abundance, and proximity to *in vivo* RNS generation^{48, 52}. It is currently not possible to predict which proteins are susceptible to S-nitrosation. Adding to the controversy is the fact that reliable methods for detecting and quantifying RSNOs are lacking. Currently, only indirect assays are available for RSNO detection, including chemiluminescent-based methods and the biotin switch technique. Both of these methods are outlined later in this

chapter. As the availability of good assays increases, many of the questions concerning the role and localization of S-nitrosation within the proteome will be able to be addressed.

Sulfenic Acids

Sulfenic acids have the general formula RS-O-H. They exist as a tautomer in both the keto and enol forms as seen in Scheme 2. Because of this, sulfenic acids exhibit both nucleophilic and electrophilic properties. RSOH is formed in proteins as the direct product of the cysteine thiolate (RS⁻) reaction with H₂O₂ (Figure 4)^{53, 54}. RSOH is the simplest organosulfur oxyacid; unlike sulfinic (RSO₂H) and sulfonic (RSO₃H) acids, it is highly reactive and fairly unstable^{2, 55}. In vitro, RSOHs are formed upon reaction of some proteins with small amounts of H₂O₂ (1 - 2 eq.). At higher H₂O₂ concentrations, proteins are readily over-oxidized to RSO₂H and RSO₃H.



Scheme 2. Tautomerization of cysteine sulfenic acids.

Sulfenic acids exhibit both electrophilic and nucleophilic chemical reactivity⁵⁶. Two uninhibited sulfenic acids in close proximity will dimerize and dehydrate to form a thiosulfinate (RS(O)SR), a modification which is also susceptible to over-oxidation (Scheme 3)⁴. Importantly, the electrophilic sulfur atom in R-S-OH will combine with a thiolate in close proximity, commonly termed the “resolving cysteine”, to produce a

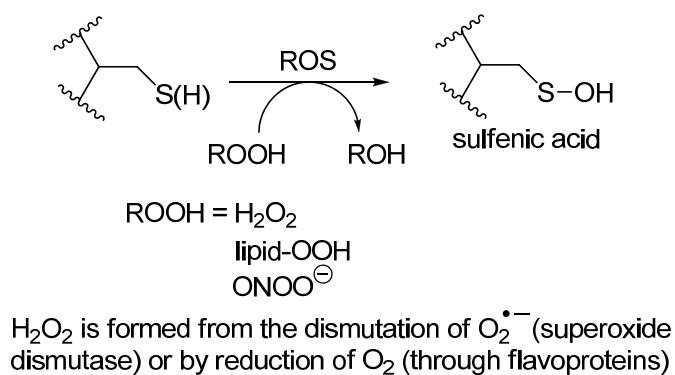
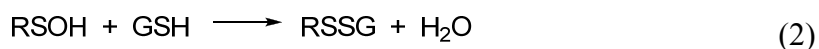
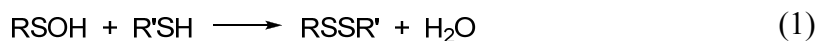
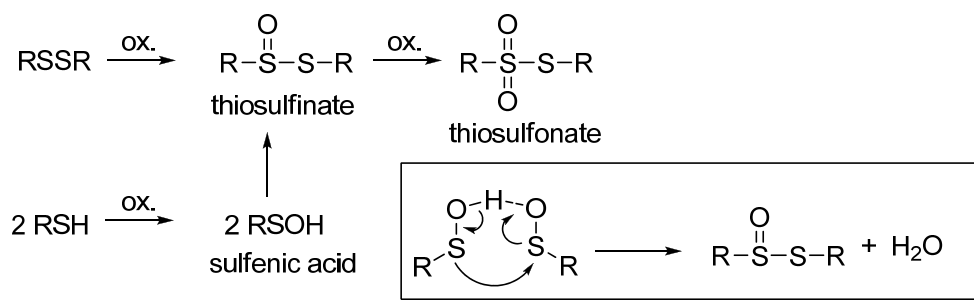


Figure 4. The formation of protein sulfenic acids. Several endogenous sources of ROS exist including peroxide (H_2O_2), lipid peroxides, and peroxynitrite. At higher levels of ROS, overoxidation of the cysteine thiolate will result in the formation of irreversible adducts including sulfinic and sulfonic acids.



disulfide and water (eq. 1). The resolving thiol can either be a nearby cysteine on the same (or another) protein, or a low molecular weight thiol that can access the site such as glutathione (GSH). A mixed disulfide would then be generated (eq. 2)⁵⁶. Moreover,



Scheme 3. Sulfenic Acid Reactivity

these disulfides are readily reversible through the action of cellular reductants like thioredoxin (Trx) or GSH.

The Importance of the Resolving Cysteine

Many of the biologically important consequences of RSOH and RSNO formation within the proteome are likely a result of increased disulfide bond formation, and thus an overall increase in oxidation. Several proteins critical to cellular transcription and homeostasis possess a Cys-X₁-X₂-Cys motif in which the oxidation-sensitive cysteine forms a sulfenic acid or S-nitrosothiol intermediate which condenses with the neighboring resolving cysteine to form a disulfide bond and release water or nitroxyl,

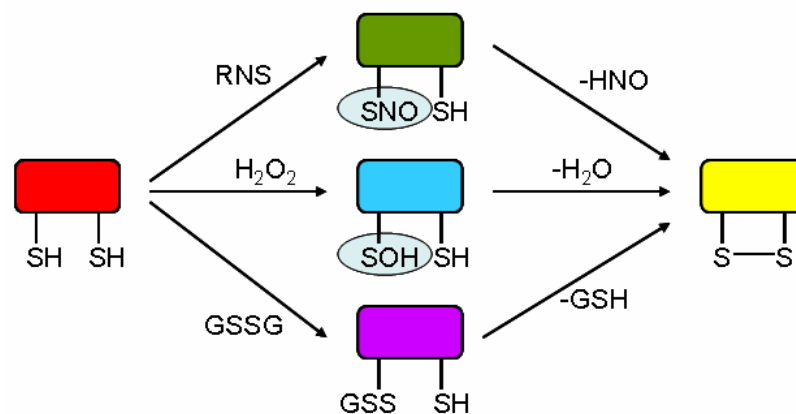


Figure 5. The role of the resolving cysteine in cell signaling and catalysis. (Adapted from Neil Hogg's webinar on 2/24/10)

respectively⁵⁶. Several enzymes use this type of cysteine thiol-switch for normal catalysis. Figure 5 demonstrates how 2-cys motifs result in disulfide bond formation of the protein, regardless of how the reactive cysteine was oxidized. The ability of an S-nitrosated intermediate to release nitroxyl as seen in Figure 5 suggests an additional role

of S-nitrosothiols as endogenous nitroxyl sources^{57, 58}. It is worth noting that this global oxidation of the proteome is what may account for the changes in protein signaling and function; however, several types of enzymes also exhibit a 1-Cys motif where the RSNO/RSOH intermediate itself accounts for the changes in catalytic activity^{59, 60}. In the context of this dissertation, however, we will discuss our efforts towards the detection of these S-nitrosothiol and sulfenic acid intermediates, which we believe will enhance our understanding of the mechanisms by which ROS/RNS stimuli effect changes in signal transduction.

Examples of Sulfenic Acid-based Oxidative Signaling

Many prokaryotic examples demonstrate that cysteine oxidation, specifically through RSNO/RSOH intermediates, leads to a reversible change in enzymatic catalysis and function. Several of the peroxiredoxins (Prx), a family of peroxidases that exist as 1-Cys and 2-Cys Prx groups, use a sulfenic acid intermediate during their catalytic cycle to detoxify cellular peroxide²⁶. The catalytic cycle for one such enzyme, alkyl hydroperoxide reductase system from *Salmonella typhimurium*, is shown below in Figure 6. It is comprised of AhpF and AhpC components and is required for cellular detoxification of oxidants^{26, 61}. AhpC contains the redox-active cysteine/disulfide center and AhpF is a flavoprotein involved in the reduction of AhpC in order to maintain normal catalysis. At low concentrations of H₂O₂, AhpC forms a cysteine sulfenic acid at Cys-46 (the peroxidatic cysteine) which condenses with the neighboring Cys-165 of another subunit, forming an inter subunit disulfide bond. This disulfide is reducible by AhpF in the presence of NADH, regenerating the catalytically active form of the enzyme.

Peroxiredoxins are a major means by which yeast, enterobacteria and trypanosomes detoxify peroxide⁶². Eukaryotes also express Prxs, but since superoxide dismutase and catalase are believed to be the major players in eukaryotic cellular detoxification, their role remains unclear.

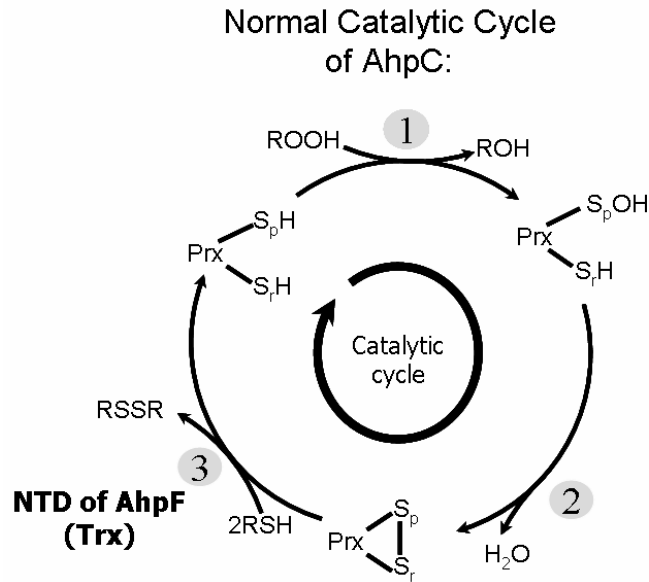


Figure 6. The catalytic cycle of AhpC. AhpC uses a cysteine sulfenic acid intermediate to detoxify cellular peroxide. (Adapted from notes by Leslie Poole)

Another example of RSOH signal transduction includes the *B. subtilis* 2-Cys OhrR protein. It is a thiol-based peroxide sensor involved in oxidative stress defense. Through a sulfenic acid intermediate, the oxidized form of this enzyme regulates an inducible OhrA^{27, 63}. Another widely studied example where a sulfenic acid intermediate had a functional outcome on signal transduction is the *E. coli* transcription factor OxyR²⁶⁻²⁸. OxyR possesses a similar cysteine/disulfide motif, which is required for the enzymatic detection of cellular peroxides. Through a sulfenic acid intermediate,

the oxidized form of OxyR initiates the transcription and translation of antioxidant enzymes⁶⁴. This mechanism is outlined in Figure 7 and shows how cysteine sulfenic acids can have functional consequences on transcriptional regulation⁶⁵. The OxyR example provides a useful paradigm for eukaryotic redox signaling, which is substantially more complex.



Figure 7. The mechanism of OxyR transcriptional regulation. Through a sulfenic acid intermediate, OxyR in its oxidized form binds DNA and initiates the transcription of antioxidant enzymes.

Numerous eukaryotic examples also exist where cysteine oxidation leads to a signaling response of the protein. Exemplary sulfenic acid examples include the yeast Yap1/Orp1 system, the protein tyrosine phosphatase PTP1B and MAP3 kinase ASK1, as well as transcription factor complex Keap1/Nrf2⁶⁶⁻⁶⁸. However, for the context of this dissertation, these examples are too complicated to be discussed in great detail.

Examples of S-Nitrosothiol-based Oxidative Signaling

S-Nitrosated cysteine residues, including SNO-hemoglobin, are believed to be stable transporters of $\cdot NO$. Nitric oxide relaxes smooth muscle cells, inhibits platelet aggregation, promotes angiogenesis, and decreases inflammation⁶⁹, but it is unclear if S-nitrosothiols exhibit the same or distinct effects relative to $\cdot NO$ itself. It is widely

accepted that the secondary messenger $\cdot\text{NO}$ plays an important role in signal transduction through guanylyl cyclase dependent and independent mechanisms. S-Nitrosation also has a profound effect on enzymatic activity. S-Nitrosation of caspase 3 inhibits apoptosis^{70, 71} and poly-S-nitrosation of the ryanodine receptor (RyR 2) reversibly activates calcium channels to enhance cardiac contractility⁷². $\cdot\text{NO}$ -induced S-nitrosation of N-ethylmaleimide-sensitive factor (NSF) inhibited platelet granule exocytosis and may be one means by which NO regulates thrombosis⁷³.

One of the most notable examples of S-nitrosation in signal transduction is the direct and indirect inhibition of NF- κB . Under normal signaling conditions, extracellular NF- κB is bound to the inhibitor complex I κB . Phosphorylation of I κB by IKK initiates the translocation of NF- κB to the nucleus where transcription occurs (Figure 8)⁷⁴. NF- κB

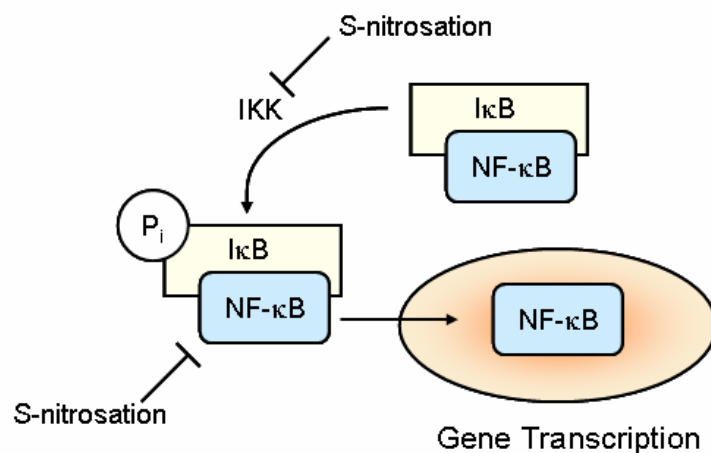


Figure 8. The effect of RSNO formation on the NF- κB pathway. S-nitrosation of NF- κB blocks the pathway by inhibiting binding of NF- κB to DNA. The S-nitrosation of the IKK β subunit of IKK inhibited phosphorylation of I κB , preventing translocation of NF- κB to the nucleus. This example shows both direct and indirect effects of S-nitrosation on the inhibition of the NF- κB pathway. (Adapted from Martínez et al.¹⁰²)

is a critical element in the immune and anti-inflammatory response and also regulates the transcription of the NOS enzymes. Direct S-nitrosation of NF- κ B at Cys-62 inhibits DNA binding and transcription⁷⁴. Reynaert et al. showed the indirect inhibition of the NF- κ B pathway through the S-nitrosation of the IKK β subunit of IKK, which inhibited phosphorylation of I κ B⁷⁵. Additionally, arsenite inhibits the TNF- α activation of IKK β , suggesting that an oxidized form of the enzyme is required for IKK β activation⁷⁵. These data are further supported by the fact that H₂O₂ is known to stimulate the pro-growth anti-apoptotic pathways of NF- κ B and growth factors are now known to initiate these ROS/RNS signaling pathways (as will be discussed in the next section of this introduction).

Growth Factor stimulation of ROS

Cellular stimulation by peptide growth factors and cytokines leads to an enhanced production of H₂O₂ by inducing NADPH oxidases^{76, 77}. ROS at moderate concentrations participate in the regulation of vascular tone, oxygen sensing, cell growth and proliferation, apoptosis, and inflammatory responses^{78, 79}. Vascular endothelial growth factor (VEGF) stimulates angiogenesis in part through the activation of NADPH oxidases (Nox-1, Nox-2, Nox-4), generating ROS signaling events which mediate this biology^{80, 81}. Platelet-derived growth factor (PDGF) activates Nox-5 which, in human aortic smooth muscle cell (HASMC), resulted in an ROS-induced activation of the JAK/STAT pathway⁸². Insulin-like growth factor 1 (IGF-1) also stimulates the production of ROS and leads to insulin resistance⁸³.

1.2 Methods for Detecting Cysteine and its Oxidized Derivatives

Cysteine Detection and Quantification

Several methods have been devised for the quantification of reduced cysteine residues in pure proteins or complex mixtures. Spectroscopic methods include the use of 5,5'-dithio-*bis*-(2-nitrobenzoic acid) (DTNB), commonly termed Ellman's Reagent, and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)^{61, 84}. Figure 9 shows the reaction of DTNB and NBD-Cl with thiols to give products with quantifiable absorption spectra.

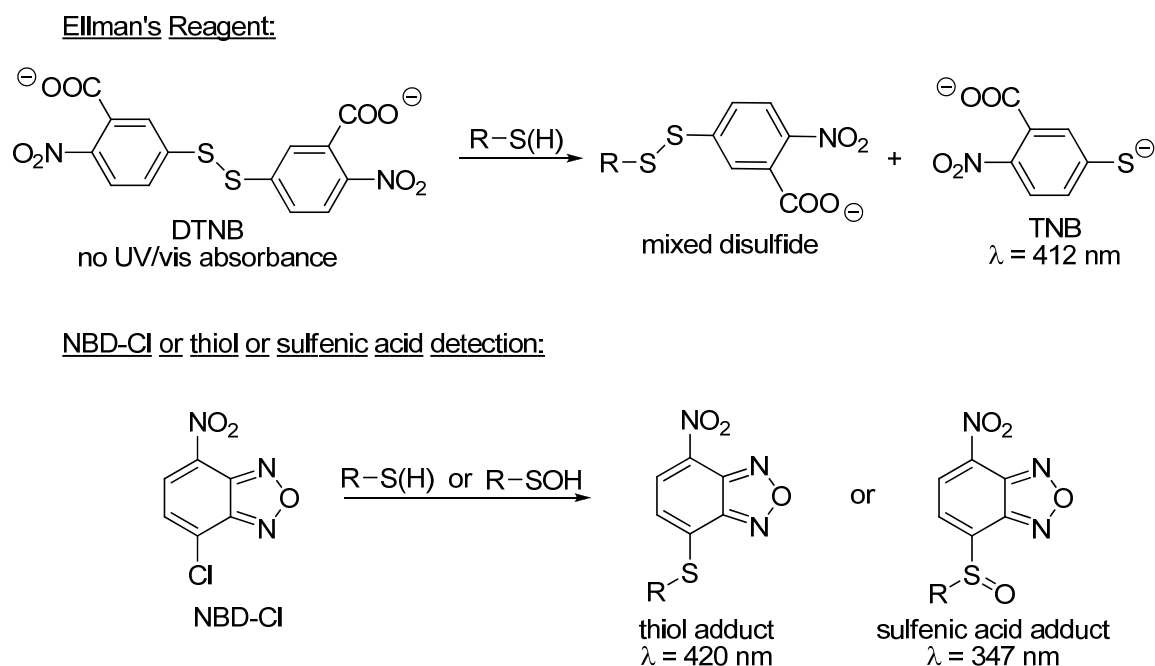
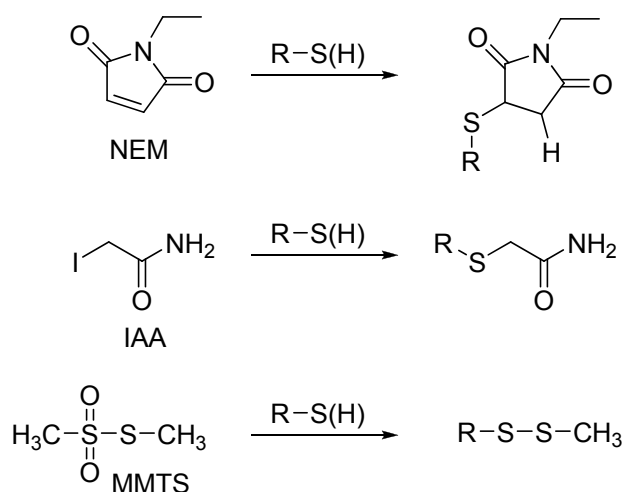


Figure 9. Spectroscopic methods for thiol detection. DTNB, otherwise known as Ellman's reagent, reacts with protein thiols to give stoichiometric amounts of TNB which is quantified spectroscopically based on its absorbance at 412 nm. NBD-Cl reacts with thiols and sulfenic acids to give adducts which absorb at 420 nm and 347 nm respectively.

DTNB does not have a strong UV/vis absorbance; however, upon reacting with thiols DTNB produces a near-UV absorbing mixed disulfide adduct and 1 equivalent of 2-nitro-5-thiobenzoic acid (TNB), which has a molar absorption coefficient of $14,150 \text{ M}^{-1}\text{cm}^{-1}$ at 412 nm in dilute buffer solutions⁸⁴. NBD-Cl is unique in that it reacts with both thiol and sulfenic acid residues on proteins to give covalent adducts, which are distinguishable by their spectroscopic absorbances at 420 nm (thiol adduct) versus 347 nm (sulfenic acid adduct)⁶¹.

Several alkylating agents react rapidly with thiols and can be visualized using mass spectrometry-based techniques (MS). The most commonly used alkylating agents are presented in Scheme 4 and include N-ethyl maleimide (NEM), iodoacetamide (IAA) and methyl methanethiosulfonate (MMTS). These reagents are also available as fluorescent or biotinylated derivatives, which enhance their utility.



Scheme 4. Common thiol alkylating agents

S-Nitrosothiol Detection

Organic and small molecule S-nitrosothiols are easily quantified because of their characteristic absorbance patterns at roughly 336 nm and 545 nm and are usually bright green, red, or pink in color⁵⁰. Concentrations typically obtained in biological assays (high nM – low μ M) are too low for detection using UV/ Vis spectroscopy, which presents a challenge for researchers looking to correlate protein RSNOs to their *in vivo* function. To date, no affinity label exists that specifically reacts with RSNOs. Therefore, only indirect methods are available for S-nitrosothiol detection.

One assay currently cited in the literature for RSNO detection is the Biotin Switch Technique (BST)⁸⁵⁻⁸⁷. The BST consists of three basic steps: (1) blocking all the free Cys thiols, (2) reduction of RSNOs with ascorbate and (3) *in situ* labeling of the nascent thiols with a biotin label (Figure 10). RSNO concentrations are then determined based on a biotin pull-down method or through Western blot analysis. The advantage of this technique is that it can be performed without specialized equipment and can detect low μ M concentrations of biotinylated protein. Recently however, the validity of the BST has

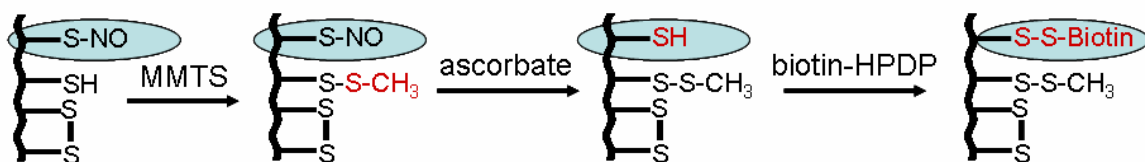


Figure 10. A schematic of the Biotin Switch Technique. This 3-step assay is used for the detection of protein RSNOs and involves the specific reduction of the S-NO bond using sodium ascorbate.

been under intense scrutiny. The reliability of this technique hinges on the claim that ascorbate will specifically reduce RSNO, despite some evidence that it reacts with sulfenic acids as well as disulfide bonds^{88, 89}. Another obvious disadvantage is that this is at best an indirect method to quantify RSNO levels. Since these modifications are inherently unstable, it is unlikely that all protein SNOs can survive the first two steps of this assay intact (in order to be biotinylated and quantified successfully).

A complementary technique for S-nitrosothiol detection is reductive chemiluminescence using reagents designed specifically to promote NO release from RSNOs (CuCl/ cysteine based assay)⁹⁰⁻⁹². Chemiluminescence-based assays correlate NO concentration to the generated luminescence ($\text{NO}_2^* \rightarrow \text{NO}_2$) when NO reacts with ozone, producing NO_2 in the excited state (NO_2^*). Excess cysteine in the CuCl/ cysteine assay serves to drive the transnitrosation equilibrium in favor of cysteine-SNO (Cys-SNO) and maintains the Cu^+ redox state of the metal⁹⁰. These conditions selectively reduce the S-NO bond by transferring NO^+ to free cysteine, allowing for an alternative (but still indirect) method to detect RSNOs. This method also fails to indicate which cys was modified.

Another method for indirectly detecting S-nitrosothiols is the Saville assay⁹³. It is an absorbance-based technique involving the use of mercuric chloride to disrupt the S-NO bond followed by the quantitative detection of nitrite using the diazotization assay (Griess Reagent)^{94, 95}. The difference in nitrite concentrations of complex mixtures +/- HgCl_2 is then taken as the total [RSNO] content for the sample⁹⁰. Unfortunately this assay has a limit of detection of approximately 500 nM and is therefore not as useful for many biological SNOs.

Sulfenic Acid Detection

Unlike RSNOs, there is no characteristic absorbance that can be used to detect RSOH formation. As discussed earlier, Ellis and Poole have demonstrated that the reaction of RSOHs with NBD-Cl gives an adduct that can be distinguished by its absorbance at 347 nm, versus 420 nm for the adduct of NBD-Cl with thiol groups⁶¹. This method is practical for the quantification of RSOH formation on pure proteins; however, it lacks the ability to profile proteome-level changes in RSOH.

The small molecule dimedone (Figure 11A) is known to react specifically and directly with RSOH modifications and has been used for many years as an –SOH-directed alkylating agent^{36, 96, 97}. Recently, Poole and others have created a series of dimedone-like fluorescent and biotinylated probes (Figure 11B) that are highly specific for RSOH⁹⁸⁻¹⁰². The probes shown in Figure 11B, and derivatives thereof, have been vital in identifying global sulfenic acid formation within cells. Several surprising targets of RSOH formation were identified using these dimedone-based chemical probes including Hsp-90- α , GAPDH, and PTP1B^{101, 103}.

Antibody-based methods for detecting the dimedone modifications of sulfenic acids have also been explored. An anti-dimedone antibody generated from α -haptens was used to visualize and profile RSOH formation in human cancer cell lines¹⁰. Eaton et al. used a similar anti-dimedone antibody to show that GAPDH exists basally as an RSOH in rat ventricular myocytes¹⁰⁴. While antibody-based approaches are cost effective and widely used, these anti-dimedone antibodies are still an indirect means for RSOH detection.

A sulfenic acid biotin switch method is also available for the indirect detection of RSOH^{87} . This method is analogous to the S-nitrosothiol biotin switch assay outlined in

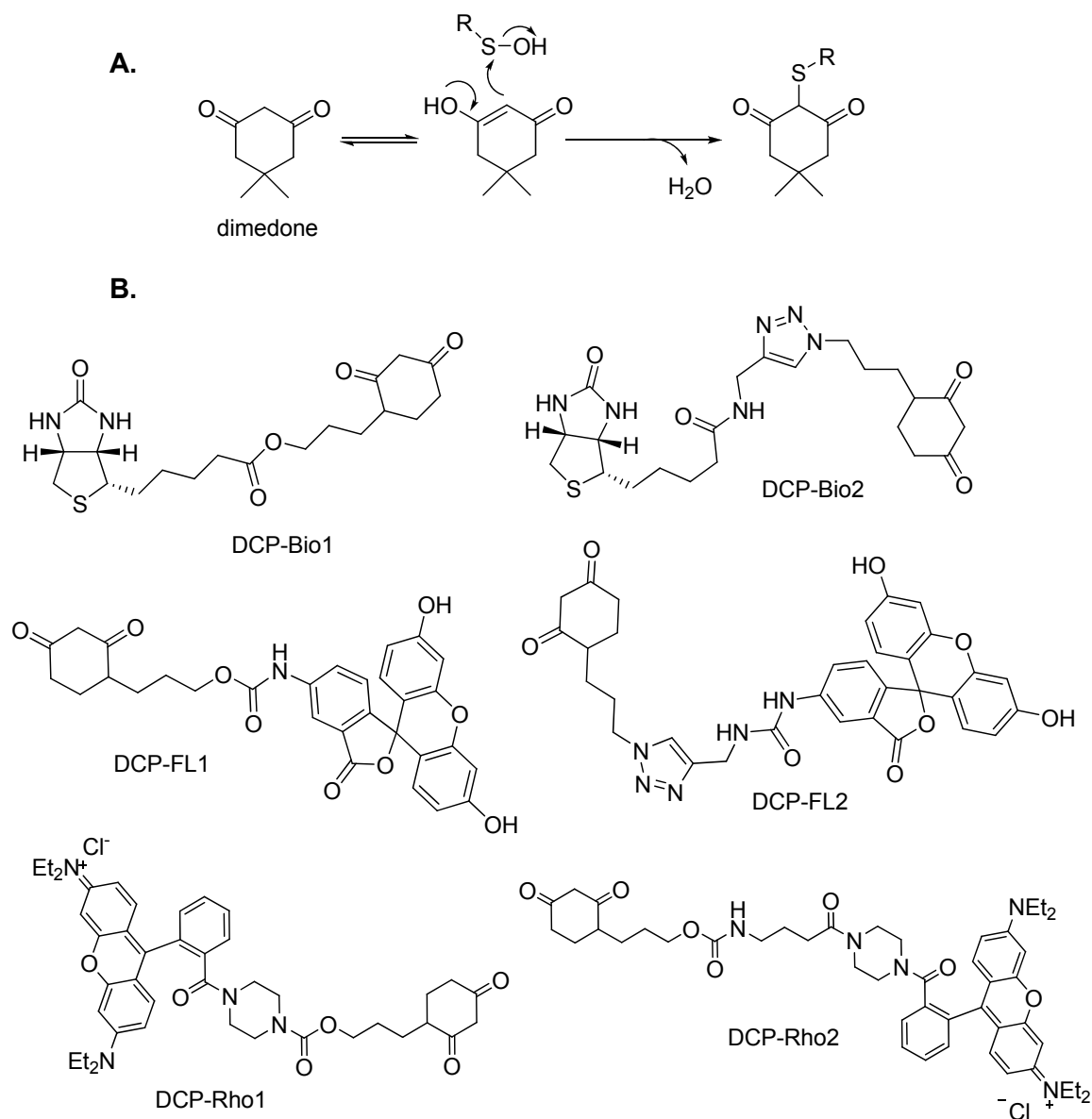


Figure 11. Dimedone-based probes for labeling protein sulfenic acids. (A) A schematic of how dimedone covalently labels RSOHs through the displacement of water. (B) A series of dimedone derivatives synthesized by Poole et al.^{96, 97} for tracking cellular sulfenic acid formation.

Figure 10 but relies on an arsenite-specific reduction of protein sulfenic acid under denaturing conditions (as opposed to an ascorbate-specific reduction for S-nitrosothiols).

1.3 The Role of Cysteine Oxidation in Disease Progression

Consequences of S-nitrosation in Disease

Cellular RNS induce a variety of reactions including S-nitrosation and tyrosine nitration. While we have highlighted the role of RSNOs as beneficial signaling events crucially important for cellular signaling and homeostasis, it is important to recognize that signaling depends on the concentration of RNS as well as the cellular target of S-nitrosation. At higher concentrations, many cysteine residues that are not typically targets of oxidation may become S-nitrosated. A few examples where S-nitrosation can have a negative effect on the cell are outlined below.

Ion channels are susceptible targets of RNS. One of the most well studied examples is the activation/inactivation of cardiac and skeletal RyRs¹⁰⁵. As mentioned above, low concentrations of RNS lead to an activation of RyR 2 through an S-nitrosated intermediate, which enhances Ca^{2+} release and cardiac contractility⁷². However, higher concentrations of RNS were shown to inhibit several RyRs and inhibit contraction and induce cell death¹⁰⁵.

Several RNS-dependent mechanisms contribute to the onset and development of Parkinson's disease. In Parkinson's models, an upregulation of iNOS occurs, which accounts for the increased nitrosative damage in dopaminergic neurons¹⁰⁶. Increased RNS may lead to S-nitrosation of Complex I in the mitochondria, resulting in an increase

in superoxide (O_2^-)¹⁰⁷. The addition of GSH reversed this RSNO-mediated inhibition of complex (I)¹⁰⁸. As seen in Figure 12, GSH can reduce RSNOs by first forming an S-glutathionylated intermediate, which is reduced by the Grx systems. These data highlight the importance of redox balance within neuronal cells. Without proper cellular detoxification, this increase in ROS/RNS will lead to the dysfunction observed during Parkinson's disease.

S-Nitrosation is also believed to play a crucial role in insulin secretion and resistance. S-Nitrosation of Akt/PKB affects glucose metabolism and leads to insulin resistance in mice¹⁰⁹. $\cdot NO$ donors directly inhibit insulin secretion by S-nitrosation of key enzymes in the stimulus-secretion pathway which suggests that RNS and S-nitrosation could modulate insulin resistance and diabetes¹¹⁰.

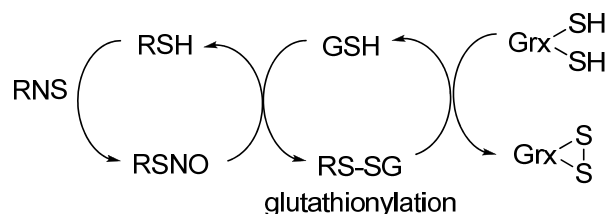


Figure 12. The antioxidant ability of glutathione. Glutathione can detoxify cellular RSNOs via S-glutathionylation which is reversible by the Grx system.

1.4 Synopsis

Herein we have summarized the importance of S-nitrosothiols and sulfenic acids in cellular signal transduction and their multiple roles in cell growth, proliferation, and detoxification. Since both of these cysteine-based modifications are reversible in nature,

we believe that they represent a specific signaling event, which is critically important for normal homeostasis. Unfortunately, since RSOHs and RSNOs are intermediates, a functional outcome of cellular ROS/RNS is often easier to quantify than the involvement of either sulfenic acids or S-nitrosothiols during the signal transduction process. The work in this dissertation aims to synthesize and evaluate new chemical probes for the detection of RSOH and RSNO modification in cells in order to elucidate their *in vivo* roles.

Chapter 2 summarizes work on the use of triarylphosphines to quantify protein S-nitrosation. The triarylphosphine tris(4,6-dimethyl-3-sulfonatophenyl) phosphine trisodium salt hydrate (TXPTS) forms a covalent adduct with both peptide and protein RSNOs, which is both NMR and MS detectable (Figure 13). Possible mechanisms are presented based on NMR kinetics studies and an evaluation of the TXPTS by-products. This chapter, published earlier this year¹¹¹, also addresses the utility of phosphine-based probes as a means for tracking *in vivo* S-nitrosation.

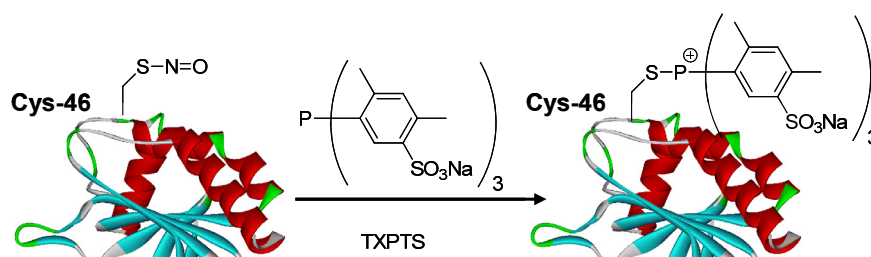
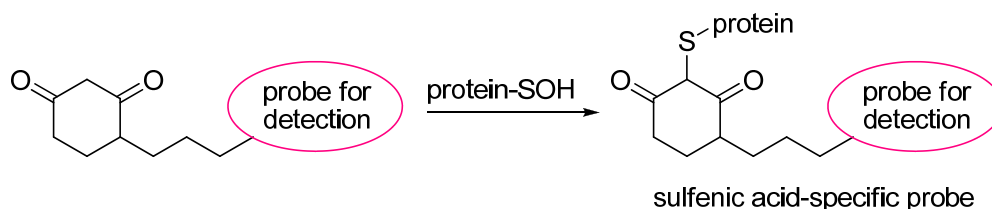


Figure 13. The reaction of TXPTS with C165S AhpC-SNO. The triarylphosphine TXPTS reacted specifically with a protein SNO, forming a covalent adduct which was quantified using ESI-TOF-MS.

In Chapter 3, a series of dimedone-based chemical probes were prepared, which are highly selective for protein sulfenic acids (Scheme 5). Improvements to the method for synthesizing DCP-Bio1 (Figure 11) are discussed, as well as the synthesis of a new fluorescent probe (DCP-DABCYL) and an alkyne probe (DCP-alkyne). Additionally, new approaches towards the synthesis of an acid-cleavable biotin-azide are presented. We hypothesize that this new biotin-azide will provide a useful means for tracking RSOH labeling with our DCP-alkyne probe using click chemistry. Our insertion of an acid-cleavable group into the biotin-azide component is expected to efficiently remove the biotin moiety prior to LC-MS analysis of the labeled proteins. The starting materials used in the synthesis of this new biotin-azide have been carefully planned and based on the availability of commercial ^{13}C starting materials, providing a platform for the future synthesis of an isotope-coded affinity tagged (ICAT) biotin-azide for the proteomic profiling of cysteine sulfenic acids.



Scheme 5. Labeling protein sulfenic acids using dimedone-based chemical probes

Chapter 4 discusses the short-falls of the biotin switch assay (Figure 10) as a means to quantify S-nitrosation. We present convincing data that the ascorbate reduction of S-nitrosothiols is promiscuous and non-specific. SDS-PAGE shows formation of covalent dimers for C165S AhpC-SOH when incubated with sodium ascorbate, a feature that is not observed with C165S AhpC-SNO. Further analysis of the BST also revealed

specificity concerns of the thiol alkylating agents (Scheme 4) used in this assay and found that they react with sulfenic acids as well. Both ESI-MS and MALDI-MS evidence suggests that NEM, IAA, and MMTS react with C165S AhpC-SOH to form a covalent sulfoxide adduct which is distinguishable from the thiol adduct based on a mass difference of 16 amu. Based on this work, we discuss ways to improve the specificity of the BST and make it a viable means for quantifying cellular S-nitrosation. We also address the implications of this cross-reactivity of the alkylating agents during normal sulfenic acid labeling experiments.

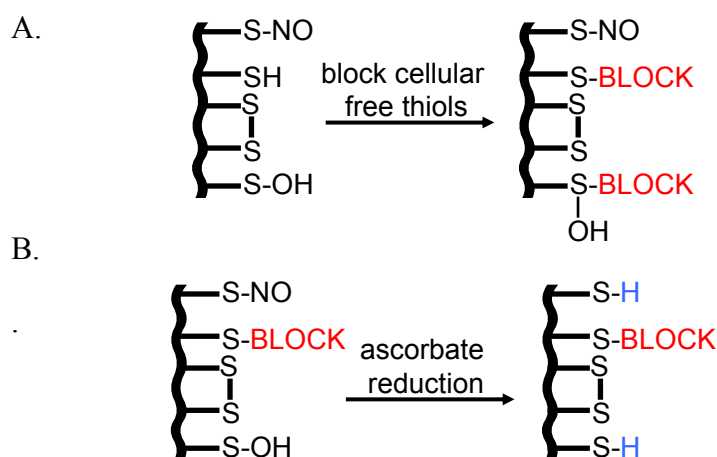


Figure 14. Specificity concerns of the Biotin Switch Technique. **(A)** The initial step of the BST involves the blocking of cellular free thiols using traditional alkylating agents. MS evidence suggests that these reagents would also block sulfenic acids, but at a slower rate. **(B)** The reduction of the RS-NO bond by ascorbate is also non-specific, resulting in the reduction of the RS-OH bond as well. This would be expected to produce false-positive results in the assay.

CHAPTER 2

WATER-SOLUBLE TRIARYLPHOSPHINES AS BIOMARKERS FOR PROTEIN S-NITROSATION

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Wright[†], Leslie B. Poole[‡], Cristina M. Furdui[§], S. Bruce King[†]

[†]Department of Chemistry, Wake Forest University, Winston-Salem, NC 27109, [‡]Center for Structural Biology, Department of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, NC 27157, and [§]Department of Molecular Medicine, Wake Forest University School of Medicine, Winston-Salem, NC 27157

The work contained in this chapter was initially published in *ACS Chemical Biology* in 2010. The manuscript, including figures and schemes, was drafted by Erika Bechtold and edited by Bruce King and Leslie Poole. Since the publication, changes in both format and contents were made to adapt this work for the dissertation format. The research described herein was performed by Erika Bechtold. The crystal structure determination was contributed by Dr. Cynthia S. Day. The protein MS work was done in collaboration with Dr. Cristina M. Furdui and the NMR work, specifically the 2-D correlation spectroscopy and time-course NMR data, were done in collaboration with Dr. Marcus Wright.

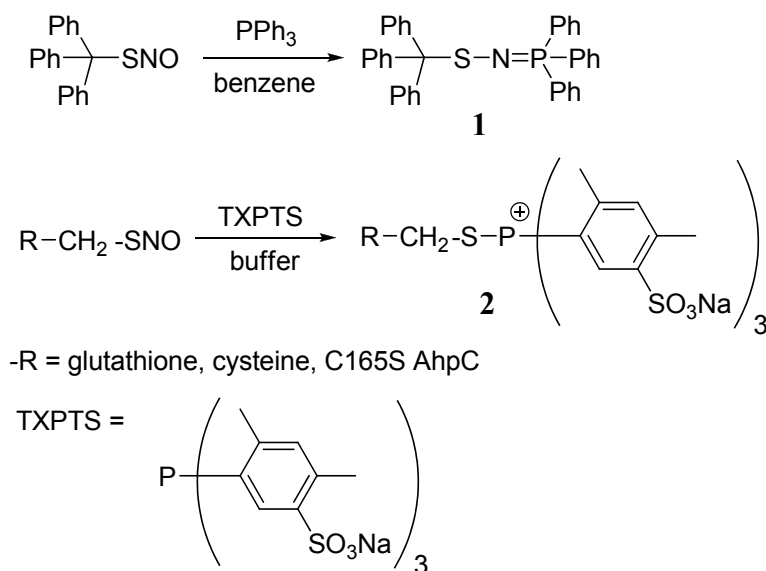
2.1 The use of Phosphines as S-nitrosothiol Labels

As discussed in the Introduction of this dissertation, reactive oxygen and nitrogen species (ROS/RNS) affect cellular signaling processes at concentrations far below those required to inflict oxidative damage^{45, 53, 112, 113}. The over-oxidation and metabolism of nitric oxide (NO), an endogenous cell signaling agent, produces several RNS including nitrogen dioxide and nitrite, which can generate competent nitrosation agents^{114, 115}. Nitrosation of cysteine sites yields S-nitrosothiols (RSNOs) that preserve, attenuate, and amplify the actions of NO. This selective and reversible process represents an important post-translational modification in cell signaling and regulation^{45, 115-117}. Several proteins have been identified as targets of cellular S-nitrosation including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), papain, hemoglobin (Hb), and several caspases^{114, 118-121}. Inflammatory stimuli, including RNS, increase overall cellular RSNO formation, which further mediates the inflammatory process^{1, 118, 122-125}.

As was summarized in the introduction, the direct detection and specific identification of S-nitrosated residues in proteins is essential to better understand RSNO-mediated signaling. Current techniques, such as the biotin switch assay, Saville assay, and chemiluminescence-based methods, are indirect methods of detection and may not exclusively label RSNOs^{85, 86, 90-92, 94}. Also, variability in these assays has challenged their accuracy and driven the search for alternative detection systems^{88, 126}. Deciphering the role and importance of RSNOs in biology requires direct and specific methods of quantification. We report a new strategy to covalently label biological RSNOs using the

water-soluble phosphine tris(4,6-dimethyl-3-sulfonatophenyl) phosphine trisodium salt hydrate (TXPTS) ¹²⁷.

Earlier work showed the reaction of trityl-S-nitrosothiol with triphenylphosphine yields an S-substituted aza-ylide (**1**, Scheme 6) and provided inspiration for developing an SNO-specific label ¹²⁸. Recently, Xian revealed that the treatment of various organic SNOs with derivatized triarylphosphines provides products arising from similar S-substituted aza-ylides ¹²⁹⁻¹³¹. In the presence of properly situated electrophiles on the triarylphosphine, the S-substituted aza-ylide intermediates undergo ligation to form stable



Scheme 6. The reactivity of triarylphosphines with organic and aqueous RSNOs

sulfenamides or disulfide iminophosphoranes ¹²⁹⁻¹³¹. Treatment of S-nitrosocysteine ester derivatives with various phosphines yields the corresponding dehydroalanines through intramolecular elimination via a similar S-substituted aza-ylide ¹²⁹. These results encouraged our use of the water-soluble phosphine TXPTS (Scheme 6) as a potential RSNO trap. We report the reactions of TXPTS with S-nitrosocysteine (Cys-SNO), S-

nitrosoglutathione (GSNO), and a mutated peroxiredoxin, S-nitrosated C165S alkyl hydroperoxide reductase (C165S AhpC-SNO) yield the covalent S-alkylphosphonium salt (**2**, Scheme 6).

2.2 Results and Discussion

S-Nitrosated protein thiols play important roles in cellular signaling as nitric oxide (NO) storage molecules and as reversible post-translationally modified species that alter protein structure and function^{1, 116, 132}. Despite their wide acceptance in biology, the chemical instability of S-nitrosothiols derived from primary thiolates, such as L-cysteine, limits their direct detection^{86, 90, 94}. A more complete understanding of RSNOs' biological effects requires improved detection methods that allow for a method for trapping and quantifying S-nitrosated protein intermediates and a means of identifying specific S-nitrosated cysteine residues.

Haake's observation that the reaction of trityl S-nitrothiol and triphenylphosphine gives an S-substituted aza-ylide (**1**, Scheme 6) reveals that organic S-nitrothiols can act in a similar manner as the organic azide in Staudinger-type reductions/ligations^{128, 133}. Based upon this work, we hypothesized that protein and other biologically relevant S-nitrosothiols could potentially be labeled as structurally unique S-substituted aza-ylides through their reaction with the water soluble triarylphosphine (TXPTS). Recent work by Xian shows that a variety of S-nitrosothiols react with triaryl and trialkylphosphines to give S-substituted aza-ylides, which undergo further reactions to yield a variety of different products depending on both RSNO and phosphine structure

¹³⁰, ¹³¹. In the presence of an electrophilic trap, these S-substituted aza-ylide intermediates ligate to generate sulfenamides or rearrange to disulfide iminophosphoranes ¹³⁰, ¹³¹. In the absence of an electrophilic trap, the intermediate S-substituted aza-ylide eliminates to form dehydroalanine-containing products ¹²⁹. This work reveals rich reaction chemistry between S-nitrosothiols and phosphines that may form the basis of new labeling methods, but in general, these processes have been limited to protected versions of cysteine and glutathione in organic or organic/buffer mixtures. Using a combination of NMR and MS-based techniques, we sought to critically evaluate the reaction between TXPTS and several physiologically relevant S-nitrosothiols. We report that the reaction between S-nitrosothiols of cysteine, glutathione and a mutant of the bacterial peroxiredoxin, AhpC with the water soluble triarylphosphine (TXPTS) yield a unique set of products that include TXPTS oxide (**3**), the stable aza-ylide of TXPTS (**4**) and an S-alkylphosphonium adduct (RS-P⁺ connection, **5-6**, Schemes 7 and 8).

The Reaction of Triphenylphosphine with Trityl S-Nitrosothiol

Treatment of trityl S-nitrosothiol with two equivalents of triphenylphosphine in benzene yields the S-substituted aza-ylide (**1**, 92% yield, Scheme 6), that corroborates previous results and provides the first X-ray crystallographic and ³¹P NMR characterization of this compound (Figure 15).

The Reaction of TXPTS and S-Nitrosoglutathione (GSNO)

The reaction of TXPTS and S-nitrosoglutathione (GSNO, a commercially available/readily prepared stable pink solid), allowed us to evaluate the formation of S-

substituted aza-ylides in water or buffer. Ultraviolet-visible spectroscopy shows the rapid loss of the S-nitrosothiol functional group in GSNO upon addition of TXPTS as evidenced by the decrease in absorbance at 545 nm (Figure 16). ^{31}P NMR

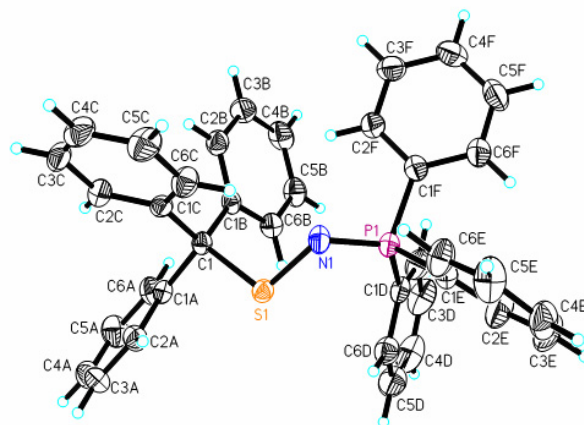


Figure 15. X-ray structure of **1** showing 50% probability ellipsoids; for crystallographic details see Appendix.

spectroscopy provides detailed information regarding this reaction. A mixture of products results when freshly prepared GSNO (1 equivalent) and TXPTS (2 equivalents) are combined in buffer (50 mM HEPES, 1mM DTPA, pH 7.1) in the dark. The peak for phosphine ($\delta = -28.8$ ppm) decreases with time and three new peaks at $\delta = 34.5$, 39.7 and 47.2 ppm emerge over 30 min (Figure 17). No other phosphorus-derived species are observed on this time scale and control experiments show that TXPTS does not react with GSH and only to a small extent (~3%) with GSSG to form the same product with the resonance at 47.2 ppm as judged by ^{31}P NMR (Figure 18). Comparison to known standards allows identification of the peak at 39.7 ppm as TXPTS oxide (**3**, Scheme 7)

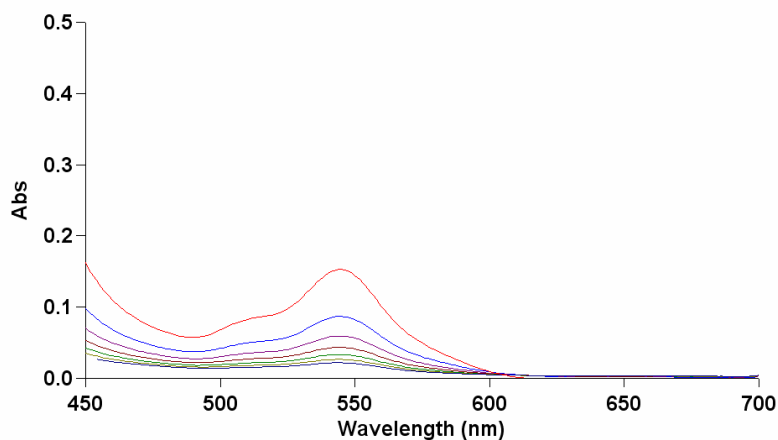
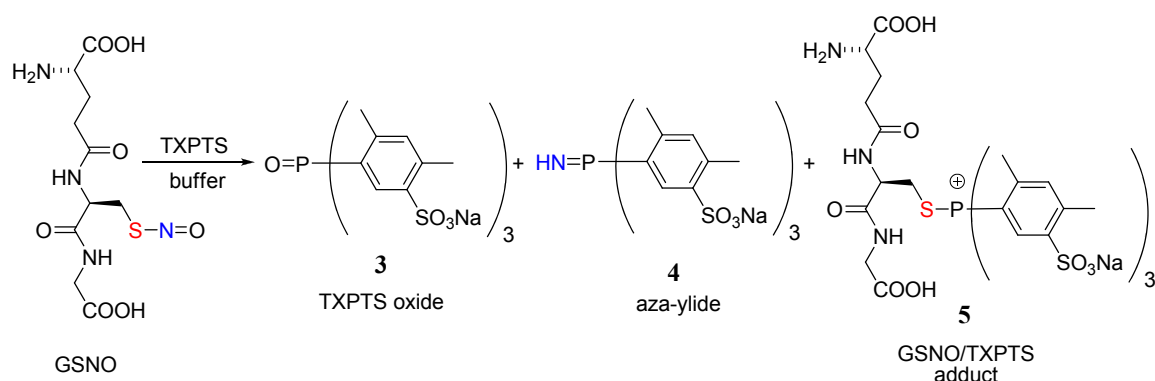


Figure 16. UV/Vis showing the decomposition of GSNO in the presence of TXPTS. GSNO (16 mM) and TXPTS (32 mM) were combined and monitored at 545 nm to observe the decomposition of the S-NO bond with 1 scan taken every 30 sec.



Scheme 7. The reaction of TXPTS with S-nitrosogluthathione at pH 7

and the peak at 34.5 ppm as the TXPTS-derived ylides (**4**, Scheme 7). Monitoring this reaction mixture over time shows the immediate formation of the species at 47.2 ppm, while **3** and **4** emerge more gradually over the course of 2 h. (Figure 17). Integration of the ^{31}P NMR spectrum after 1 h indicates the formation of these three products in roughly a 1:1:1 ratio, which gives an estimated yield of 67% for **5** (based on starting GSNO). The

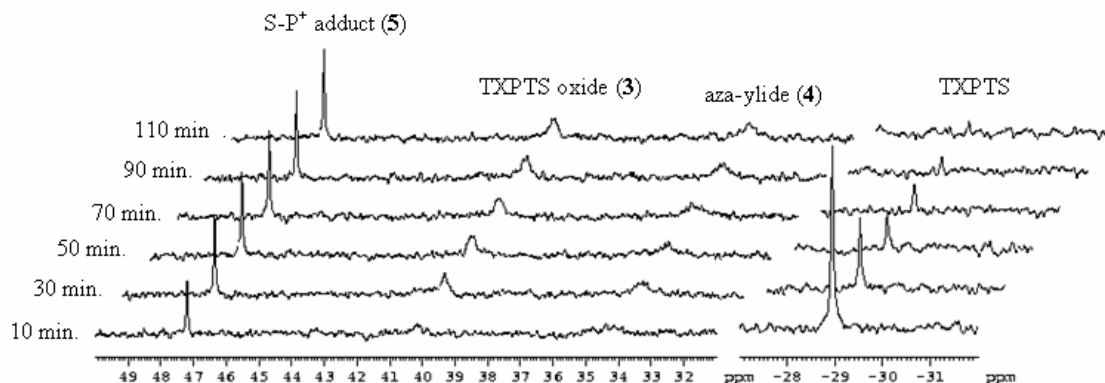


Figure 17. ^{31}P NMR of the GSNO and TXPTS reaction. ^{15}N -GSNO (1 eq.) and TXPTS (2 eq.) were incubated in a HEPES/DTPA buffered solution (pH 7.1) with 10 % D_2O and the reaction was monitored using ^{31}P NMR with 1 scan every 20 min. The peaks at 47.2, 39.7, and 34.5 ppm correspond to **5**, **3**, and **4**, respectively and TXPTS starting material at -28.8 ppm. All peaks were referenced to a 3% H_3PO_4 ($\delta = 0$ ppm) external standard which was omitted for clarity.

use of ^{15}N -labeled GSNO results in an apparent coupling ($^1J_{\text{N-P}} = 23$ Hz) in the peak at 34.5 ppm, which corresponds to **4**, but not in any of the other peaks. This observed splitting varies over time suggesting some dynamic changes of **4** under these conditions (possibly water addition to the ylide). Treatment of this reaction mixture with dithiothreitol regenerates TXPTS ($\delta = -28.8$ ppm) at the expense of the species at 47.2 ppm with no change in the peaks that correspond to **3** and **4** (Figure 19). The addition of a sodium hydroxide solution to the reaction mixture results in the conversion of the peaks at 34.5 ppm and 47.2 ppm to the phosphine oxide (**3**, 39.7 ppm). The ^{13}C and ^1H NMR spectra of this reaction show complicated mixtures that do not allow for the clear identification of the phosphorus-containing species responsible for the resonance at 47.5

ppm or definitive estimates of the total product yields. Gas chromatographic headspace analysis and chemiluminescence nitric oxide detection reveal that no nitrous oxide (evidence for HNO) and only a trace amount of nitric oxide form during the reaction of TXPTS and GSNO.

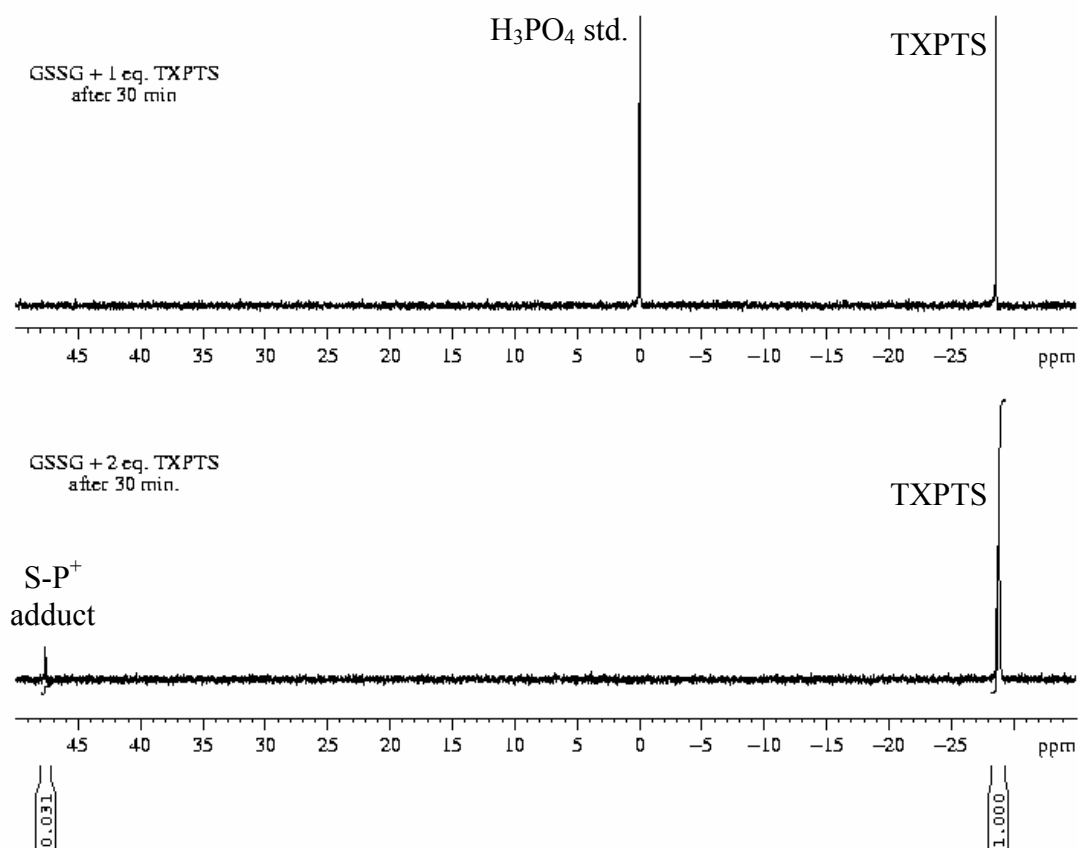


Figure 18. ^{31}P NMR of the reaction between GSSG (20 mM) and TXPTS. (**top**) with 20 mM TXPTS (**bottom**) with 40 mM TXPTS (2 eq.) roughly 3% reacts to form 5. Both spectra were taken after 30 min.

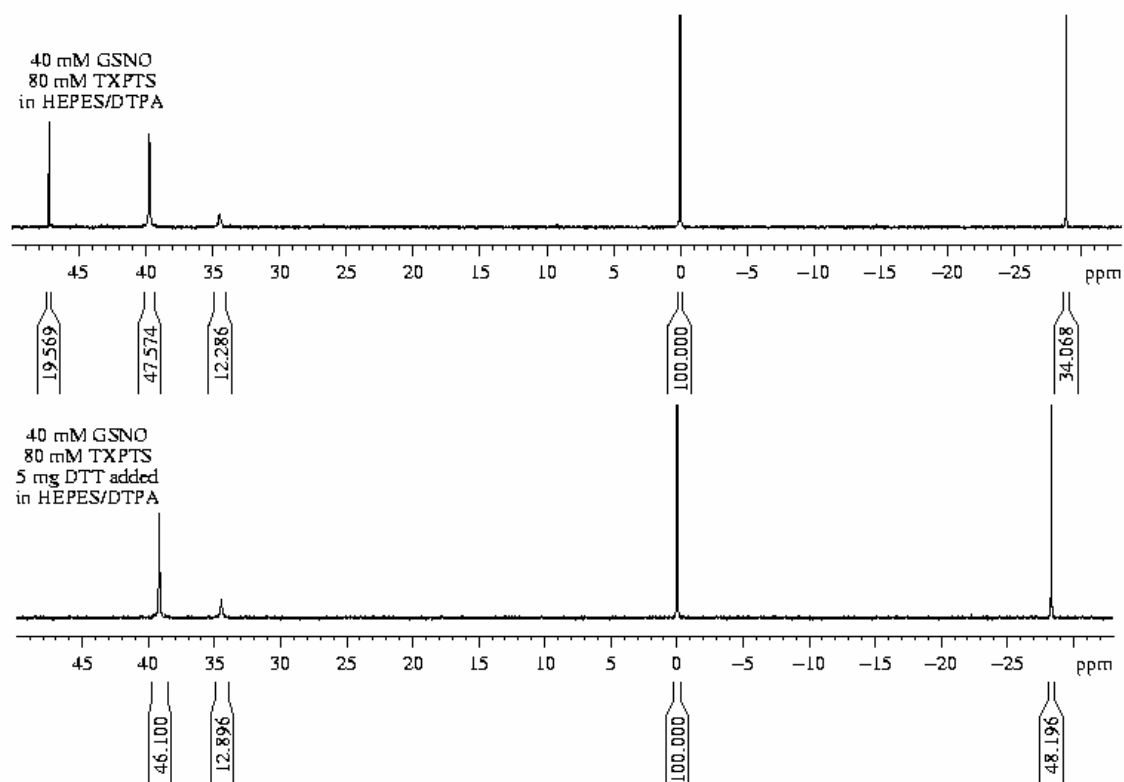


Figure 19. ^{31}P NMR showing the treatment of the GSNO/TXPTS mixture with DTT. **(Top)** ^{31}P NMR of the GSNO/TXPTS reaction mixture showing TXPTS (-28.8 ppm), ylide (34.5 ppm), oxide (39.7 ppm), S-P⁺ adduct (47.2 ppm), and the external H₃PO₄ standard (0 ppm). **(Bottom)** The addition of DTT to this mixture completely reduces the S-P⁺ adduct, regenerating TXPTS starting material as seen by the changes in integration.

Liquid chromatography-mass spectrometry (LC-MS) further confirms the identity of these products and provides insight into the structure of the compound responsible for the peak at 47.2 ppm in the ^{31}P NMR spectrum. Analysis of the GSNO/TXPTS reaction mixture validates the formation of **3** and **4** given their comparison to known standards

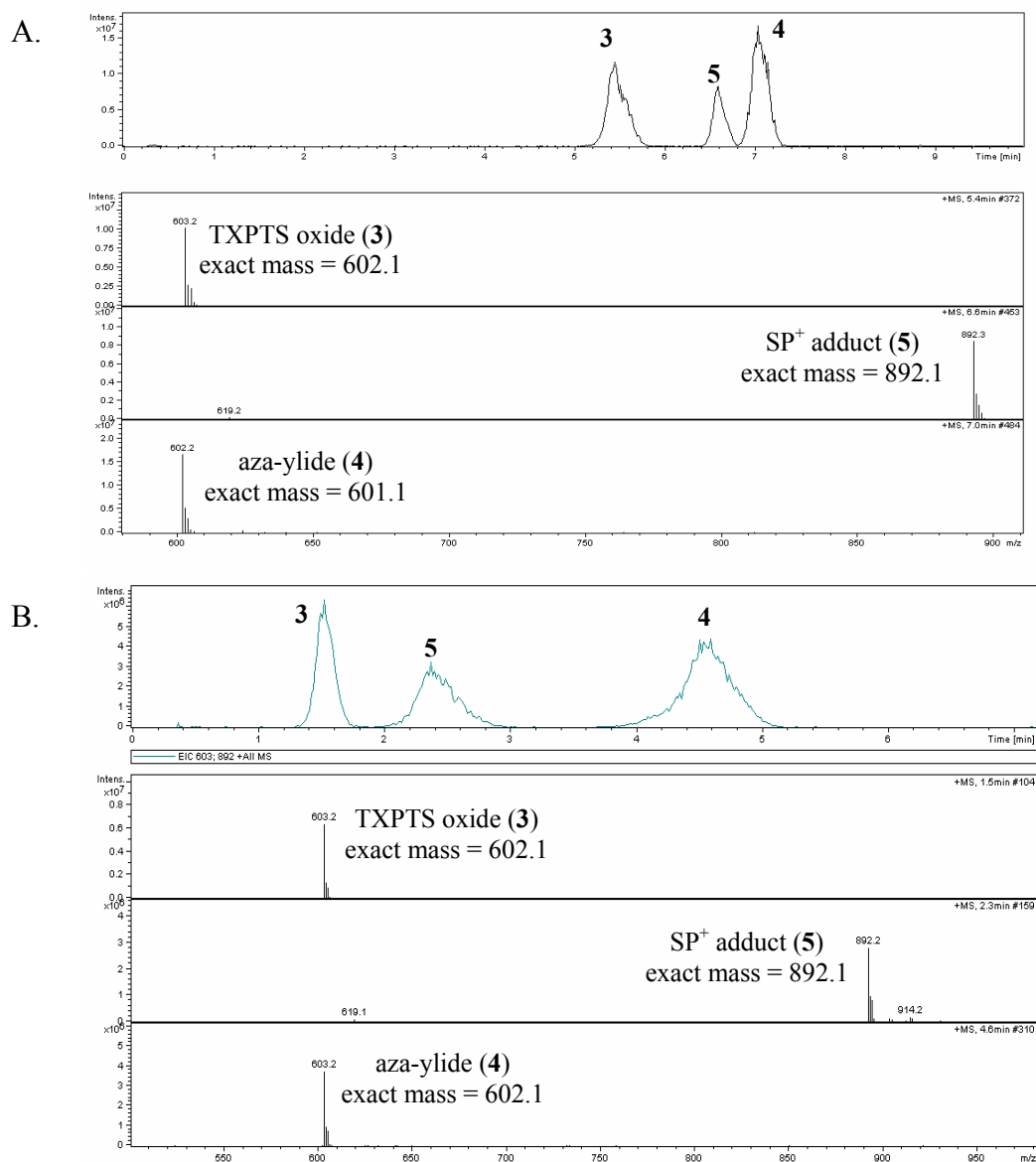


Figure 20. (A) LC-MS analysis of the reaction between ^{14}N -GSNO and TXPTS. LC retention times and observed masses are as follows: TXPTS oxide (3) = 5.4 min ($M + 1$) $m/z = 603.2$; SP⁺ adduct (5) = 6.5 min (M^+) $m/z = 892.3$; aza-ylide (4) = 7.1 min ($M + 1$) $m/z = 602.2$. All observed m/z are in agreement with the reported exact masses for each compound. (B) LC-MS for the ^{15}N -GSNO/TXPTS reaction. LC retention times are identical as well as observed masses, except aza-ylide (4) which has an $m/z = 603.2$ due to the use of ^{15}N -GSNO-derived ylide formation.

(Figure 20A). The observed m/z of the final compound (892.1 m/z) suggests an adduct of GSNO and TXPTS with the loss of NO as opposed to the initially predicted $-S-N=PR_3$ motif leading to the proposal of an S-alkylphosphonium product (**5**, Scheme 6)¹²⁸⁻¹³¹. The use of ^{15}N -labeled GSNO results in the formation of ^{15}N -labeled **4** indicating the transfer of the $-S-N=O$ nitrogen atom to the phosphorus atom of TXPTS (Figure 20B). Similar LC-MS experiments do not support the formation of GSH or GSSG during this reaction.

A series of 2-D NMR experiments confirm the proposed $S-P^+$ bonding motif of **5**. A ^1H - ^{31}P COSY indicates that the ^{31}P signal of **5** (47.2 ppm) correlates to three protons in the ^1H NMR spectrum (3.85, 3.42, and 3.01 ppm, presumably the $-\text{CH}$ and diastereotopic $-\text{CH}_2$ of the cysteine side chain of **5**), in the reaction mixture (Figure 21A). Using this

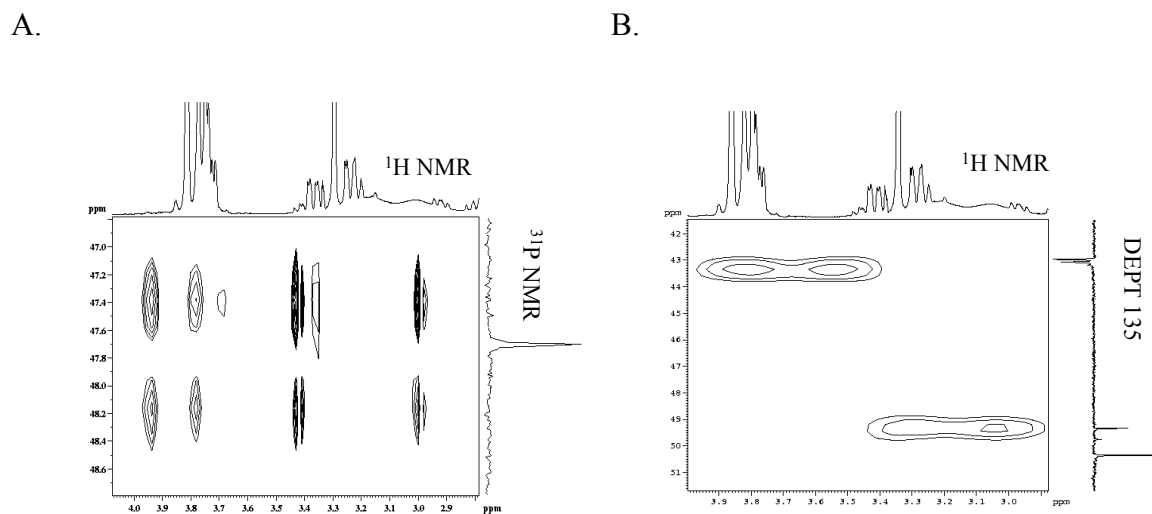
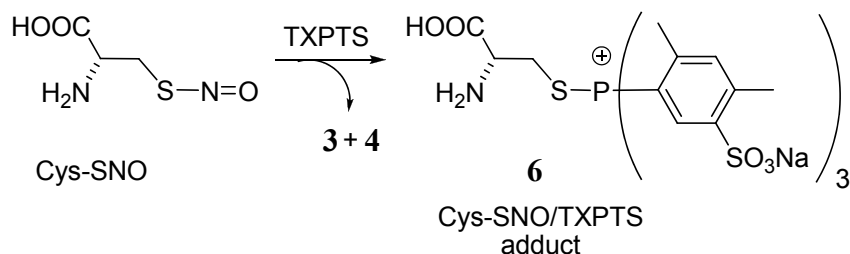


Figure 21. 2-D correlation spectroscopy for the GSNO/TXPTS reaction. **(A)** ^1H - ^{31}P COSY of the GSNO/TXPTS reaction mixture in D_2O . **(B)** ^1H - ^{13}C HMQC of the GSNO/TXPTS reaction mixture in D_2O

obtained ^1H correlation, the ^1H - ^{13}C HMQC of the same reaction mixture reveals the proximity of the P atom of the S-P^+ moiety to nearby $-\text{CH}_2$ and $-\text{CH}$ carbons. Coupling constant analysis shows splitting of the $-\text{CH}_2$ carbon ($^2J_{\text{P-C}} = 14.6$ Hz, Figure 21B), providing evidence for attachment of the phosphine through an S-P^+ linkage.

The Reaction of TXPTS and S-nitroso-L-cysteine (Cys-SNO)

Similar ^{31}P NMR experiments show the formation of three phosphorus-derived products from the mixture of freshly prepared Cys-SNO (1 equivalent) with TXPTS (2 equivalents) in buffer (50 mM HEPES, 1 mM DTPA, pH 7.1) in the dark that correspond to TXPTS oxide (**3**, $\delta = 39.7$ ppm) and the TXPTS-derived ylide (**4**, $\delta = 34.5$ ppm) and a presumed cysteine-derived S-alkylphosphonium adduct, (**6**, Scheme 8, $\delta = 46.1$ ppm) after 30 min (Figure 22). No other phosphorus-derived species were observed and control experiments show that TXPTS does not react with cysteine but partially reacts with cystine to form the same product with a resonance at 46.1 ppm (~15%, Figure 23). Integration of the ^{31}P NMR spectrum after 1 h indicates the formation of compounds **3:4:6** in roughly a 3:1:1 ratio which suggests a more rapid hydrolysis of **6** compared to **5**.



Scheme 8. Reaction of TXPTS with S-nitrosocysteine at pH 7

Analogous to the GSNO reaction, treatment of this mixture with dithiothreitol regenerates TXPTS ($\delta = -28.8$ ppm) at the expense of the species at 46.1 ppm with no change in the peaks that correspond to **3** and **4** (data not shown). LC-MS analysis of the Cys-SNO/TXPTS mixture also confirms the presence of **3** and **4** as well as the expected mass for the S-P⁺ adduct **6** (Figure 24).

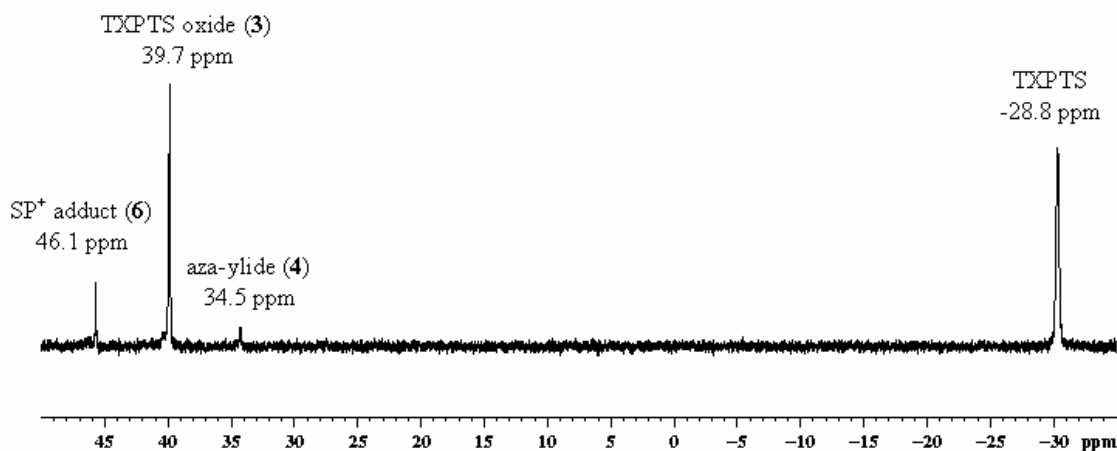


Figure 22. ³¹P NMR of the Cys-SNO and TXPTS reaction. Cys-SNO (1 eq.) and TXPTS (2 eq.) were incubated in a HEPES/DTPA buffered solution (pH 7.1) with 10 % D₂O. The peaks at 46.1, 39.7, and 34.5 ppm correspond to **6**, **3**, and **4**, respectively and TXPTS starting material at -28.8 ppm.

The commercial availability of (3-¹³C) Cys provides the opportunity to further confirm the structure of the S-P⁺ adduct (**6**) in solution. Treatment of (3-¹³C) Cys-SNO with TXPTS followed by ³¹P NMR spectroscopy showed through-bond coupling of the ³¹P atom to the ¹³C with an observed ²J_{C-P} coupling = 3.7 Hz, which confirms the connectivity and covalent nature of the S-alkylphosphonium adduct (**6**, Figure 25). Importantly, LC-MS analysis revealed a 1 amu increase in mass for the S-P⁺ species (**6**),

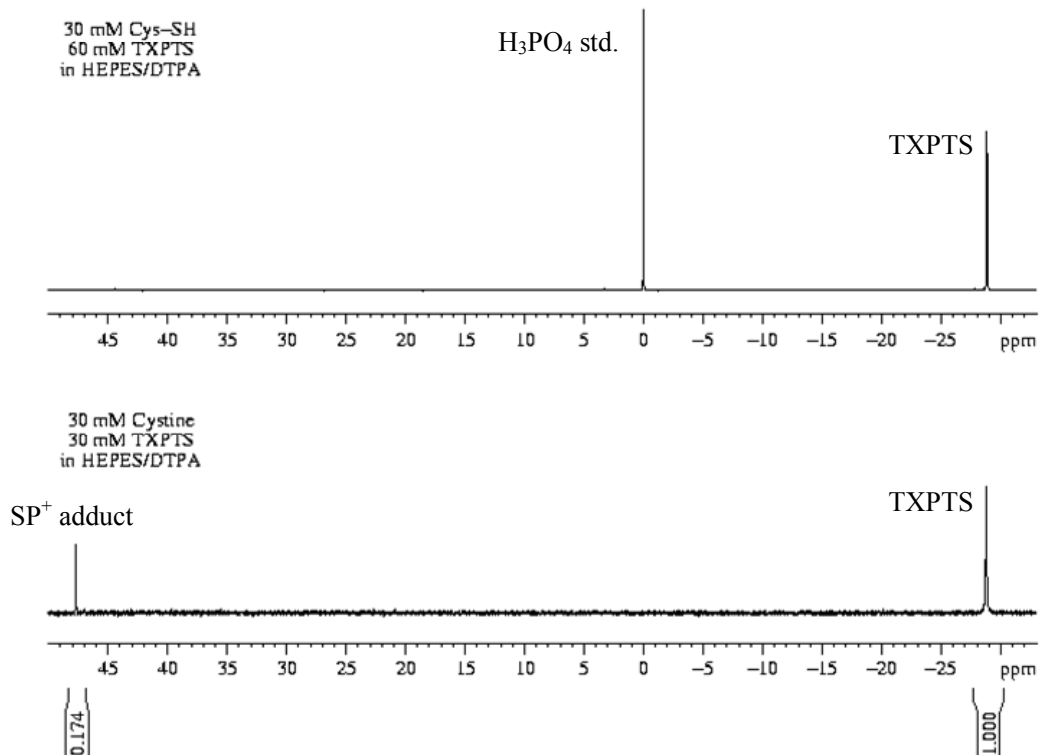


Figure 23. ^{31}P NMR of cysteine and cystine with TXPTS. **(Top)** ^{31}P NMR of a reaction mixture containing Cys-SH (30 mM) and TXPTS (60 mM) in HEPES buffer (100 mM). Only TXPTS (-28.8 ppm) and the external H₃PO₄ standard (0 ppm) are observed. **(Bottom)** ^{31}P NMR of a reaction mixture containing Cys-S-S-Cys (30 mM) and TXPTS (30 mM) in HEPES buffer (100 mM). Based on integration, roughly 15% of the TXPTS reacts to form the SP⁺ adduct (46 ppm).

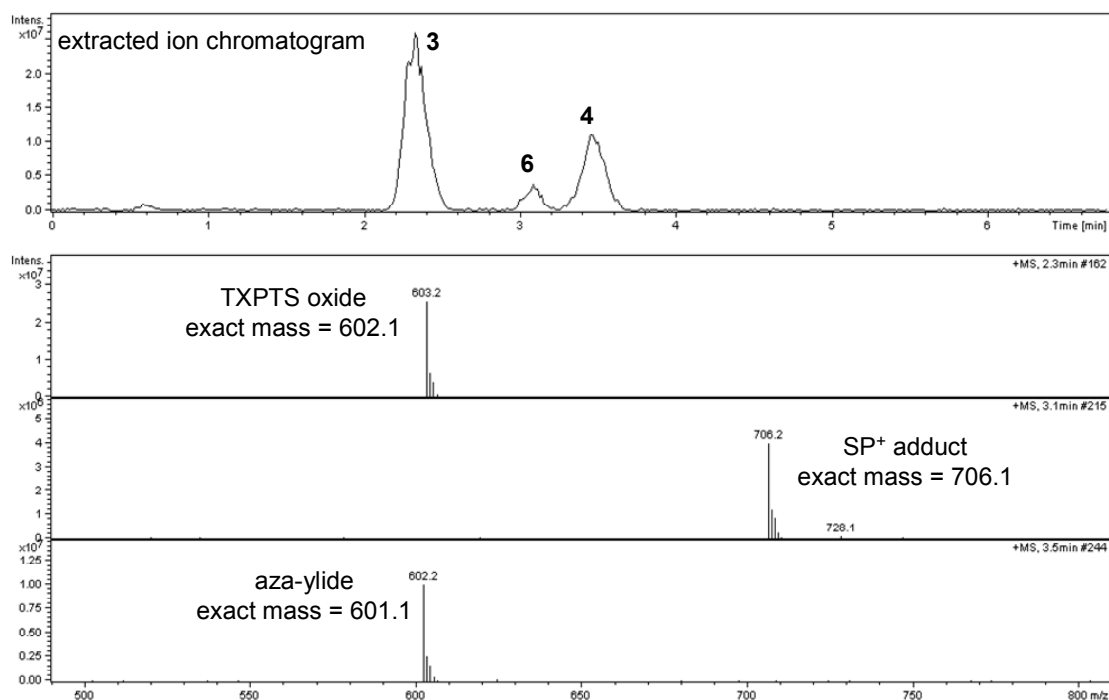


Figure 24. LC-MS for the (¹²C)-Cys-SNO + TXPTS reaction. TXPTS oxide (**3**) = 2.4 min ($M + 1$) m/z = 603.2; SP⁺ adduct (**6**) = 3.1 min (M^+) m/z = 706.2; aza-ylide (**4**) = 3.5 min ($M + 1$) m/z = 602.2. All observed m/z are in agreement with the reported exact masses for each compound.

further validating the covalent attachment of TXPTS in the adduct (data not shown). The ¹³C NMR spectrum of the reaction between TXPTS and (3-¹³C) Cys-SNO shows four peaks at 42.1, 42.4, 60.8 and 62.7 ppm (Figure 26). The resonance at 42.1 ppm is a doublet (² J_{P-C} = 8.6 Hz) indicating coupling and further corroborating the ³¹P NMR results. Comparison to standards reveals that the peak at 60.8 ppm corresponds to the methylene carbon of L-serine. The other two resonances (42.4 and 62.7 ppm) currently remain unidentified but appear during the preparation of Cys-SNO. This spectrum also indicates the absence of cysteine, cystine and dehydroalanine (no resonances between

100-150 ppm) in the reaction mixture, in contrast to recent work that shows dehydroalanine formation in the reaction of Cys-SNO derivatives with phosphines¹²⁹.

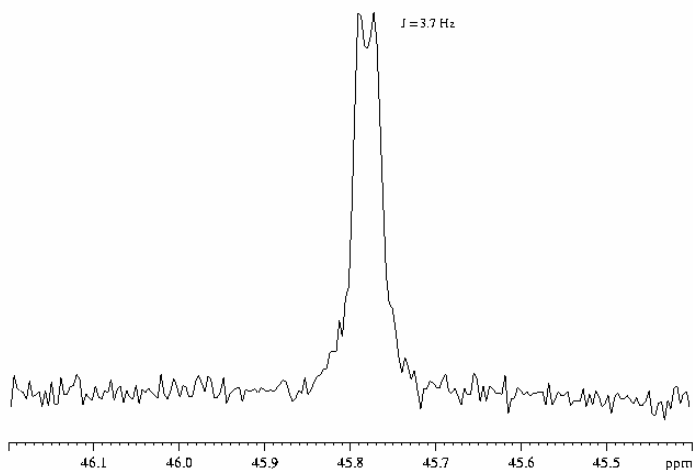


Figure 25. ³¹P NMR for the (¹³C)-Cys-SNO/TXPTS reaction mixture. The experiment was run in HEPES buffer with 10% D₂O. Shown above is the 203 MHz field ³¹P NMR zoomed in showing **6** from the reaction mixture (²J_{P-C} = 3.7 Hz).

While providing strong evidence of S-alkylphosphonium ion formation, the identification of other non-phosphorus containing products (by NMR) and our inability to completely account for the GSNO and Cys-SNO reveal the complexity of these reactions. Both NMR and LC-MS examination of these reaction mixtures do not show the presence of disulfides (GSSG/cystine), the glutathione-derived sulfenamide (GSNH₂) and only small amounts of thiol (GSH/cysteine), eliminating these likely products. The identification of serine from the reaction of Cys-SNO, which may form from the hydrolysis of **6**, demonstrates the complexity of these reactions. These results indicate

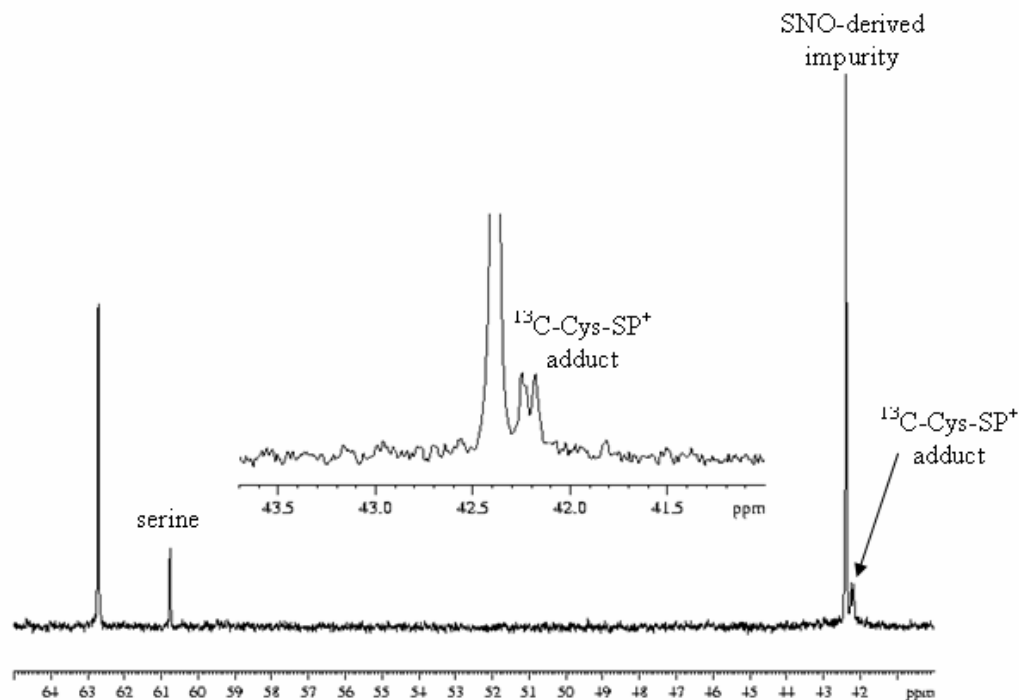


Figure 26. ^{13}C NMR for the $(^{13}\text{C})\text{-Cys-SNO/TXPTS}$ reaction mixture. The peak at 42.2 ppm corresponds to **6** with a weak 2J coupling of 8.6 Hz. The peak at 42.4 ppm emerges during the Cys-SNO preparation, 60.8 ppm corresponds to the serine CH_2 , and the identity of the 62.7 ppm peak is currently unknown.

the likelihood of other processes during S-alkylphosphonium ion formation and breakdown that may include reactions of sulfenamide intermediates, other rearrangements/dehydrations of intermediate aza-ylides and various reactions of the S-alkylphosphonium ions (**5** and **6**).

Literature values for S-alkylphosphonium ion ^{31}P NMR shifts are roughly 40-50 ppm, consistent with the observed resonances for **5** and **6**¹³⁴⁻¹³⁶. These SP^+ ions also represent known intermediates in phosphine-mediated disulfide reductions. In general, phosphines, including tris(2-carboxyethyl)phosphine (TCEP), the reagent of choice for

protein disulfide reduction, rapidly reduce disulfides to the S-alkylphosphonium intermediate that hydrolyzes to give free thiol and phosphine oxide¹³⁷⁻¹⁴⁰. Control experiments show that TXPTS reacts with GSSG and cystine to a limited extent to yield the S-P⁺ intermediates (**5** and **6**). The unique structure of TXPTS appears to slow the hydrolysis of these SP⁺ species, allowing them to be isolated. Compounds **5** and **6** react with thiols to yield the phosphine and hydrolyze in base to give the phosphine oxide, consistent with known reactivity of S-alkylphosphonium ions.

The Reaction of TXPTS with AhpC-SNO

Given the reproducible formation of S-alkylthiophosphonium adducts from the reactions of TXPTS with GSNO and Cys-SNO, we examined the reaction of the S-nitrosated 2-Cys peroxiredoxin mutant, C165S alkyl hydroperoxide reductase C (AhpC-SNO) with TXPTS. As C165S AhpC forms a relatively stable sulfenic acid at the remaining peroxidatic cysteine (Cys-46) this mutant was an ideal candidate for examining the interaction of C165S AhpC-SNO with TXPTS¹⁴¹. Incubation of freshly prepared AhpC-SNO with 25-fold excess TXPTS in buffer (50 mM HEPES, 1 mM DTPA) at 25°C forms an S-alkylphosphonium (SP⁺-type) adduct over several hours (Figure 27) as shown by electrospray ionization-time of flight (ESI-TOF) MS experiments. As seen in Figure 27, both AhpC-SNO (exact mass = 20629.38) and the S-thiolation product with cysteine (AhpC-S-S-Cys, exact mass = 20719.11) form upon mixture of AhpC (reduced) and Cys-SNO. Upon incubation of this sample with TXPTS, a time-dependent decrease of AhpC-SNO occurs with the emergence of the corresponding AhpC-SP⁺ adduct (exact mass = 21184.69). Based on the m/z difference

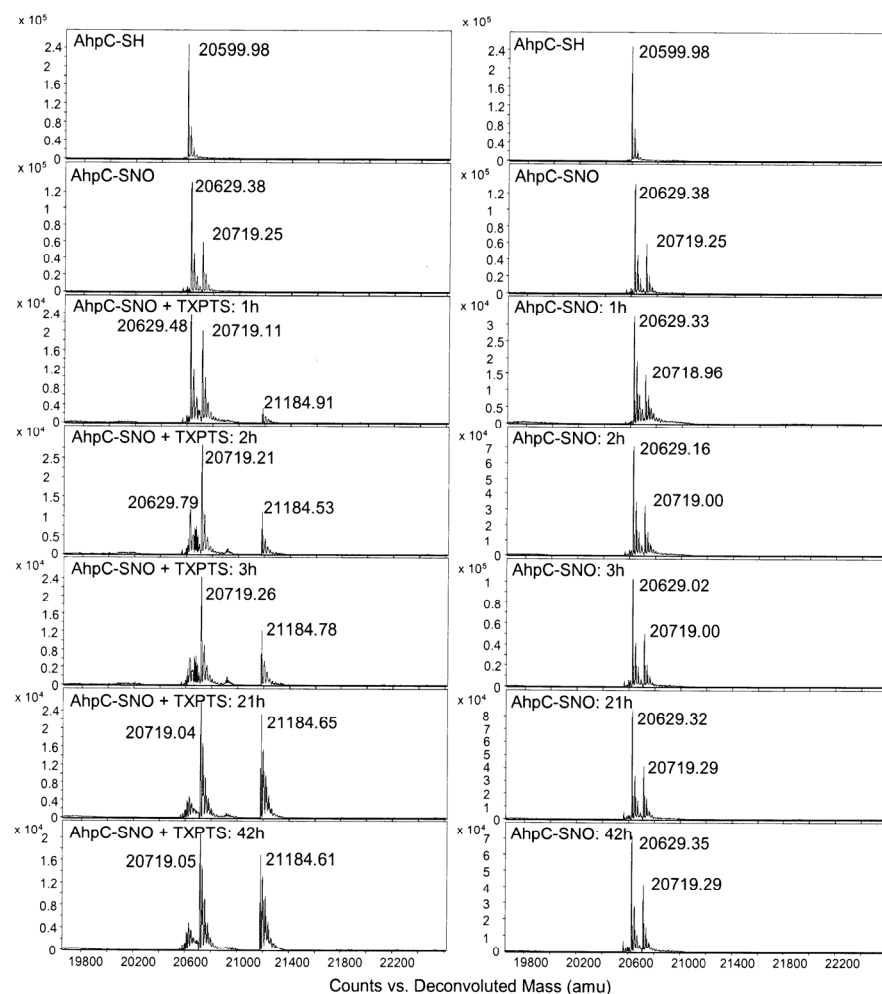


Figure 27. ESI-TOF-MS data showing TXPTS covalently labeling the S-nitrosated mutated peroxiredoxin (C165S AhpC-SNO). The exact mass of the covalent adduct is 21184.69, MW of the AhpC-SNO is 20629.55. The MW 20719.14 corresponds to the mixed disulfide that forms between AhpC-SNO and Cys-SNO (AhpC-S-S-Cys). **(left)** C165S AhpC-SNO with 25-fold excess TXPTS over 42 hours **(right)** control showing C165S AhpC-SNO in the absence of TXPTS under the same conditions

of 555.1 amu and the lack of reaction between AhpC (reduced) and TXPTS, the phosphine (exact mass = 652 as the tri-sodium salt) appears to be covalently bound to the protein in an S-P⁺ fashion. Direct treatment of the free thiol form of C165S AhpC (AhpC-SH) with TXPTS does not form this product by ESI-TOF MS (data not shown). Figure 27 also indicates that TXPTS does not react with the AhpC-S-S-Cys mixed disulfide over 42 h. The AhpC-derived S-P⁺ species demonstrates excellent stability over several days at room temperature and under denaturing conditions (40% acetonitrile, Figure 28).

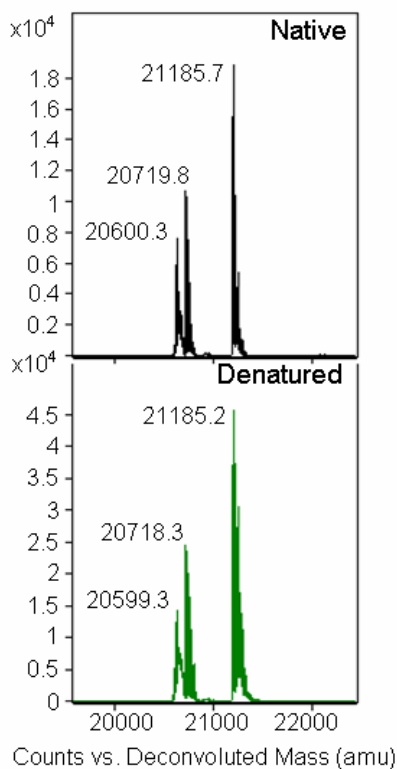


Figure 28. A comparison of SNO-labeled AhpC under normal and denaturing (40% acetonitrile) conditions. Based on the above ESI-TOF MS data, no decomposition is observed.

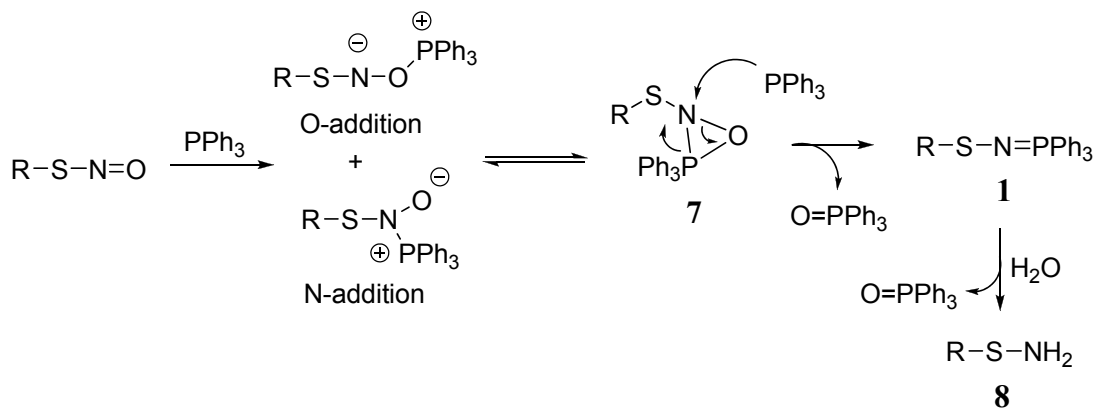
To the best of our knowledge, these results describe the first covalent labeling of an S-nitrosated protein in buffer and demonstrate the potential of phosphines as protein SNO labels. While TXPTS rapidly reacts with small molecule RSNOs, Figure 27 shows that the reaction with AhpC-SNO occurs much more slowly. Steric differences in the environment of the –SNO group likely dictate the rates of these reactions and thus rates may vary among proteins with different thiol accessibility, and the cysteine of AhpC is known to react relatively sluggishly with alkylating agents¹⁴². Evaluation of the rate and selectivity of this reaction with protein SNOs, particularly oxidized sulfur species, is warranted and will be required for the development of this reagent as a labeling tool.

2.3 Evaluating the Mechanism of SNO-Derived Adduct Formation.

Scheme 9 depicts a proposed mechanism for the formation of S-substituted azaylides from an S-nitrosothiol with a triarylphosphine. This reaction could yield products through phosphorous-addition to either nitrogen or oxygen, and both could exist as a three-membered ring (**7**, Scheme 9)^{128, 131}. Addition of a second phosphine to **7** (or one of the initial addition products) would give the phosphine oxide and S-substituted azaylide in equal proportions (as observed for **1**, Scheme 6)¹²⁸.

The described spectroscopic and LC-MS studies show that the reaction of GSNO or Cys-SNO with TXPTS in buffer rapidly consumes the S-nitrosothiol. While ³¹P NMR provides evidence of TXPTS phosphine oxide (**3**) formation as expected, ³¹P NMR chemical shift comparison to **1**, the lack of observed splitting in the ³¹P NMR spectrum in the reaction using ¹⁵N-GSNO and LC-MS work do not support formation of the expected

S-substituted aza-ylide. Hydrolysis of any S-substituted aza-ylide product to the corresponding sulfenamide (RSNH_2 , **8**, Scheme 9) and **3** would explain the absence of

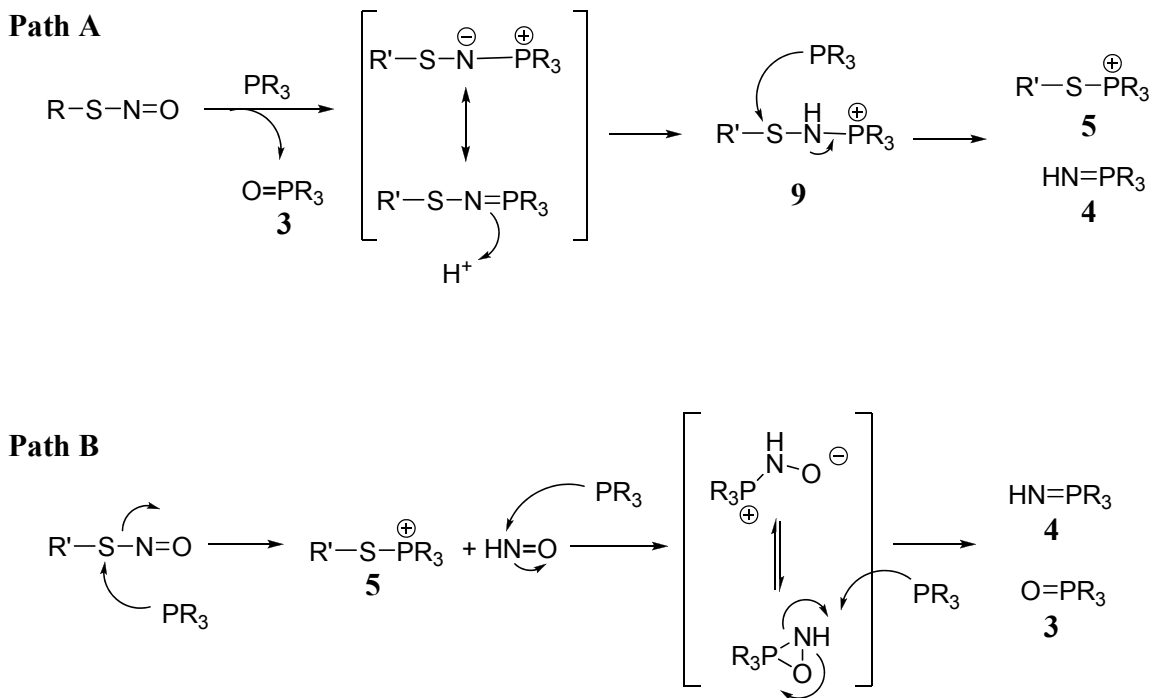


Scheme 9. Proposed mechanism for the formation of the S-N=P type aza-ylide (**1**)

the S-substituted aza-ylide, but the ^{31}P NMR identification of two other phosphorus-containing species clearly indicates a different mechanism; direct formation of the S-substituted aza-ylide followed by hydrolysis would produce phosphine oxide (**3**) as the only phosphorus containing product. This work identifies the two other phosphorus-containing products as the aza-ylide (**4**) and the corresponding S-alkylphosphonium salts (**5-6**, Schemes 7 and 8). Aza-ylide (**4**), which has been characterized during the reaction of TXPTS and nitroxyl (HNO), demonstrates considerable water stability, presumably due to stabilization of the phosphonium ion by the electron-donating ortho and para methyl groups of TXPTS¹⁴³. The reaction of TXPTS with ^{15}N -labeled GSNO results in ^{15}N incorporation in **4** and reveals the formal 6 e^- reduction of the S-nitrosothiol nitrogen atom to ammonia (consistent with the absence of NO/HNO formation). This reaction

finds some precedence in the GSNO reductase (aka alcohol dehydrogenase) mediated reduction of GSNO to ammonia¹⁴⁴.

Scheme 10 shows two potential mechanisms that account for the observed phosphorus-containing products. Path A proceeds through a mechanism that includes an S-substituted aza-ylide ($-S-N=P$), presumably formed through a mechanism as seen in Scheme 9. In aqueous conditions, protonation of this S-substituted aza-ylide gives a new intermediate (**9**) that rapidly reacts with another equivalent of phosphine to simultaneously yield equal amounts of the S-alkylphosphonium ion (**5** or **6**) and the aza-ylide (**4**). This mechanism utilizes 3 equivalents of phosphine and predicts a 1:1:1 ratio



Scheme 10. Two proposed mechanisms for the formation of an SNO-derived adduct from TXPTS

of **3:4:5**, as experimentally observed for the GSNO reaction. While 2 equivalents of phosphine were used in these experiments, the inherent instability of GSNO provided for excess phosphine during the reaction. The lack of experimental evidence of an S-substituted aza-ylide (**1**) and previous work showing that TXPTS reacts with HNO to form equal amounts of **3** and **4** suggest a more direct mechanism for observed product formation¹⁴³. Path B (Scheme 10) depicts the direct nucleophilic attack of TXPTS on the sulfur atom of an S-nitrosothiol to form the S-alkylphosphonium ion (**5**, for GSNO) with release of HNO. The reaction of 2 equivalents of TXPTS with nascent HNO would yield **3** and **4**. This mechanism also requires three equivalents of phosphine and predicts a 1:1:1 ratio of **3:4:5**. The ³¹P NMR time-course experiment (Figure 17) supports Path B with the rapid initial formation of **5** followed by the emergence of **3** and **4** while Path A would initially produce **3** followed by **4** and **5**. Given the rapid reaction of TXPTS and HNO, the failure to detect N₂O (the HNO dimerization/dehydration product) does not eliminate Path B. While both mechanisms account for the observed products and ratios, further experiments, particularly a detailed kinetic analysis of the constituent reactions of this sequence, are required to delineate the mechanisms.

2.4 Conclusion

In summary, the triarylphosphine TXPTS reacts directly with S-nitrosothiol residues to produce stable S-alkylphosphonium ions (**5-6**, or AhpC-SP⁺) as well as TXPTS oxide (**3**) and the TXPTS-derived aza-ylide (**4**). This work details the first reaction of triarylphosphines with RSNOs in water. Mechanistically, this reaction may

proceed through an S-substituted aza-ylide or through the direct displacement of HNO from the RSNO group. This reaction, which is amenable to MS detection and ^{31}P NMR spectroscopy, provides a new method for identifying S-nitrosated species in solution. This unique reactivity suggests that phosphines may be useful tools for understanding the complex physiological roles of biological S-nitrosation and its implications in cell signaling and homeostasis.

2.5 Materials and Methods

Tris(4,6-dimethyl-3-sulfonatophenyl)phosphine trisodium salt hydrate (TXPTS) was purchased from Strem Chemicals. Glutathione (reduced and oxidized), L-cysteine, L-cystine, 1,4-dithiothreitol, sodium nitrite, sodium hydroxide, triphenylphosphine, triphenylmethane thiol, butyl nitrite, hydrogen peroxide solution (10 M), deuterium oxide, and deuterated chloroform were purchased from Sigma Aldrich. Hydrochloric acid solution (1 N) was purchased from TCI. (3- ^{13}C) L-Cysteine and ^{15}N -sodium nitrite were purchased from Cambridge Isotope Laboratory. All reagents were used directly from suppliers without further purification.

Preparation of S-substituted aza-ylide (1).

A solution of trityl thionitrite in CHCl_3 (0.1905 g, 0.62 mmol, (λ_{max} , (CHCl_3) 335, 545 nm)¹⁴⁵ was added to a solution of triphenylphosphine (0.33 g, 1.25 mmol) in anhydrous benzene (5 mL) at room temperature. After 30 minutes, the solids were

filtered to give **1** as a bright yellow solid ¹²⁸. Recrystallization in CH₃CN/toluene afforded **1** as bright yellow crystals. ³¹P NMR (121 MHz, CDCl₃) δ 17.3.

Preparation of S-nitrosocysteine (Cys-SNO)

A solution of L-cysteine (0.06 g, 0.34 mmol) in 0.75 N HCl (4 mL) with 15 mM EDTA was added to a solution of sodium nitrite (0.035g, 0.5 mmol) in H₂O (5 mL) with 0.5 mM EDTA at 4°C. After 10 minutes, the reaction turned deep red and was neutralized with 1 N NaOH (2.5 mL) to afford a solution of L-Cys-SNO (H₂O, $\epsilon_{335\text{nm}}$ 503 M⁻¹cm⁻¹). (3-¹³C) L-Cys-SNO was made using the same procedure. (¹⁵N)-S-Nitrosocysteine was prepared using ¹⁵N-sodium nitrite.

*Preparation of S-nitrosoglutathione (GSNO)*⁵⁰

Sodium nitrite (0.345 g, 5 mmol) was added to an ice-cold solution of glutathione (1.53 g, 5 mmol) in 2N HCl (5 mL). After 40 minutes at 4°C, the red solution was treated with acetone (10 mL) and stirred for an additional 10 minutes. The resulting pink solid was filtered off and washed successively with ice-cold water (5 mL), acetone (3 mL), and ether (3 mL) to afford S-nitrosoglutathione (1.29 g, 76%) (H₂O, $\epsilon_{335\text{nm}}$ 922 M⁻¹cm⁻¹, $\epsilon_{545\text{nm}}$ 15.9 M⁻¹cm⁻¹). (¹⁵N)-S-Nitrosoglutathione was prepared using ¹⁵N-sodium nitrite.

Preparation of S-nitroso (C165S) AhpC

The mutant form of *Salmonella typhimurium* AhpC, C165S, was overexpressed and purified from *Escherichia coli* as previously described.^{142, 146} A solution of reduced

protein (0.5 mg/mL, 500 μ L) in HEPES (50 mM), DTPA (1 mM) buffer (pH 7.1) was incubated with freshly prepared S-nitrosocysteine (1 mM) at 25°C for 60 min in the dark. Removal of S-nitrosocysteine was performed using BioRadTM spin columns with Bio-Gel P6TM equilibrated in HEPES (50 mM), DTPA (1 mM) buffer (pH 7.1). The formation of C165S AhpC-SNO (as well as a lesser amount of C165S AhpC/Cys mixed disulfide) was monitored by ESI-TOF MS as described below.

Labeling of AhpC-SNO using TXPTS

A solution of TXPTS (2 μ L, 250 mM) was added to a solution of freshly prepared AhpC-SNO (200 μ L, 100 μ M) in HEPES (50 mM), DTPA (1 mM) buffer. Aliquots (50 μ L) were taken at different time points and diluted with ammonium bicarbonate buffer (50 μ L, 50 mM, pH 7.5), then TXPTS was removed using BioRad spin columns packed with Bio-Gel P6 (BioRad) and samples were equilibrated with ammonium bicarbonate buffer (50 mM, pH 7.5). The samples were analyzed by ESI-TOF MS as described below.

NMR Analysis

1-D Analyses: ¹H NMR spectra were recorded on Bruker Avance DPX-300 and DRX-500 instruments at 300.13 and 500.13 MHz, respectively. ¹³C and DEPT NMR spectra were recorded on a Bruker DRX-500 instrument operating at 125.76 MHz. ³¹P NMR spectra were recorded on Bruker DPX-300 and DRX-500 instruments operating at 121.49 MHz and 202.46 MHz respectively and ³¹P chemical shifts are relative to 3% H₃PO₄ (δ = 0 ppm) contained in a concentric internal capillary (Wilmad).

2-D Analyses: The ^1H - ^{31}P gradient selected COSY spectrum was acquired with 2048 complex points in t2, 256 points in t1 and 40 transients with a pulse repetition delay of 2 s. A sweep width of 10 ppm in ^1H (centered at 4.5 ppm) and 100 ppm in ^{31}P (centered at 10 ppm) was used. Data sets were multiplied with 90° phase shifted squared-sinebell apodization function and zero-filled to 512 x 512 data points before Fourier transformation. Chemical shifts were referenced to the residual H_2O signal and the observed SP^+ chemical shift (^{31}P NMR shift of 47.7 ppm). The 2-D HMQC spectrum was collected with 256 complex points in t2, 256 points in t1 and 32 transients with a pulse repetition delay of 1.5s. A sweep width of 7.78 ppm in ^1H (centered at 5 ppm) and 250 ppm in ^{13}C (centered at 110 ppm) was used. Data sets were multiplied with 90° phase shifted squared-sinebell apodization function and zero-filled to 1024 x 1024 data points before Fourier transformation.

LC-MS Analysis

LC-MS experiments were performed on an Agilent Technologies 1100 LC/MSD Trap instrument. Separations were achieved using an Agilent Zorbax Rapid Resolution SB-C18 reverse phase column (2.1 X 30 mm/3.5 μm) at 25°C with solvent A = 0.1% ammonium formate in water and solvent B = HPLC-grade acetonitrile. *Flow rate:* 0.4 mL/min. *Gradient for conditioning of column:* 100% B to 15% B over 90 minutes. *Gradient for GSNO + TXPTS analyses:* 1% B for 1 min, ramp to 3% B over 2 min, ramp to 5% B over 3 min, and hold for 1 min, for a total run time of 7 min. The post-run time was 3 min at 5% B. *Gradient for Cys-SNO + TXPTS analyses:* 3% B for 3 min, ramp to 10% B for 3 min, and lower to 5% B for 1 min giving a total run time of 7 min. The post-

run time was 3 min at 5% B. The ion trap mass spectrometer was equipped with an atmospheric pressure electrospray ionization source, and was operated in smart mode. Nebulization was achieved with a N₂ pressure of 50 psi, and solvent evaporation assisted by a flow of He drying gas (11 L/min, 325°C). Mass spectra were obtained in positive ion mode, and target masses (m/z) were set at 603 (TXPTS oxide, **3**), 602 (aza-ylide, **4**), and 892 (S-alkylphosphonium adduct, **5**). Retention times and masses of extracted ions were compared to standards on the same instrument. TXPTS oxide (**3**) was prepared by incubating H₂O₂ and TXPTS in a 1:1 mole ratio in H₂O for 5 min. Aza-ylide (**4**) was prepared by incubating hydroxylamine-*O*-sulfonic acid and TXPTS in a 1:1 mole ratio in H₂O for 1h. as previously reported ¹⁴³.

ESI-TOF-MS Analysis

All ESI-TOF MS data was collected on an Agilent MSD TOF system. The operating conditions for MS analysis were as follows: positive ion mode, capillary voltage (VCap) 3500 V, nebulizer gas 30 psig, drying gas 5.0 L/min; fragmentor 140 V; gas temperature 325°C. The samples were injected into the ion source using a syringe pump (KD Scientific) and a 250 µL Hamilton syringe connected to the ion probe with a 50 µm ID fused silica capillary. The injection flow rate was 10 µL/min. The averaged MS spectra were deconvoluted using the Agilent MassHunter Workstation Software vs B.01.03.

CHAPTER 3

THE SYNTHESIS OF DIMEDONE-BASED CHEMICAL PROBES FOR THE DETECTION AND QUANTIFICATION OF PROTEIN SULFENIC ACIDS

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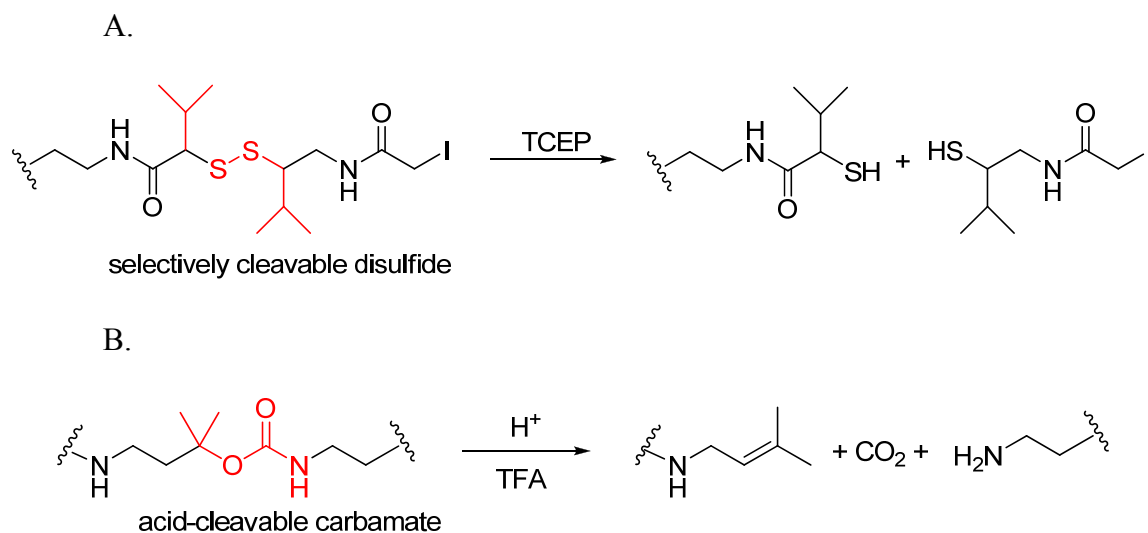
The work contained in this chapter is still on-going and had not been published at the time this Dissertation was prepared. This chapter, including figures and schemes, was drafted by Erika Bechtold and edited by S. Bruce King. The research described herein was performed by Erika Bechtold with contributions from Rajeswari Mukherjee. The Click chemistry experiments and Western blot analysis were contributed by Kimberly J. Nelson and all of the work was done in collaboration with Leslie B. Poole.

3.1 Chemical Probes for the detection of Cysteine Sulfenic Acids

Previous work by our group has focused on the synthesis of dimedone-based chemical probes for the detection of protein sulfenic acids. A series of fluorescent and biotinylated derivatives of 1,3-cyclohexadione (the reactive core of dimedone) were synthesized and have been used to characterize global sulfenic acid formation in a variety of cell lines⁹⁸⁻¹⁰⁰. Many of these new probes (including DCP-Bio1 and DCP-Rho1) are cell permeable and have been quite successful at gaining proteome-level information on cellular sulfenic acid content. One of the most widely used probes, DCP-Bio1 (Figure 11), was used to show that intracellular lipid raft H₂O₂ production promoted VEGF signaling and angiogenesis by inactivating DEP1/PTP1B through the formation of a sulfenic acid intermediate¹⁰³. In a separate experiment, endogenous cellular oxidation following PDGF stimulation regulated Akt2 signaling through a sulfenic acid intermediate which was detected by DCP-Bio1 as well¹⁴⁷.

The limitation of the DCP-Bio1 probe is our inability to remove the biotin moiety from the probe prior to LC-MS analysis of our sample. Removing biotin would improve the MSMS spectra as well as significantly increase the number of proteins identified and quantified through higher confidence scores in a single experiment^{148, 149}. This is due to the complex fragmentation of biotin during the MS ionization process which can complicate spectra and decrease signal-to-noise. The concept of using an acid-labile group to remove biotin from a chemical probe prior to the LC-MS experiment is well established. Several cysteine-targeting probes, many of which use iodoacetamide (IAA) to study the global cysteine proteome, contain cleavable linker regions within the

molecule to remove biotin prior to MS/MS evaluation. As one example, a unique disulfide linker was engineered to IAA which is stable against DTT but sensitive to TCEP-induced cleavage and allows for the controlled removal of the biotin moiety from the parent molecule (Figure 11A)¹⁵⁰. Another type of linker is the acid-labile carbamate (Scheme 11B) which is resistant to intracellular proteases but cleaves under mild acidic conditions with TFA^{151, 152}. In this chapter, we will present our work on the incorporation of a similar acid-labile carbamate for the removal of biotin from our sulfenic acid probes.



Scheme 11. Cleavable chemical linkers for the removal of biotin

Additionally, the incorporation of stable isotopes into the DCP-Bio1 structure would enhance its utility as a MS-based quantitative tool. Stable isotope-labeling methods, such as isotope coded affinity tags (ICAT) or isobaric tags for relative and absolute quantitation (iTRAQ), rely on the incorporation of stable isotopes (typically ^2H , ^{13}C , or ^{15}N) into chemically identical probes which allows them to be differentiated by their molecular weight using MS-based techniques^{153, 154}. These new technologies allows

for the quantitative comparison of protein abundances between complex proteomes and streamlines the analysis process to reduce human error¹⁵³. Importantly, they circumvent the need to rely on 2D PAGE and protein-staining methods for quantification. To date, no ICAT-based reagents have been made to detect changes in sulfenic acid expression levels of complex mixtures. The general procedure to do so is outlined below and in Figure 29B. The full assay involves: 1) labeling two different complex proteomes (to compare relative sulfenic acid content) with a “light chain” (LC) or “heavy chain” (HC) ICAT reagents which would bind protein sulfenic acids (the only difference being their MW), 2) combine and digest the samples with trypsin or an equivalent protease, 3) affinity purify the sulfenic acid-containing peptides on an avidin column, 4) treat samples with dilute TFA to remove biotin, and 5) use LC-MS to analyze changes in RSOH content in identical proteins between samples. LC-MS-MS can be used to identify the peptides at this point as well. With ¹³C incorporation, the ICAT probes are chemically identical and they chromatograph with identical retention times, allowing for MS-based comparison of changes in RSOH formation on each peptide within two different samples. The development of ICAT-based sulfenic acid probes provides a method to quantify changes in protein sulfenic acid content within the whole proteome in a single MS experiment.

Our plan towards the synthesis of the ICAT-based sulfenic acid probe is outlined in Figure 29A. Cellular sulfenic acids will be labeled with a dimedone-based alkyne to which an ICAT-containing biotin-azide will be attached using click-type reactions. The incorporated acid-cleavable carbamate linker will allow for the efficient removal of biotin prior to LC-MS analysis. The reagents used towards the synthesis of this biotin-azide

(Figure 29A) should be available in ^{13}C -based starting materials as well, in order to prepare “light chain” (LC) and “heavy chain” (HC) derivatives of the probe.

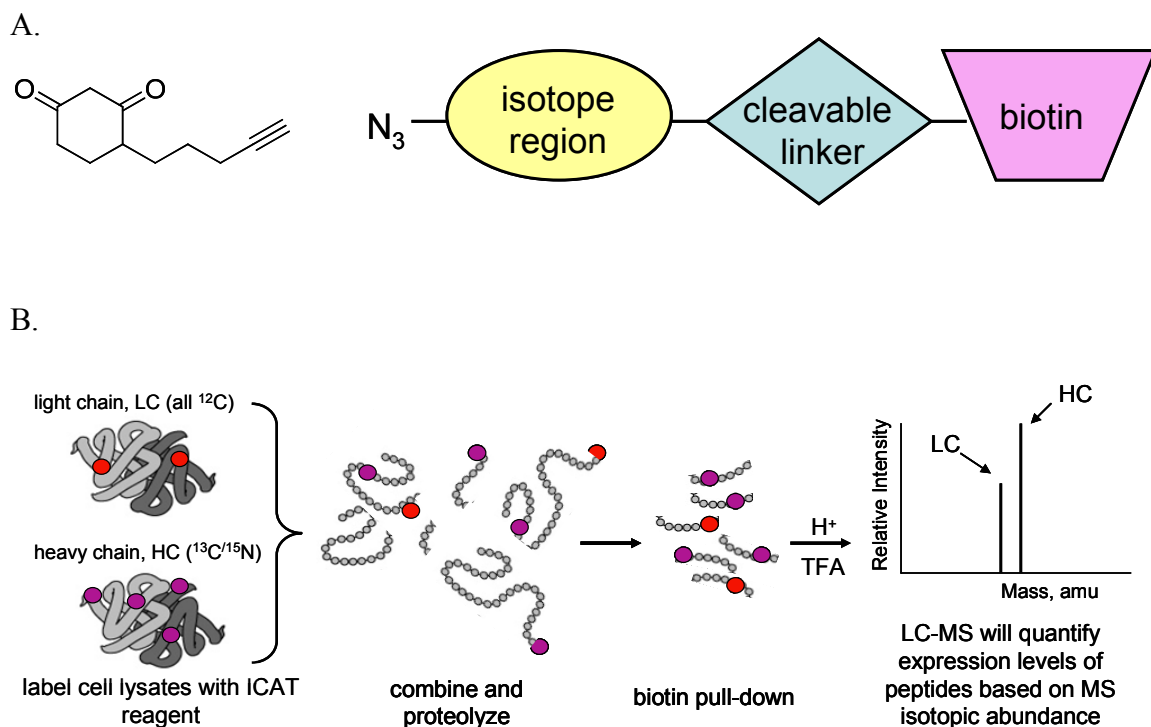


Figure 29. The use of ICAT technology for the differential detection of protein sulfenic acids in complex mixtures. **(A)** General structural design requirement for a sulfenic acid-specific ICAT reagent. Proteins will be labeled using the DCP-alkyne probe and biotinylated with a biotin-azide with an isotope-coded region of varied $^{12}\text{C}/^{13}\text{C}$ composition, and an acid-cleavable carbamate for the removal of biotin. **(B)** General procedure for using ICAT technology to study differential expression levels of the same protein in two different systems.

In this chapter, we present the synthesis of several new sulfenic acid probes for the cellular detection of protein sulfenic acids, including the synthesis of a new fluorescent probe in which 4-(4-dimethylaminophenylazo)benzoic acid (DABCYL) was attached to the cyclohexadione core structure. Additionally, the synthesis of a cyclohexadione-derived alkyne (DCP-alkyne) probe (Figure 29A) is reported, is our progress towards the synthesis of an acid-cleavable, ICAT-based biotin-azide.

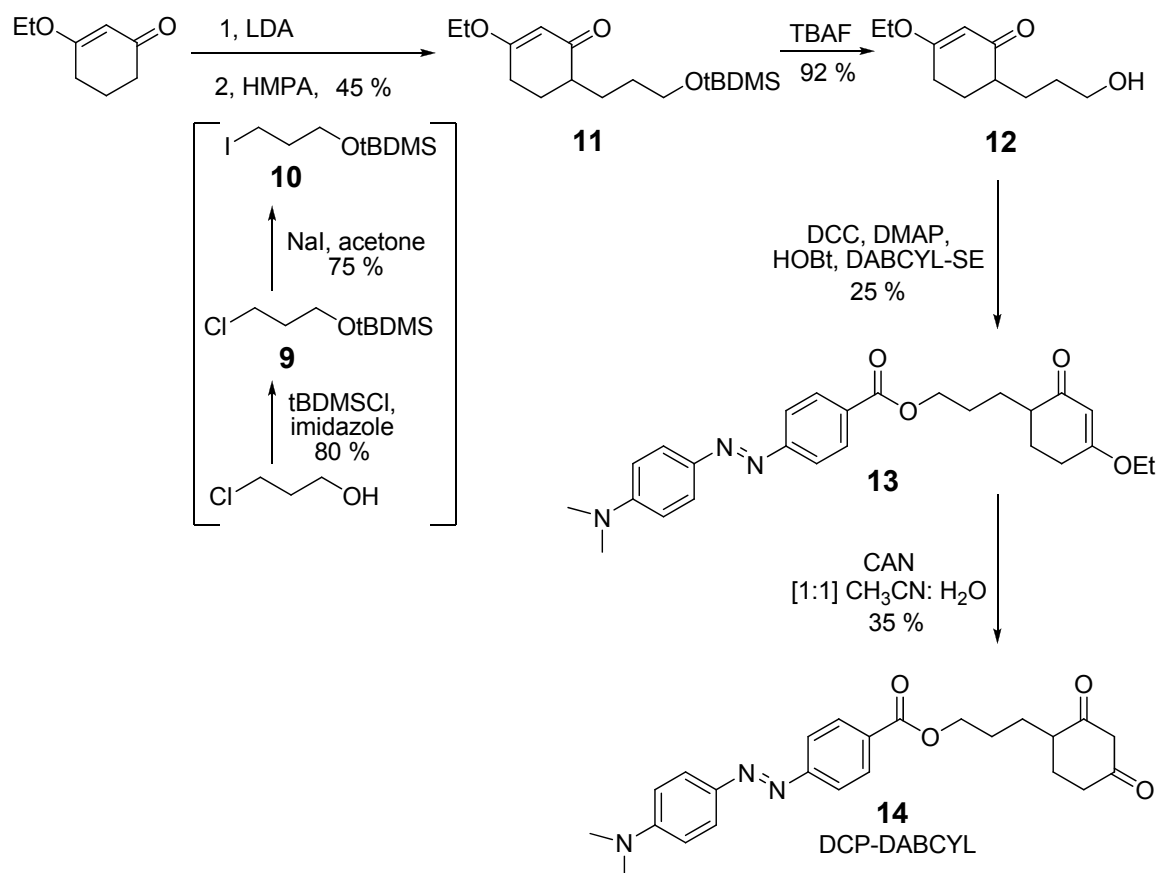
3.2 Results and Discussion

We have now expanded our arsenal of dimedone-based tagging reagents to include alkyne- and DABCYL-conjugated probes. Our synthetic strategy was based on well-established methods used previously in our lab for the synthesis of fluoresceine-, rhodamine-, and biotin-conjugated compounds^{99, 100}.

The synthesis of DCP-DABCYL

To provide an expanded array of fluorescent tools to quantify cellular RSOH, we have prepared a cyclohexadione-based DABCYL probe (**14**, Scheme 12). DABCYL is a widely used fluorescence resonance energy transfer (FRET) acceptor with a unique MS fragmentation pattern and could provide different fluorescent properties from the rhodamine- and fluoresceine-conjugated probes already created^{99, 155}. DCP-DABCYL (**14**, Scheme 12) is synthesized in high purity in a two-step reaction starting with a N,N'-dicyclohexylcarbodiimide (DCC) coupling of alcohol **12** with commercially available

DABCYL-SE to yield **13**. In the presence of aqueous cerium ammonium nitrate (CAN), **13** is readily converted to the desired product (**14**) in moderate yield.

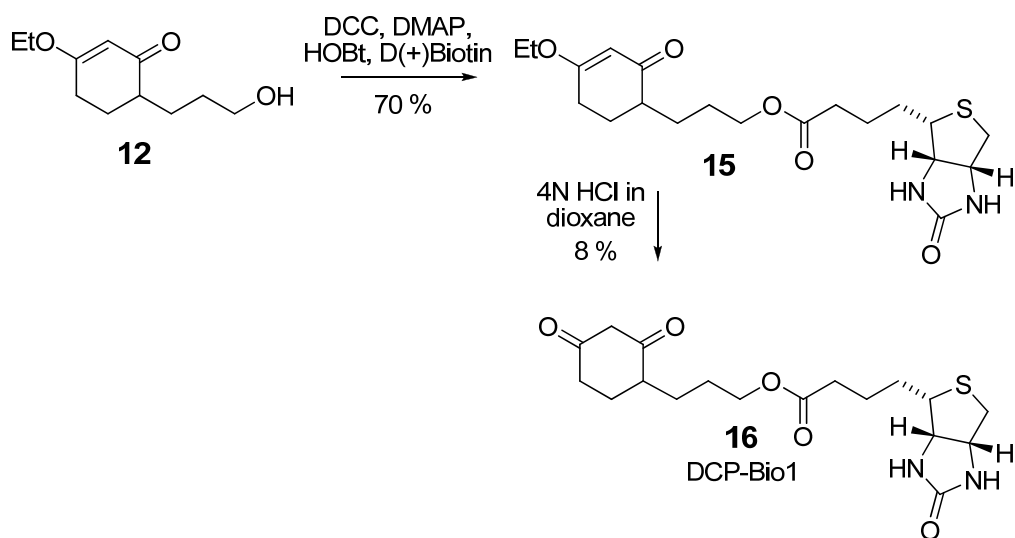


Scheme 12. The synthesis of DCP-DABCYL

The synthesis of DCP-Bio1

The synthesis of DCP-Bio1 (**16**, Scheme 13) has already been reported by our group and follows a similar synthetic strategy to DCP-DABCYL. D(+)Biotin is attached to **12** using a DCC coupling reaction to yield **15** (Scheme 13). Incubation with 4 N HCl in dry dioxane generates **16** in relatively poor yield due to difficulties in purification. Unfortunately, CAN cannot be used to deprotect the cyclohexenone due to an oxidation

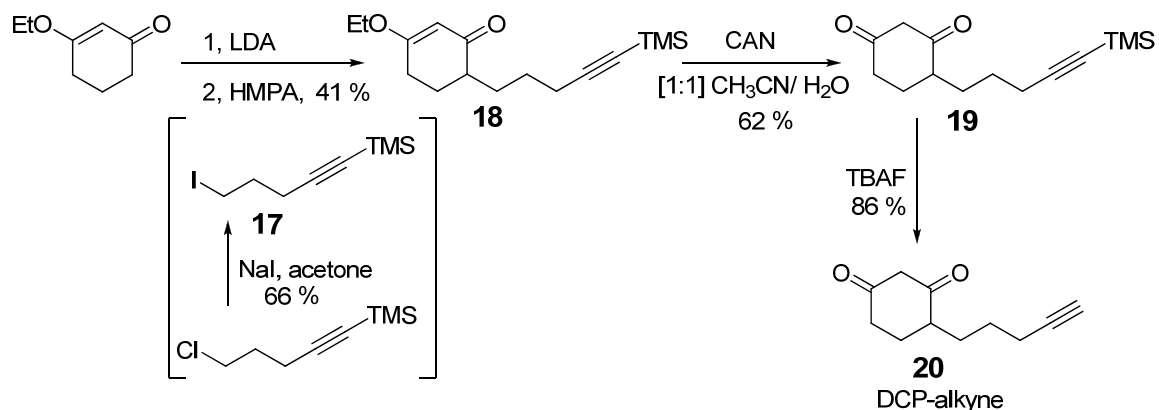
of the sulfur atom on biotin. Efforts have been made to optimize the conversion of **15** to **16**, but without good results. The effective removal and neutralization of the HCl during the work-up helps prevent the decomposition of **16**, as methanol used during chromatography (in the presence of HCl) will initiate the conversion of the dione to 3-methoxy cyclohexenone.



Scheme 13. The synthesis of DCP-Bio1

The synthesis of DCP-alkyne

Scheme 14 shows the preparation of the DCP-alkyne (**20**). In this case, treatment of **18** with CAN generates the cyclohexadione **19** in good yield, without interfering with the TMS-protected alkyne. Tetrabutyl ammonium fluoride (TBAF) deprotection of **19** yields DCP-alkyne (**20**) in good yield. Western blot analysis of NIH3T3 cells following PDGF stimulation and treatment with DCP-alkyne or DCP-Bio1 show that the alkyne probe is also cell-permeable and labels equally to DCP-Bio1 (Figure 30). Preliminary work to optimize the click reaction with a commercially available biotin azide (K.J.



Scheme 14. Synthesis of ALKYNE

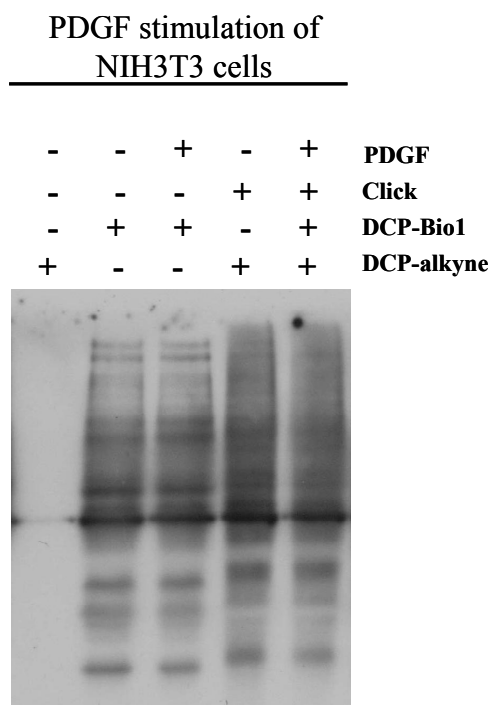


Figure 30. Western blot analysis of NIH3T3 cell lysate following PDGF stimulation. Lysates were treated with DCP-alkyne or DCP-Bio1 to quantify RSOH formation. For the alkyne, click chemistry was initiated by the addition of commercially available biotin-azide (0.5 mM), TCEP or sodium ascorbate (1 mM), TBTA (0.1 mM), and CuSO_4 (1 mM) in that order (work done by K.J. Nelson).

Nelson) also showed that the click reaction is more efficient when sodium ascorbate (as opposed to TCEP) is used as the Cu^{2+} reducing agent (data not shown).

The synthesis of a biotin-azide

An ideal biotin-azide label (Figure 29A) would contain all of the following: 1) a region of the molecule that could be made from ^{13}C starting material for ICAT labeling, 2) an acid-labile carbamate linker, 3) an azide moiety to “click” to the DCP-alkyne following protein labeling, and 4) the incorporation of biotin to allow for peptide

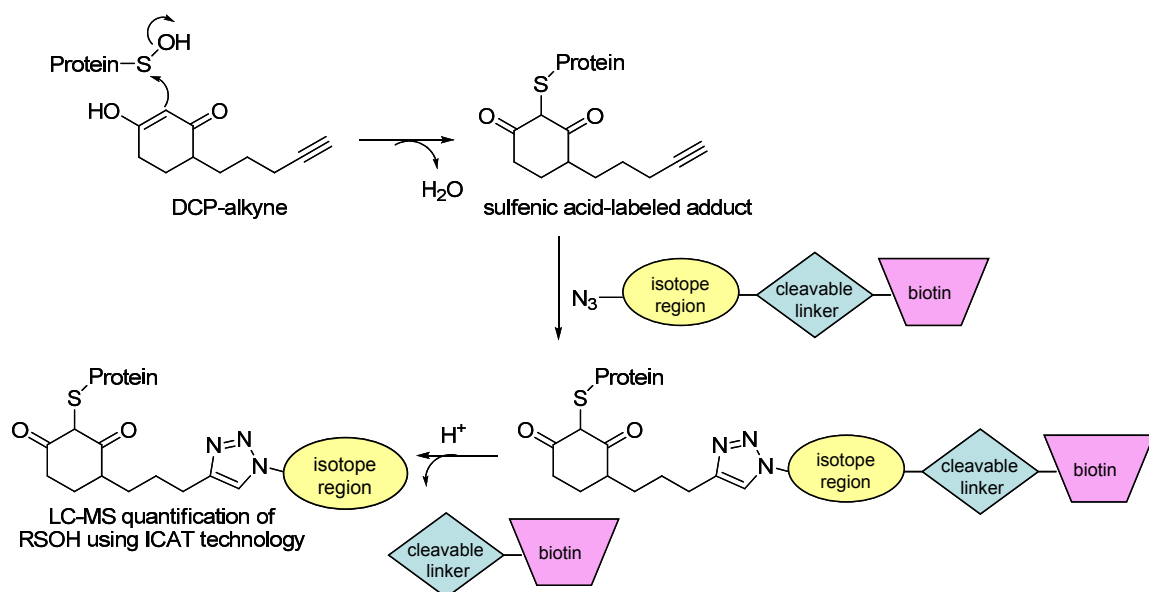
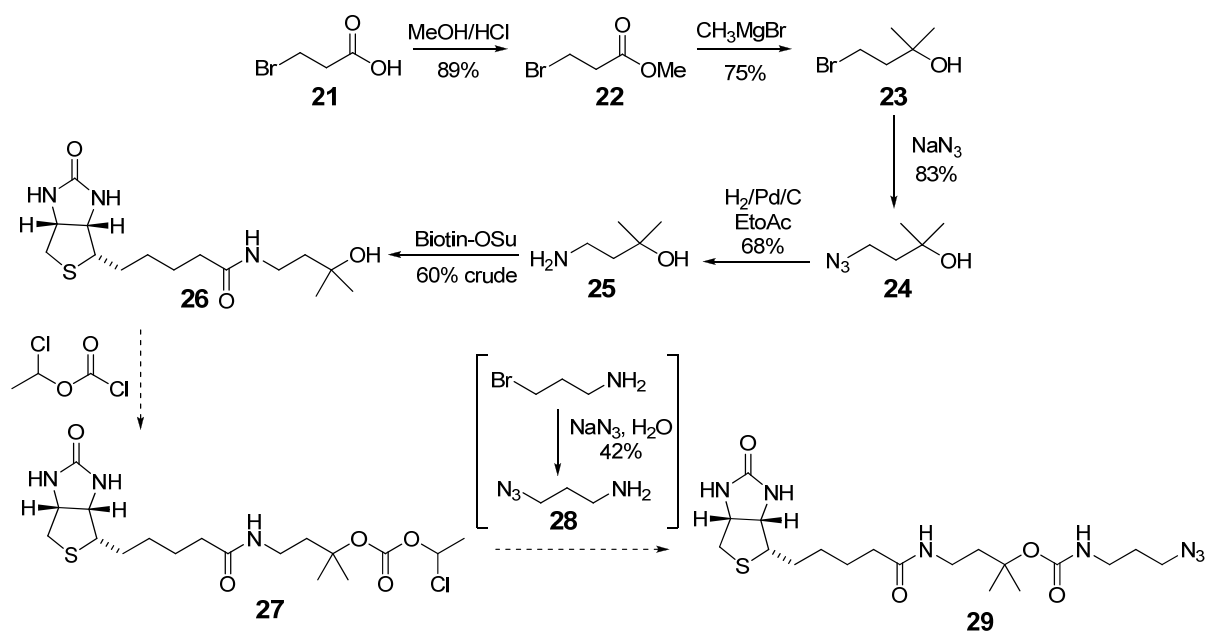


Figure 31. The utility of an ICAT-based biotin-azide as a chemical biology tool. RSOH modifications will be “trapped” using the DCP-alkyne probe followed by the attachment of a biotin-azide using click chemistry. Prior to LC-MS analysis of the sample, affinity capture followed by acid treatment will remove the biotin moiety and elute the tagged protein or peptide, retaining only the ICAT region of the molecule which allows for LC-MS quantification of individual RSOH expression between samples.

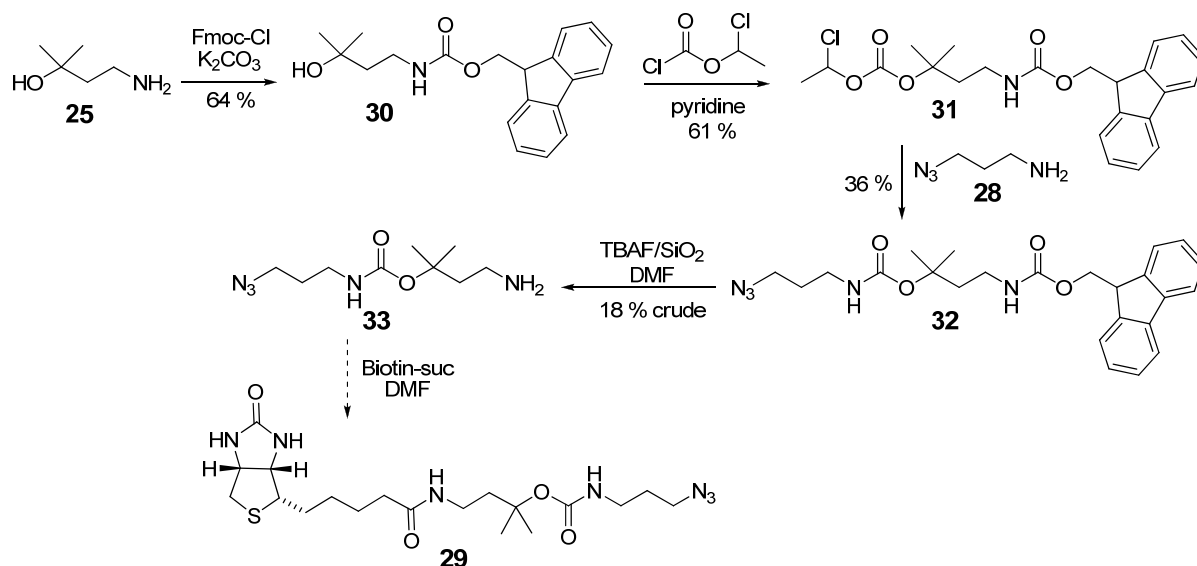
purification through streptavidin binding following the labeling. Figure 31 outlines how this new biotin-azide, in conjunction with the DCP-alkyne, would provide a new method for quantifying cellular RSOH using stable isotopes.

Our initial strategy for making a biotin-azide with an acid-labile carbamate linker is outlined in Scheme 15. The amino alcohol (**25**) is prepared from the commercially available 3-bromopropionic acid (**21**) as previously reported^{152, 156}. An attempt to couple **25** to biotin succinimide appeared to give **26**, but the desired product could not be isolated using flash column chromatography. C₁₈-reverse phase chromatography gave the desired product (**26**) as a crude mixture with a 20 % impurity based on ESI MS (data not shown). The attachment of **25** to D(+)Biotin using a DCC-coupling strategy was also attempted, but this reaction resulted in a low yield (< 6 %) of **26** which could not be isolated from the added DMAP during flash column chromatography (data not shown).



Scheme 15. Initial attempt to synthesize biotin-azide

To circumvent the chromatographic issues associated with biotin-based products, the synthesis of biotin-azide (**29**) was altered to incorporate biotin in the last step of the reaction (Scheme 16). In this case, the amino alcohol (**25**) was N-protected with 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) to give **30**. Treatment of **30** with chloroethyl chloroformate gave **31** in good yield, which was then reacted with a freshly prepared azido-amine (**28**) to give **32**. Deprotection of the Fmoc group to generate **33** was problematic. Traditional cleavage of Fmoc with a piperidine solution (5 - 20 % in DMF) resulted in a mixture of products based on ESI MS and NMR. The use of TBAF (10 eq.) efficiently removed Fmoc from **32**, generating **33** in good yield (as seen by ESI MS)¹⁵⁷ but purification was not possible using flash column chromatography

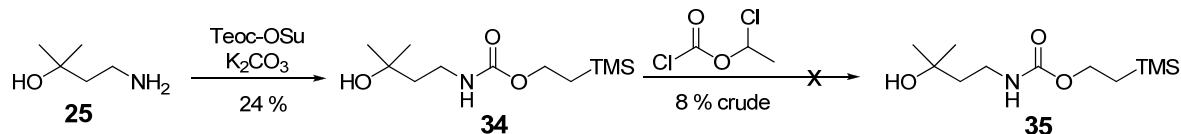


Scheme 16. The synthesis of biotin-azide

as the product could not be separated from the TBAF salt. The reaction was then attempted with TBAF/ SiO_2 and the TBAF was removed by filtration. In this case, the

crude mixture showed the formation of the desired product (based on ESI MS) but **33** could not be isolated and decomposed during flash column chromatography. In the future, crude **33** will be reacted directly with biotin-OSu without further purification to circumvent this issue.

Other protecting groups were also used to try to avoid the problematic TBAF deprotection of Fmoc. 1-[2-(Trimethylsilyl)ethoxycarbonyloxy]pyrrolidin-2,5-dione (Teoc-OSu) was reacted with **25** to generate the Teoc-protected amine **34** in moderate yield (Scheme 17). However, the reaction of **34** with chloroethyl chloroformate to produce **35** was low yielding and not a viable method for the synthesis of **29**.



Scheme 17. Teoc protection of **25**

While our synthetic approaches towards the preparation of an ICAT-based acid-cleavable biotin-azide remain ongoing, the use of Fmoc for protecting **25** followed by building the acid-labile carbamate is most promising (Scheme 16). We propose that the use of crude **33** to synthesize **29** in a one-pot reaction will circumvent the chromatographic issues associated with the purification of **33**. Additionally, attachment of biotin to this molecule will allow it to be visualized by UV during chromatography and will aid in its purification.

The use of **28** for the attachment of the azide to the final target compound will provide a synthetic platform for which multiple ^{13}C azido-amine derivatives will be

synthesized and incorporated in a similar fashion. In the future, this method for preparing ICAT-based derivatives of **29** will allow for the quantification of cellular sulfenic acids in complex proteomes.

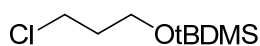
3.3 Conclusions

Herein we have presented the synthesis of two novel dimedone-based chemical probes (DCP-DABCYL and DCP-alkyne) for the detection of protein sulfenic acids. The synthesis of DCP-Bio1 has been reproduced and optimized in an effort to improve the overall yield of the reaction. A series of reactions towards the synthesis of an acid-cleavable biotin-azide were also investigated. While incomplete, the reactions proposed in Scheme 16 show a promising means for the synthesis of our target compound.

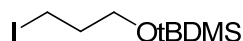
3.4 Materials and Methods

General: All reagents were purchased from Sigma Aldrich unless otherwise noted. Dry solvents were obtained from a commercial source and passed through a Meyer Solvent SystemTM to remove water prior to use. In all cases, glassware was oven-dried and cooled to room temperature under N₂. Analytical thin layer chromatography was performed on 200 μ m G TLC plates w/UV 254 from Sorbent Technologies. Visualization was accomplished with UV light, ethanolic phosphomolybdic acid (PMA) solution or ethanolic ninhydrin solution followed by heating. Purification of the reaction products was carried out by flash column chromatography using silica gel 60 (32–63 μ m).

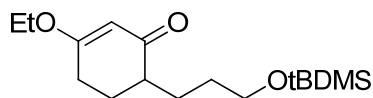
NMR spectra were recorded on a Bruker DPX 300 spectrometer, operating at 300 MHz (^1H NMR) and 75 MHz (^{13}C NMR), with chemical shifts referenced to the residual solvent peak. ^1H NMR data are reported as follows: chemical shift, integration, multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet). Mass spectra of purified compounds were obtained on a Agilent 1100 Series and reported in m/z .



Tert-butyl(3-chloropropoxy)dimethylsilane (9)¹⁰⁰. Imidazole (4.32 g, 63.5 mmol) was added to a solution of 3-chloro-propan-1-ol (3.54 mL, 42.3 mmol) in CH_2Cl_2 (100 mL). After 5 min, the solution was cooled to 0°C and a solution of *tert*-butyl dimethylsilyl chloride (9.57 g, 63.5 mmol) in CH_2Cl_2 (60 mL) added dropwise over 10 min. The reaction warmed to rt and stirred for an additional 16 h at which time the reaction was diluted with diethyl ether (100 mL) and washed with sat. NH_4Cl (50 mL). The aqueous phase was further washed with diethyl ether (3 x 50 mL), the organic phases combined and washed with brine (100 mL), dried over anhydrous MgSO_4 and concentrated. The resultant syrup was purified by flash column chromatography to yield a clear liquid (13.96g, 80%). R_f 0.34 (hexanes/ CH_2Cl_2 9:1); ^1H NMR (300 MHz, CDCl_3) δ 3.75 (2H, t, J = 5.7 Hz), 3.65 (2H, t, J = 6.3 Hz), 1.95 (2H, pentet, J = 6.3 Hz), 0.90 (9H, s), 0.06 (6H, s); ^{13}C NMR (75 MHz, CDCl_3) δ 59.6, 41.9, 35.6, 26.1, 18.5, -5.3.

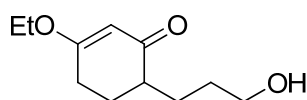


3-Iodo-1-*tert*-butyldimethylsiloxypropane (10)¹⁰⁰. A solution of 3-chloro-1-*tert*-butyldimethylsiloxypropane (7 g, 34 mmol) and NaI (40 g, 268 mmol) in acetone (200 mL) was refluxed for 18 h. Upon cooling, the reaction mixture was diluted with diethyl ether (150 mL), washed with a thiosulfinate solution (100 mL) and brine (100 mL). The organic layer was dried over anhydrous MgSO₄ and concentrated. The resultant syrup was used for the next reaction without further purification (6.91 g, 70 %). ¹H NMR (300 MHz, CDCl₃) δ 3.67 (2H, t, J = 5.7 Hz), 3.28 (2H, t, J = 6.6 Hz), 2.03-1.95 (2H, m), 0.90 (9H, s), 0.06 (6H, s); ¹³C NMR (300 MHz, CDCl₃) δ 62.5, 36.3, 26.1, 18.5, 3.8, -5.2.

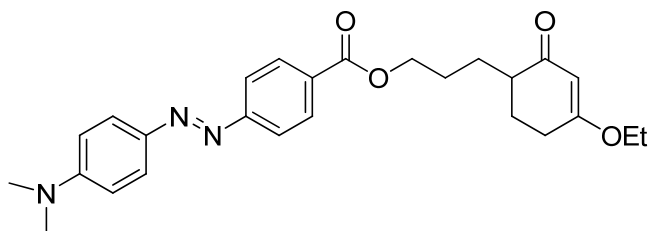


3-Ethoxy-6-(3-*tert*-butyldimethylsiloxypropyl)-cyclohex-2-enone (11)¹⁰⁰. A solution of 3-ethoxy-2-cyclohexen-1-one (2.7 mL, 20 mmol) in THF (6 mL) was added dropwise over 30 min to an LDA solution at -78 °C [freshly prepared from diisopropylamine (3.8 mL, 30 mmol) and nBuLi (13.8 mL of a 1.6 M solution in hexanes, 18 mmol) in THF (13 mL)]. After stirring for an additional 30 min at -78 °C, HMPA (3.5 mL, 20 mmol) was added followed by the dropwise addition of 3-iodo-1-*tert*-butyldimethylsiloxypropane (6 g, 20 mmol) in THF (8 mL). The resultant mixture was allowed to warm to rt, stirred for 18 h, and then quenched by the addition of water (12 mL) and dissolved in CH₂Cl₂ (110 mL). The CH₂Cl₂ layer was washed with sat. NH₄Cl (50 mL) and the aqueous phase was extracted with CH₂Cl₂ (2 x 60 mL). The organic phases were combined and washed with brine (60 mL), dried over anhydrous MgSO₄ and

concentrated and purified by flash column chromatography to give the product as a yellow oil (2.66 g, 43 %). *R_f* 0.21 (hexanes/EtOAc 8/2); ¹H NMR (300 MHz, CDCl₃) δ 5.26 (1H, s), 3.83 (2H, q, 7.0 Hz), 3.59 (1H, t, 6.5 Hz), 3.58 (H, t, 6.5 Hz), 2.38 (2H, t, 6.2 Hz), 2.20-1.99 (2H, m), 1.85-1.35 (5H, m), 1.31 (3H, t, *J* = 7.0 Hz), 0.84 (s, 9H), 0.00 (s, 6H); ¹³C NMR (75 MHz, CDCl₃) δ 201.6, 176.7, 102.3, 64.2, 63.4, 45.0, 30.5, 28.0, 26.4, 26.0 (x 2), 18.4, 14.2, -5.2.

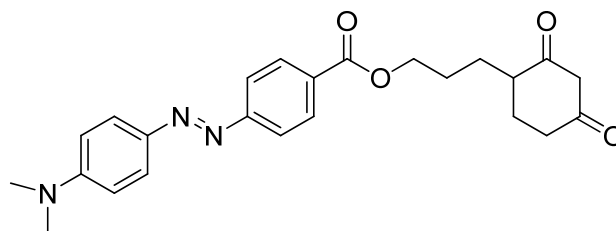


3-Ethoxy-6-(3-hydroxypropyl)-cyclohex-2-enone (12)¹⁰⁰. TBAF (16 mL of a 1.0M solution in THF, 16 mmol) and NEt₃ (2.2 mL, 16 mmol) were added to a solution of **11** (2 g, 6.4 mmol) in THF (16 mL). After 1 h at rt, the reaction was quenched with water (80 mL) and extracted with CH₂Cl₂ (3 x 150 mL). The combined organic phases were dried over anhydrous MgSO₄ and concentrated. The resultant oil was purified by flash column chromatography on a short pad of silica to yield the product as pale yellow oil (1.18 g, 92%). *R_f* 0.22 (EtOAc); ¹H NMR (300 MHz, CDCl₃) δ 5.32 (1H, s), 3.89 (2H, q, *J* = 7.1 Hz), 3.64 (2H, t, *J* = 6.2 Hz), 2.44 (2H, 2d, *J* = 7.1 Hz), 2.24 (1H, m), 2.06 (1H, m), 1.92-1.49 (5H, m), 1.36 (3H, t, *J* = 7.1 Hz); ¹³C NMR (75 MHz, CDCl₃) δ 202.0, 177.2, 102.3, 64.4, 62.6, 44.9, 30.3, 28.4, 26.8, 25.8, 14.3.

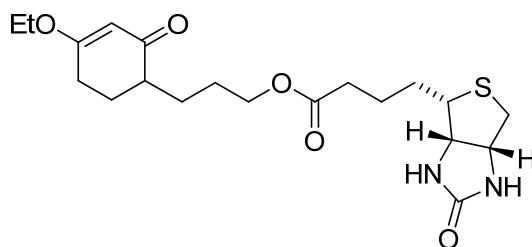


(E)-3-(4-ethoxy-2-oxocyclohex-3-enyl)propyl 4-((4-(dimethylamino)phenyl)diazenyl) benzoate (13).

NOTE: DABCYL benzoic acid was made fresh by treating DABCYL sodium salt with 6 N HCl for 3 h at rt. The reaction mixture was filtered and the DABCYL benzoic acid was extracted from the filtrate using MeOH. A solution of DCC (1.7 mL of a 1 M solution in CH₂Cl₂) was added dropwise to a solution of DABCYL benzoic acid (0.4 g, 1.5 mmol), hydroxybenzotriazole (HOBT, 50 mg, 0.5 mmol), and 4 Å molecular sieves in anhydrous CH₂Cl₂ (40 mL). The reaction was given 2 h at rt after which a solution of **12** (0.36 g, 1.8 mmol) and dimethylaminopyridine (DMAP, 0.22g, 1.83 mmol) in CH₂Cl₂ (5 mL) was added and the mixture was heated to 60 °C. After 12 h, the sieves were filtered, the filtrate concentrated, and the crude solid purified using column chromatography to give the desired product as a red solid (0.16 g, 25 %). *R*_f 0.27 (Hex/EtOAc 1/1); ¹H NMR (300 MHz, CDCl₃) δ 8.11 (2H, d, *J* = 8.6 Hz), 7.89 (2H, d, *J* = 9.1 Hz), 7.85 (2H, d, *J* = 8.6 Hz), 6.75 (2H, d, *J* = 9.2 Hz), 5.32 (1H, s), 4.36 (2H, t, *J* = 5.9 Hz), 3.88 (2H, q, *J* = 7.1 Hz), 3.10 (6H, s), 2.44 (2H, t, *J* = 5.4 Hz), 2.28 – 1.57 (m, 7H), 1.35 (3H, t, *J* = 7.1 Hz); ¹³C NMR (75 MHz, CDCl₃) δ 201.15, 176.86, 166.38, 155.99, 152.90, 143.72, 130.53, 125.52, 121.98, 111.48, 102.24, 65.17, 64.27, 44.79, 40.30, 36.68, 33.96, 28.17, 26.22, 24.72, 14.18.

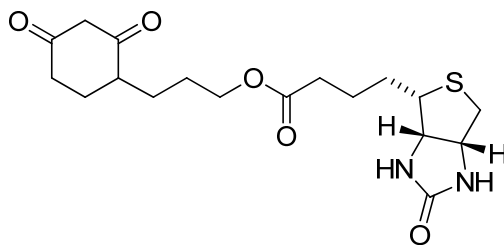


(E)-3-(2,4-dioxocyclohexyl)propyl 4-((4-(dimethylamino) phenyl) diazenyl) benzoate, DCP-DABCYL (14). CAN (20 mg, 0.05 mmol) was added to a solution of **13** (200 mg, 0.45 mmol) in CH₃CN/H₂O (1:1, 24 mL) and the reaction was heated to 90 °C for 3 h, quenched by the addition of brine (21 mL), and extracted with Et₂O (2 x 45 mL). The combined organic phases dried over anhydrous MgSO₄, and concentrated to give a red solid that was purified by column chromatography to yield the product as a red solid (0.032 g, 17%). *R_f* 0.19 (EtOAc/Hex 8/2); ¹H NMR (300 MHz, CDCl₃) δ 8.13 (2H, d, *J* = 8.5 Hz), 7.90 (2H, d, *J* = 9.1 Hz), 7.86 (2H, d, *J* = 8.5 Hz), 6.76 (2H, d, *J* = 9.1 Hz), 4.38 (2H, t, *J* = 5.6 Hz), 3.45 (m, 1H), 3.11 (s, 6H), 2.59 (m, 2H), 2.20 – 1.26 (m, 11 H); ¹³C NMR (75 MHz, CDCl₃) δ 204.16, 203.68, 166.35, 156.10, 152.96, 143.74, 130.53, 130.16, 125.56, 125.04, 111.50, 64.74, 58.38, 48.98, 40.32, 39.84, 26.36, 25.79, 24.67; ESI-MS 422 *m/z* (*M* + H)⁺.



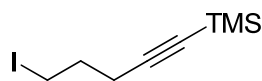
3-(4-Ethoxy-2-oxocyclohex-3-enyl)propyl 5-((3a*R*,6*S*,6a*S*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)pentanoate (15)¹⁰⁰. D(+)-Biotin (600 mg, 2.46 mmol) and hydroxybenzotriazole (HOBt; 66 mg, 0.49 mmol) were suspended in anhydrous

DMF (20 mL) with 4 Å molecular sieves and heated until a clear solution was obtained. Upon cooling, a solution of DCC (2.7 mL of a 1.0 M solution in CH₂Cl₂, 2.70 mmol) was added dropwise and the mixture stirred at rt for 3 h (solution goes cloudy). 3-Ethoxy-6-(3-hydroxypropyl)-cyclohex-2-enone (584 mg, 2.95 mmol) and *N,N*-dimethylaminopyridine (DMAP; 30 mg, 0.025 mmol) were added and the mixture stirred at 60 °C for 4 h, and then at rt for 24 h. The mixture was filtered, washed with CH₂Cl₂/MeOH (1/1 v/v, 20 mL), and the filtrate reduced to dryness and purified by column chromatography. The pure product was obtained as a white foam (805 mg, 77.2%). *R_f* 0.43 (CH₂Cl₂/MeOH 9/1); ¹H NMR (300 MHz, CDCl₃) δ 6.14 (1H, s), 5.70 (1H, s), 5.29 (1H, s), 4.49–4.45 (1H, m), 4.27 (1H, dd, *J* = 4.7 Hz, 7.2 Hz), 4.04 (2H, t, *J* = 6.5 Hz), 3.85 (2H, q, *J* = 7.0 Hz), 3.12 (1H, q, *J* = 7.2 Hz), 2.87 (1H, dd, *J* = 4.9 Hz, 12.8 Hz), 2.71 (1H, d, *J* = 12.7 Hz), 2.40 (2H, t, *J* = 5.8 Hz), 2.29 (2H, t, *J* = 7.4 Hz), 2.23–2.12 (1H, m), 2.09–2.00 (1H, m), 1.93–1.81 (1H, m), 1.76–1.57 (6H, m), 1.46–1.36 (4H, m), 1.32 (3H, t, *J* = 7.1 Hz); ¹³C NMR (75 MHz, CDCl₃) δ 201.5, 177.3, 174.2, 164.3, 102.6, 64.7 (x 2), 62.3, 60.5, 55.9, 50.4, 45.1, 34.4, 28.7, 28.6, 28.5, 26.8, 26.6 (x 2), 26.4, 25.2, 14.5; ESI MS *m/z* 425 (*M* + *H*)⁺.

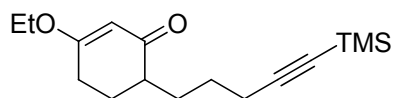


3-(2,4-dioxocyclohexyl)propyl 5-((3a*R*,6*S*,6a*S*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl) pentanoate, DCP-Bio1 (16). A solution of HCl in 1,4-dioxane (4 M, 3.3 mL) was added to a solution of **15** (1 g, 2.35 mmol) in acetone/CH₂Cl₂ (3/1 v/v;

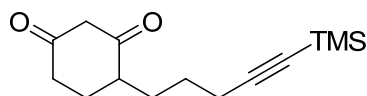
22 mL). The mixture was stirred at rt for 5 hr, then carefully neutralized by the addition of solid Na₂CO₃ (1.4 g, 13.2 mmol). After 10 min, the inorganic solids were filtered off, washed with 10% MeOH in CH₂Cl₂, and the combined filtrates were concentrated. The crude yellow oil was dried under vacuum for 18 h to remove trace HCl from the sample and then purified by flash column chromatography to yield the pure product as a glassy solid (73 mg, 8 %); *R_f* 0.25 (CH₂Cl₂/MeOH 9/1); ¹H NMR (300 MHz, MeOH-d₄) δ 4.74 (1H, dd, *J* = 5.0 Hz, 7.4 Hz), 4.55 (1H, dd, *J* = 4.4 Hz, 7.6 Hz), 4.35 (2H, t, 6.1 Hz), 3.46 (m, 1H), 3.18 (1H, dd, *J* = 4.9 Hz, 12.8 Hz), 2.96 (1H, d, *J* = 12.7 Hz), 2.69–2.52 (m, 4H), 2.44–2.29 (m, 1H), 2.16–1.68 (m, 12H); ¹³C NMR (75 MHz, MeOH-d₄) δ 175.48, 175.06, 166.12, 65.57, 63.42, 61.65, 57.04, 41.09, 36.92, 34.92, 33.88, 29.77, 29.53, 27.73, 27.47, 27.03, 26.69, 26.21, 26.04; ESI MS *m/z* 397 (M + H)⁺, *m/z* 395 (M – H)[–].



(5-Iodo-1-pentynyl)-trimethylsilylsilane (17). Sodium iodide (21.4 g, 143.1 mmol) was added to a solution of 5-chloro-1-pentynyl)-trimethylsilylsilane (5 g, 28.6 mmol) and acetone (100 mL) and the reaction was heated to 60 °C. After 16 h, the mixture was cooled to rt, brine (75 mL) was added, and the mixture was extracted with Et₂O (2 x 100 mL). The combined organic phases were dried over MgSO₄ and concentrated to give the crude product as a clear oil (5.14 g, 68 %). ¹H NMR (300 MHz, CDCl₃) δ 3.14 (2H, t, *J* = 6.8 Hz), 2.21 (2H, t, *J* = 6.8 Hz), 1.85 (2H, p, *J* = 6.8 Hz), 0.00 (s, 9H).

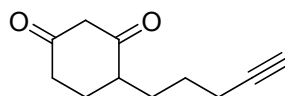


3-ethoxy-6-(5-(trimethylsilyl)pent-4-ynyl)cyclohex-2-enone (18). 3-ethoxy-2-cyclohexen-1-one (2.8 mL, 21 mmol) in THF (6 mL) was added dropwise at -78 °C to a LDA solution [prepared from diisopropylamine (3.13 mL, 31 mmol) and nBuLi (13.5 mL of a 1.6 M solution in hexanes, 22 mmol) in THF (14 mL)] over 30 min. After stirring for an additional 30 min at -78 °C, HMPA (3.6 mL, 21 mmol) was added followed by the dropwise addition of **17** (5.5 g, 21 mmol) in THF (6 mL). The resultant mixture was allowed to warm to rt, stirred for an additional 18 h, and quenched by the addition of water (11 mL); the mixture was dissolved in CH₂Cl₂ (110 mL) and washed with sat. NH₄Cl (55 mL). The aqueous phase was extracted with CH₂Cl₂ (2 x 55 mL), the organic phases combined and washed with brine (60 mL), dried over anhydrous MgSO₄, and concentrated. The crude oil was purified by flash column chromatography to give the product as a yellow oil (2.34 g, 41 %). *R*_f 0.17 (Hex/EtOAc 8/2); ¹H NMR (300 MHz, CDCl₃) δ 5.28 (1H, s), 3.86 (2H, q, *J* = 7.1 Hz), 2.41 (2H, m), 2.23 (m, 3H), 1.90 - 1.53 (m, 7H), 1.33 (3H, t, 7.0 Hz), 0.11 (s, 9H); ¹³C NMR (75 MHz, CDCl₃) δ 201.15, 176.51, 107.14, 102.20, 84.44, 64.03, 44.62, 28.78, 27.82, 26.22, 26.16, 19.90, 14.02, 0.03.

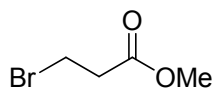


4-(5-(trimethylsilyl)pent-4-ynyl)cyclohexane-1,3-dione (19). CAN (0.19 g, 0.36 mmol) was added to a solution of **18** (1 g, 3.6 mmol) in CH₃CN/H₂O (1:1, 12 mL). The reaction was refluxed for 3h, cooled to rt, and quenched by the addition of brine (40

mL). The mixture was extracted with Et₂O (2 x 110 mL), dried over anhydrous MgSO₄, and concentrated to give the crude product which was purified by flash column chromatography to give the product as a white solid (0.56 g, 62 %). *R_f* 0.27 (CH₂Cl₂/EtOAc, 8/2); ¹H NMR (300 MHz, CDCl₃) δ 10.25 (s, 1H), 5.38 (s, 1H), 2.39 - 1.52 (m, 11H), 0.08 (s, 9H); ¹³C NMR (75 MHz, CDCl₃) δ 204.42, 203.94, 196.57, 188.88, 106.90, 103.71, 84.66, 58.06, 48.67, 41.38, 39.41, 29.72, 29.21, 28.18, 26.21, 25.85, 25.81, 24.32, 19.79, 0.03.

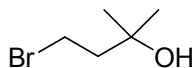


4-(pent-4-ynyl)cyclohexane-1,3-dione (20). TBAF (4.5 mL of a 1.0 M solution in THF) was added to a solution of **19** (0.56 g, 2.24 mmol) in THF (5 mL). After 30 min at rt, the mixture was concentrated to give the crude product as a glassy solid which was purified by flash column chromatography to give the final product as a white solid (0.37 g, 86 %). *R_f* 0.31 (CH₂Cl₂/MeOH, 9/1); ¹H NMR (300 MHz, CDCl₃) δ 9.96 (s, 1H), 5.36 (s, 1H), 2.58 - 1.90 (m, 6H), 1.75 - 1.51 (m, 5H); ¹³C NMR (75 MHz, CDCl₃) δ 202.92, 202.53, 195.12, 187.48, 103.23, 83.78, 68.63, 41.73, 30.14, 29.65, 26.55, 26.32, 18.98; ESI MS *m/z* 179 (M + H)⁺, *m/z* 177 (M - H)⁻; IR (cm⁻¹): 3306.36, 2942.84, 1732.73, 1708.62, 1579.41, 1455.99, 1404.89, 1341.25, 1221.68, 1191.79, 1088.62, 1044.26.

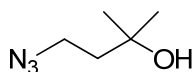


Methyl 3-bromopropanoate (22)¹⁵⁶. Chlorotrimethylsilane (TMS-Cl, 0.49 g, 4.58 mmol) was added to a solution of 3-bromopropionic acid (**21**, 7 g, 45.8 mmol) in

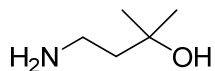
[4:1] 2,2'-dimethoxypropane/MeOH. The reaction was given 18 h at rt and concentrated to yield the crude product as a brown oil (6.75 g, 89 %). ^1H NMR (300 MHz, CDCl_3) δ 3.73 (3H, s), 3.57 (2H, t, $J = 6.8$ Hz), 2.92 (2H, t, $J = 6.8$ Hz); ^{13}C NMR (75 MHz, CDCl_3) δ 171.08, 52.13, 37.65, 25.89.



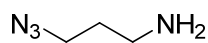
4-bromo-2-methylbutan-2-ol (23)¹⁵². Methyl magnesium bromide (50 mL of a 3 M solution in Et_2O) was added to a solution of **22** (10 g, 59.9 mmol) in Et_2O (100 mL) at 4 °C. The reaction was given 30 min, quenched with ammonium chloride (50 mL), and allowed to reach rt. The product was extracted with Et_2O (2 x 50 mL) and the Et_2O layers were combined, washed with H_2O (30 mL), dried over MgSO_4 , and concentrated to yield the crude product as a brown oil (7.6 g, 76 %). ^1H NMR (300 MHz, CDCl_3) δ 3.47 (2H, t, $J = 8.2$ Hz), 2.10 (2H, m), 1.26 (6H, s); ^{13}C NMR (75 MHz, CDCl_3) δ 71.18, 46.75, 29.41, 28.28.



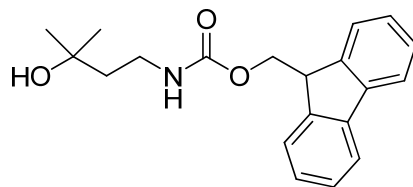
4-azido-2-methylbutan-2-ol (24)¹⁵². Sodium azide (3.25 g, 50 mmol) was added to a solution of **23** (1.39 g, 8.3 mmol) in DMF (9 mL) and the reaction was heated to 90 °C. After 18 h the reaction was cooled to rt, combined in Et_2O (45 mL), washed with H_2O (30 mL) and brine (30 mL), then dried over MgSO_4 and concentrated to yield the final crude product as an orange oil (0.89 g, 83 %). ^1H NMR (300 MHz, CDCl_3) δ 3.46 (2H, t, $J = 7.3$ Hz), 1.78 (2H, t, $J = 7.4$ Hz), 1.27 (6H, s); ^{13}C NMR (75 MHz, CDCl_3) δ 69.53, 47.15, 40.95, 29.10.



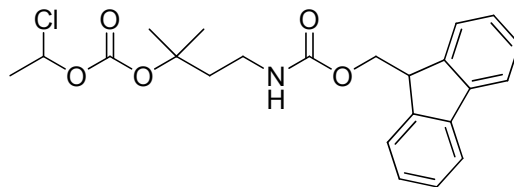
4-amino-2-methylbutan-2-ol (25). A solution of **24** (0.79 g, 6.12 mmol) in EtOAc (20 mL) was slowly added to solid palladium on carbon (10 wt%, 0.18 g) in a hydrogenation flask. This reaction mixture was subjected to hydrogenation using a Parr-hydrogenator under 1.5 atm for 3 h. The reaction mixture was then filtered over a celite pad and concentrated to yield the crude product as a yellow oil (0.43 g, 68 %). ^1H NMR (300 MHz, CDCl_3) δ 3.01 (2H, t, $J = 6.0$ Hz), 1.57 (2H, t, $J = 6.0$ Hz), 1.22 (6H, s); ^{13}C NMR (75 MHz, CDCl_3) δ 71.03, 42.65, 38.31, 29.55.



3-azidopropan-1-amine (28)¹⁵⁸. Sodium azide (4.9 g, 75.4 mmol) was added to a solution of 3-bromopropylamine (5 g, 22.8 mmol) in H_2O (16 mL) and the reaction was heated to 95 °C for 24 h. After cooling to rt, roughly 2/3 of the H_2O was removed by evaporation and the remaining residue was diluted with CH_2Cl_2 (60 mL) and cooled to 4 °C followed by the addition of KOH pellets (6 g) over 10 min. The product was extracted with CH_2Cl_2 (2 x 50 mL), dried over MgSO_4 , and concentrated to afford the crude product as an orange oil (0.96 g, 42 %). ^1H NMR (300 MHz, CDCl_3) δ 3.37 (2H, t, $J = 6.7$ Hz), 2.80 (2H, t, $J = 6.8$ Hz), 1.72 (2H, p, $J = 6.8$ Hz); ^{13}C NMR (75 MHz, CDCl_3) δ 49.13, 39.32, 32.44.

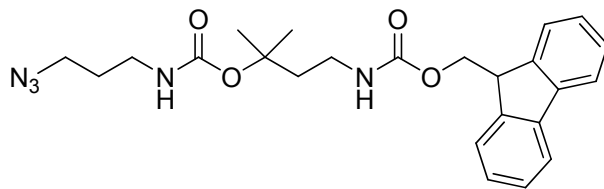


(9H-fluoren-9-yl)methyl 3-hydroxy-3-methylbutylcarbamate (30)¹⁵⁹. A solution of 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl, 1.9 g, 7.46 mmol) in THF (19 mL) was added dropwise to a solution of **25** (0.7 g, 6.79 mmol) in potassium carbonate (5 wt %, 25 mL) at 4 °C. The reaction was given 1 h, allowed to reach rt, and given an additional 1 h. The THF was removed under vacuum, the product was extracted with CH₂Cl₂ (3 x 55 mL) and washed with HCl (1 N, 55 mL) and brine (55 mL), dried over MgSO₄, and concentrated to give an oil which was purified using flash column chromatography to give the product as a clear oil (1.40 g, 64 %). *R_f* 0.13 (CH₂Cl₂/EtOAc 8/2); ¹H NMR (300 MHz, CDCl₃) δ 7.68 (2H, d, *J* = 7.4 Hz), 7.54 (2H, d, *J* = 7.3 Hz), 7.32 (2H, t, *J* = 7.2 Hz), 7.23 (2H, m), 5.65 (1H, s), 4.31 (2H, d, *J* = 6.5 Hz), 4.14 (1H, t, *J* = 6.4 Hz), 3.23 (2H, m), 1.98 (1H, s), 1.62 (2H, m), 1.19 (6H, s); ¹³C NMR (75 MHz, CDCl₃) δ 171.93, 157.53, 144.98, 142.26, 128.82, 128.21, 126.28, 121.21, 72.21, 68.49, 49.43, 44.48, 39.62, 31.88.



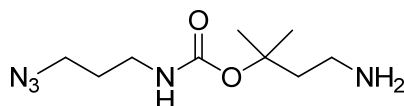
(9H-fluoren-9-yl)methyl 3-((1-chloroethoxy) carbonyloxy) -3-methyl butyl carbamate (31). A solution of chloroethyl chloroformate (0.41 g, 2.87 mmol) in CH₂Cl₂ (10 mL) was added dropwise to a solution of **30** (0.62 g, 1.91 mmol), pyridine (0.3 mL,

3.82 mmol), and CH₂Cl₂ (20 mL) at 4 °C. The reaction was allowed to reach rt, stirred for an additional 2 h, and a solution of cold HCl (0.5 M, 50 mL) was added. The mixture was extracted with CH₂Cl₂ (3 x 30 mL), washed with brine (30 mL), dried over MgSO₄, and concentrated to afford a yellow oil which was purified using flash column chromatography to afford the pure product as a white solid (0.5 g, 61 %). *R_f* 0.15 (CH₂Cl₂); ¹H NMR (300 MHz, CDCl₃) δ 7.71 (2H, d, *J* = 7.6 Hz), 7.56 (2H, t, *J* = 7.4 Hz), 7.36 (2H, m), 7.27 (2H, m), 6.36 (1H, m), 5.03 (1H, s), 4.36 (2H, d, *J* = 5.1 Hz), 4.17 (1H, t, *J* = 6.8 Hz), 3.27 (2H, m), 1.98 (2H, t, *J* = 7.4 Hz), 1.76 (3H, d, *J* = 5.7 Hz), 1.50 (6H, s); ¹³C NMR (75 MHz, CDCl₃) δ 155.18, 149.70, 142.94, 140.31, 126.87, 126.24, 124.26, 119.26, 84.55, 83.91, 66.52, 47.44, 40.51, 36.88, 26.05, 25.61; ESI MS *m/z* 432 (M + H)⁺.

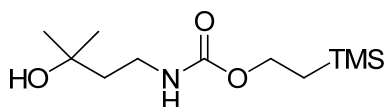


(9H-fluoren-9-yl)methyl 3-((3-azidopropan-1-amine) carbonyloxy) -3-methyl butyl carbamate (32). A solution of **31** (0.5 g, 1.2 mmol) in DMF (7 mL) was added dropwise over 1 h to a solution of **28** (0.12 g, 1.2 mmol) in DMF (5 mL) and the reaction was stirred for 38 h at rt and concentrated. The crude oil was purified using flash column chromatography to afford the pure product as a white solid (0.19 g, 36 %). *R_f* 0.11 (CH₂Cl₂/EtOAc 9/1); ¹H NMR (300 MHz, CDCl₃) δ 7.74 (2H, d, *J* = 7.3 Hz), 7.58 (2H, d, *J* = 7.3 Hz), 7.38 (2H, t, *J* = 7.1 Hz), 7.29 (2H, t, *J* = 7.4 Hz), 5.03 (1H, s), 4.90 (1H, s), 4.37 (2H, d, *J* = 6.7 Hz), 4.19 (1H, m), 3.31 (4H, m), 3.17 (2H, m), 1.97 (2H, t, *J* = 3.3 Hz), 1.72 (2H, t, *J* = 6.5 Hz), 1.44 (6H, s); ¹³C NMR (75 MHz, CDCl₃) δ 157.18, 156.52,

144.91, 142.26, 128.81, 128.18, 126.20, 121.20, 81.89, 68.44, 51.18, 49.40, 42.89, 40.31, 39.05, 38.70, 28.92; ESI MS m/z 474 ($M + Na$)⁺.



4-amino-2-methylbutan-2-yl 3-azidopropylcarbamate (33). TBAF on silica gel (1.2 g, 1.5 mmol F⁻/g resin) was added to a solution of **32** (90 mg, 0.2 mmol) in THF (20 mL) at rt. After 10 min, the reaction was filtered to remove SiO₂, the filtrate was concentrated, dissolved in H₂O (20 mL), and the mixture was extracted with CH₂Cl₂ (3 x 20 mL). The CH₂Cl₂ layers were combined, washed with brine (20 mL), dried over MgSO₄, and concentrated to afford a crude oil that was purified using flash column chromatography to give the product as a crude mixture (8.2 mg, 18 %) which could not be purified further.



2-(trimethylsilyl)ethyl 3-hydroxy-3-methylbutylcarbamate (34). A solution of Teoc-OSu (0.85 g, 3.3 mmol) in THF (5 mL) was added to a solution of **25** (0.3 g, 3 mmol) in potassium carbonate (5 % in H₂O, 10 mL) and the reaction was given 18 h at rt. The THF was removed under vacuum and the product was extracted with CH₂Cl₂ (3 x 20 mL). The CH₂Cl₂ layers were combined, washed with brine (25 mL), dried over MgSO₄, and concentrated to afford the crude product as a pale yellow oil which was purified using flash column chromatography to give the final product as a clear oil (0.18 g, 24 %). ¹H NMR (300 MHz, CDCl₃) δ 5.29 (1H, s), 4.10 (2H, t, J = 8.4 Hz), 3.27 (2H, q, J = 6.1

Hz), 2.39 (1H, s), 1.65 (2H, t, J = 7.0 Hz), 1.22 (6H, s), 0.94 (2H, t, J = 8.5 Hz), 0.01 (9H, s); ^{13}C NMR (75 MHz, CDCl_3) δ 155.96, 70.51, 62.92, 42.57, 37.59, 30.08, 18.42, -0.61.

CHAPTER 4

A COMPREHENSIVE STUDY OF PROTEIN SULFENIC ACID REACTIVITY WITH THIOL ALKYLATING AGENTS AND ASCORBATE

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The work contained in this chapter is still on-going and had not been published at the time this Dissertation was prepared. This chapter, including figures and schemes, was drafted by Erika Bechtold and edited by S. Bruce King and Leslie B. Poole. The research described herein was performed by Erika Bechtold and Channanat Klomsiri in collaboration with S. Bruce King and Leslie B. Poole.

4.1 The Specificity of Thiol Alkylating Agents and the Biotin Switch Assay

The post-translational oxidation of cysteine residues in the presence of low concentrations of reactive oxygen species (ROS) or reactive nitrogen species (RNS) as a potential mechanism for redox-based signal transduction has been extensively reviewed in the Introduction^{4, 112, 117, 160-162}. S-Nitrosothiols (RSNO) and sulfenic acids (RSOH) are two of the most common cysteine modifications that reversibly alter protein function in a variety of biological systems^{36, 46, 92, 132, 163-167}. We reviewed the current methods for detecting cysteine sulfenic acids and S-nitrosothiols in Section 1.2 including the use of 5,5-dimethyl-1,3-cyclohexanedione (dimedone) to label RSOH residues on proteins (Figure 11A)³⁶. Our group and others have pioneered the use of novel chemical probes based on this dimedone core structure which is emerging as a viable method to label and quantify cellular RSOH formation (Figure 11B)^{98-100, 102, 168}. We recently reported on the use of the triarylphosphine TXPTS for labeling S-nitrosothiols (Scheme 5)¹¹¹ and this work is summarized in chapter 2. Despite being a promising technique, no fluorescent or biotinylated derivatives of TXPTS have been reported at this time and this technology is still in its infancy.

Currently only indirect methods are available for the cellular quantification of S-nitrosothiols. One of the most widely used assays is the Biotin Switch Technique (BST), which was extensively reviewed in Section 1.2 of the Introduction (Figure 10)^{85, 86, 169}. It relies on the blocking of all cellular free thiols with MMTS, reducing the S-nitrosothiols with sodium ascorbate, and biotinylating the nascent thiols with biotin-HPDP or an equivalent alkylating agent. There are reports in the literature that the reagents used

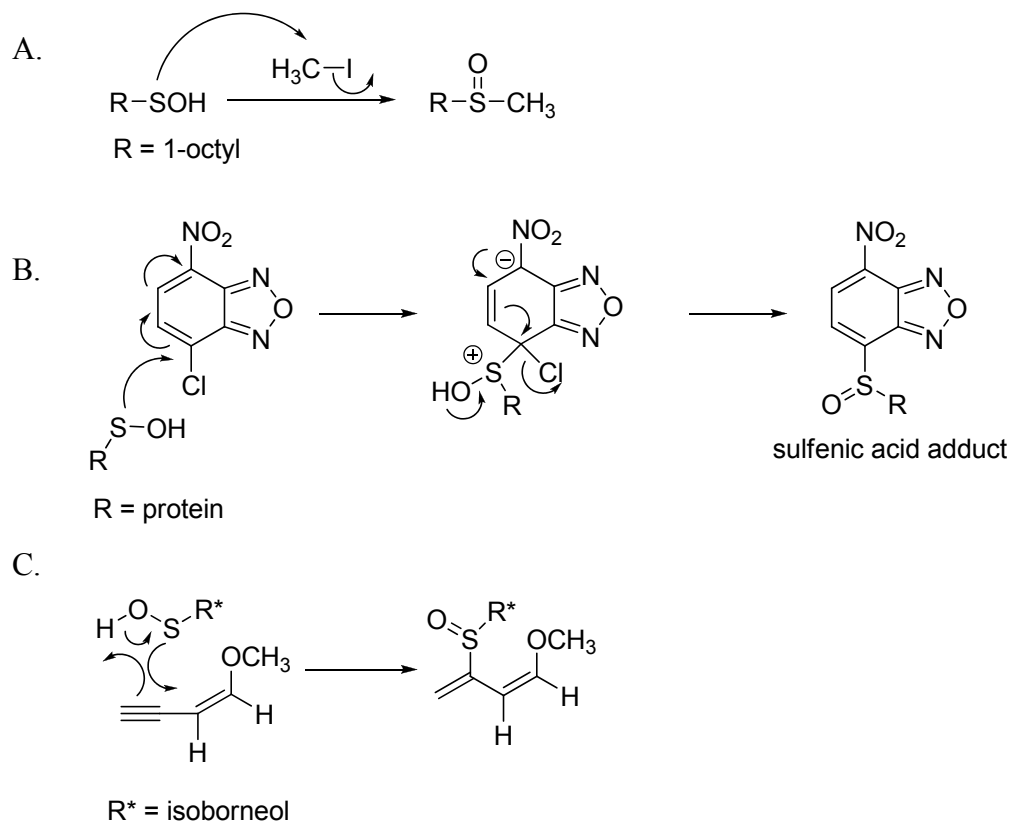
during the Biotin Switch assay, specifically the ascorbate used to reduce the S-NO bond, are non-specific and will reduce disulfides and sulfenic acids^{88, 89}. The reduction of RSNOs by ascorbate is accelerated in the presence of metal ions and is believed to be copper-dependent, a finding that also remains controversial¹⁷⁰. In a metal ion-mediated process, ascorbate oxidized Cys-149 of GAPDH to a sulfenic acid, suggesting a reaction between Fe^{3+} ions and ascorbate to produce ROS¹⁷¹.

Much of the criticism of the BST has focused on the efficiency and specificity of the ascorbate reduction of RSNOs, however the specificity of the thiol alkylating/blocking agents (specifically MMTS and biotin-HPDP) has not been addressed. Frequently, assays labeling sulfenic acids or S-nitrosothiols are accompanied by blocking all cellular free thiols using these thiol-specific reagents, many of which are shown in the Introduction (Scheme 4). These reagents are highly electrophilic and are generally assumed to be specific for thiols (RSH), due to the fact that RSHs are more nucleophilic than RSOHs or RSNOs.

The protocol in our lab for the detection of sulfenic acid-containing proteins at the time of cell lysis involves the addition of NEM and IAA to the lysis buffer for rapid alkylation of cell lysates before exposure to oxidative stress imparted by the lysis process⁹⁸. The lysis buffer used typically contains a sulfenic acid labeling reagent (DCP-Bio1 or an equivalent probe), DTPA, catalase, protease and phosphatase inhibitors, NEM and IAA⁹⁸. To our knowledge the reactivity of these thiol-specific alkylating agents with protein sulfenic acids is generally assumed to be minimal (in spite of some indications to the contrary), but this has never been significantly investigated. However, if sulfenic

acids were reacting with NEM and IAA, we would expect to see a decrease in DCP-Bio1 labeling and obtain inaccurate results.

The idea that sulfenic acids can behave as nucleophiles is well established in the literature. Organic sulfenic acids react rapidly with methyl iodide to produce methyl



Scheme 18. The reaction of sulfenic acids with electrophiles. **(A)** The reaction of 1-octyl sulfenic acid with methyl iodide produces 1-octyl sulfoxide.¹⁷² **(B)** The reaction of protein sulfenic acids with NBD-Cl produces the corresponding sulfoxide-containing adduct via nucleophilic aromatic substitution. **(C)** The reaction of isoborneol sulfenic acid with oxyalkynes produces an alkene and the corresponding sulfoxide.

sulfoxide products (Scheme 18A)¹⁷². Similarly, protein sulfenic acids react with NBD-Cl to give the corresponding NBD-Cl adduct (Scheme 18B)⁶¹. Sulfenic acids also react with oxoalkynes in a concerted manner to yield β -sulfinyl α,β -unsaturated carbonyl compounds (Scheme 18C)¹⁷³. In all of these cases, the sulfenic acid reacts to form a sulfoxide-containing adduct.

In this chapter, we will investigate the reactivity of common thiol alkylating agents with protein sulfenic acids using the mutated peroxiredoxin C165S AhpC. This

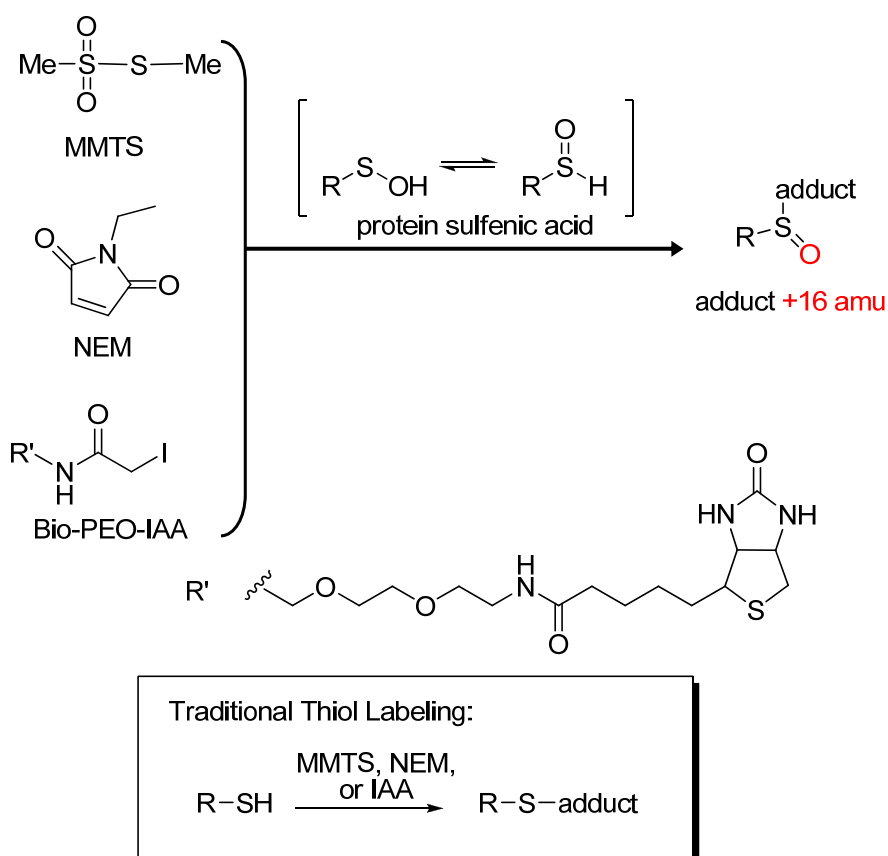


Figure 32. The reaction of thiol alkylating agents with protein sulfenic acids. Traditional thiol alkylating agents (MMTS, NEM, and bio-PEO-IAA) reacted with AhpC-SOH to produce covalent adducts which differed from the AhpC-SH adduct by at least 16 amu.

enzyme forms a relatively stable sulfenic acid and S-nitrosothiol at Cys-46 and can therefore be used as a model system to investigate sulfenic acid nucleophilicity. AhpC-SOH was incubated with NEM, biotin polyethylene oxide iodoacetamide (bio-PEO-IAA), MMTS, and N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)-propionamide (biotin-HPDP). The reaction with NEM, bio-PEO-IAA, and MMTS gave unique sulfoxide-containing adducts which are structurally different from the traditional AhpC-SH adducts (Figure 33). The reaction with biotin-HPDP did not yield a sulfenic acid adduct. In the context of the BST, we also investigated the reaction of ascorbate with AhpC-SOH and found an unexpected ascorbate-dependent dimerization of AhpC-SOH, suggesting the ability of ascorbate to act as a denaturant. We address these findings in the context of the Biotin Switch Technique (BST) and other RSOH/RSNO labeling protocols and how specificity of these assays could be improved.

4.2 Results and Discussion

The reaction between NEM and the sulfenic acid form of AhpC

NEM (1 mM, MW = 125.05 amu) was incubated with freshly prepared AhpC-SOH (MW = 20616). Based on our ESI-TOF MS results (Figure 33) it appears that maleimide reacts with the sulfenic acid, forming a covalent adduct which is differentiated from the thiol (SH)-maleimide adduct by 16 amu. While relative kinetic data could not be obtained due to air oxidation and the slow reactivity of AhpC-SOH with dimedone, the AhpC-SOH/NEM adduct forms slower than that of the thiol-maleimide adduct when compared at the same timepoints (data not shown). Two possible mechanisms exist for

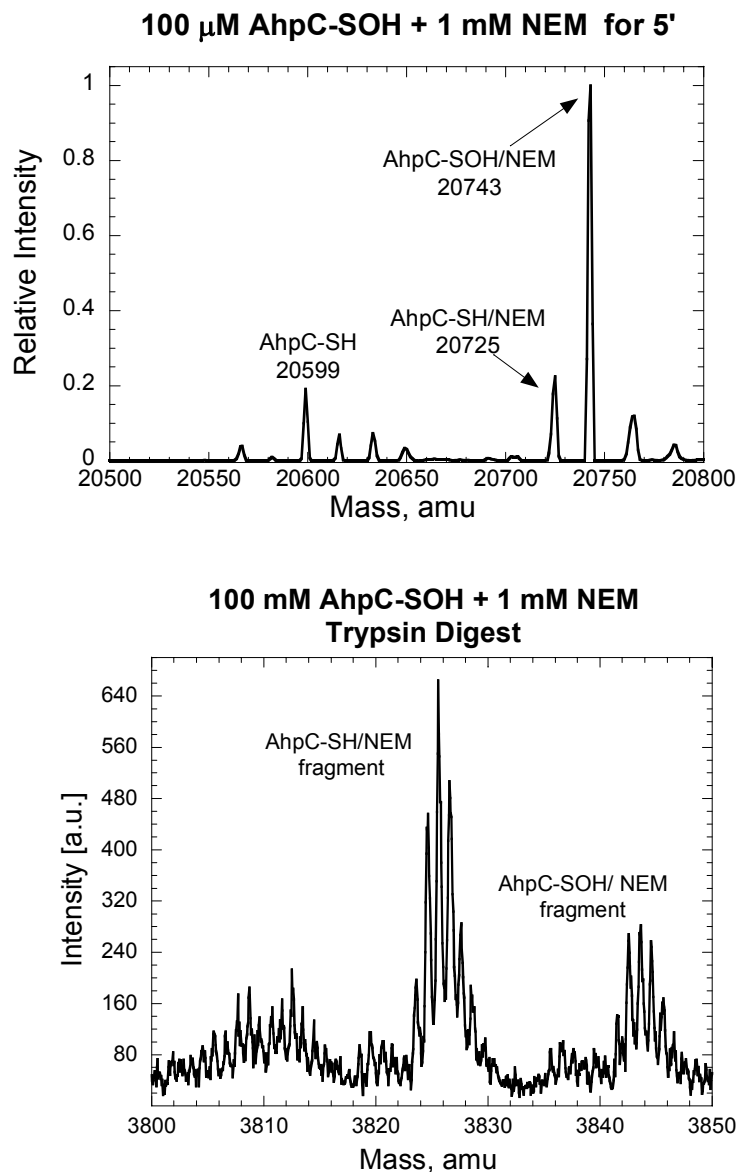
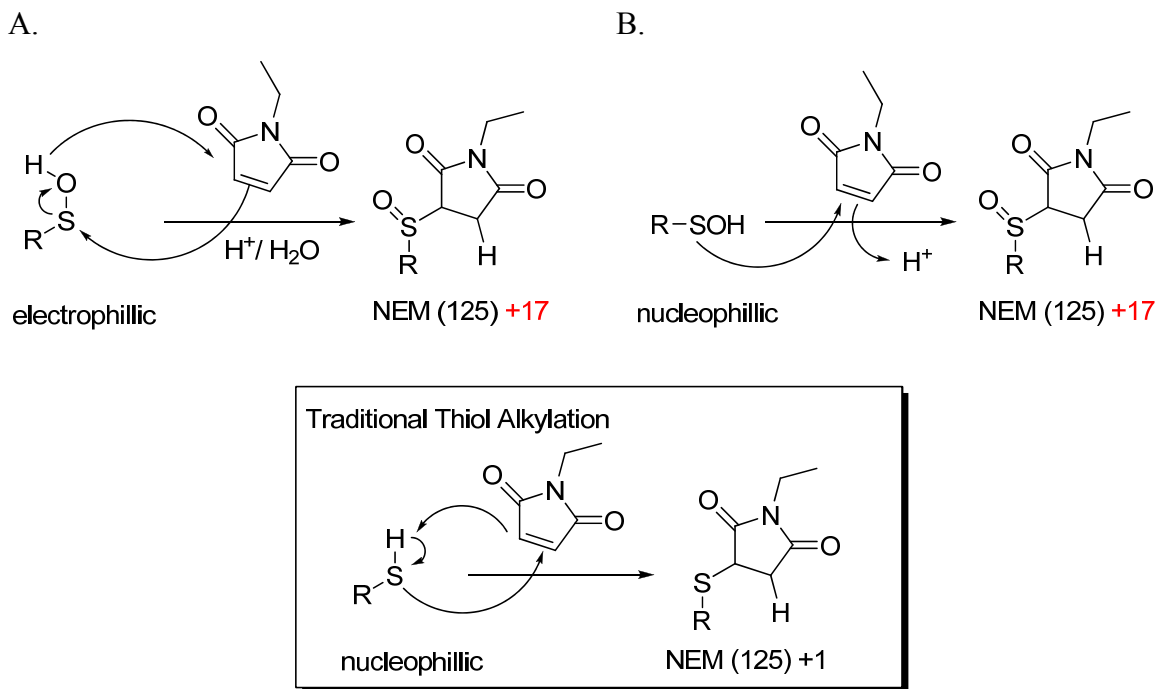


Figure 33. The reaction of AhpC-SOH with NEM **(Top)** Whole protein ESI-TOF MS of AhpC-SOH (100 μ M) with NEM (1 mM) at 5 min. gave a clear SOH-NEM adduct at 20743 amu The SH-NEM adduct was also visible due to a small % unreacted thiol. **(Bottom)** MALDI-TOF MS of the trypsin digest of this sample showing that NEM is covalently bound to the Cys-SOH residue on AhpC-SOH giving an adduct at 3842 amu which could be differentiated from the AhpC-SH/ NEM fragment at 3826 amu.

the formation of the observed AhpC-SOH/NEM adduct. The maleimide could react with AhpC-SOH through a concerted pericyclic reaction (Scheme 19A) or by direct nucleophilic addition of the AhpC-SOH to NEM (Scheme 19B). In both cases, the sulfoxide adduct would be produced.



Scheme 19. The reaction of NEM with protein sulfenic acids and thiols.

To map the NEM adduct to the sulfenic acid-containing Cys-46 residue, a trypsin digest was performed on this sample. For the digest, labeled protein (9.5 μL , 50 μM), H_2O (9.5 μL) and trypsin (1 μL of a 2.5 $\mu\text{g}/\mu\text{L}$ solution) were combined overnight at 37 $^{\circ}\text{C}$. The trypsin digestion was visualized using MALDI-TOF MS in a DHB matrix (2:1 DHB/ protein digest). As seen in Figure 33, a clear +16 amu adduct is observed in the cysteine-containing fragment of the digest. All other peptide fragments are present and

show no mass increase indicating a lack of methionine oxidation or NEM alkylation at any other region of the protein.

The reaction of AhpC-SH with NEM resulted in an adduct at 20725 amu only, which formed rapidly after 5 min of incubation (data not shown). However, a trypsin digestion of the sample shows a +16 peak in the cysteine-containing fragment (Figure 34). No Met residue exists on this fragment, and no evidence of Met oxidation at any of the Met-containing peptides elsewhere in the digest (data not shown). Since the whole protein ESI-MS for the AhpC-SH/NEM reaction showed no evidence of the +17 amu adduct, its presence in the digest suggests an air oxidation product formed during the prolonged digest conditions or from the high intensity MALDI ionization. This result warrants further investigation.

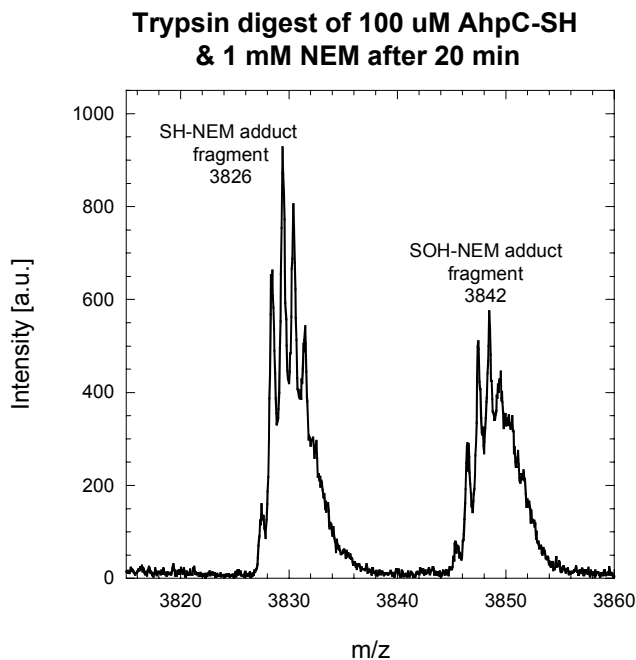


Figure 34. Trypsin digest of 100 μ M AhpC-SH + 1 mM NEM. After a 20 min incubation in AB buffer pH 7.6, the trypsin digest shows evidence of air-oxidation, generating the +16 amu adduct without the presence of AhpC-SOH.

The Reaction of bio-PEO-IAA and AhpC-SOH

Bio-PEO-IAA (8 mM, diluted from an 18 mM stock in H₂O, MW = 542.43 amu) was incubated with freshly prepared AhpC-SOH. AhpC-SOH reacted after 30 min to form a Cys46-SOH/ bio-PEO IAA adduct ($20598 + 416 + 18 = 21032$ amu) in good yield. This sulfoxide-containing adduct was 18 amu larger than the SH/ bio-PEO-IAA adduct (21014 amu) in the whole protein ESI-TOF MS (Figure 35). Additionally, there is some over-oxidized sulfinic acid (AhpC-SO₂H) observed in the whole protein ESI-MS which is un-reactive towards bio-PEO-IAA, resulting from air-oxidation of AhpC-SOH during the experiment. A trypsin digest of the sample showed a clear AhpC-SOH/bio-PEO-IAA adduct (+16 amu) on the cysteine-containing fragment in the MALDI-MS, suggesting that the +18 amu adduct in the whole protein ESI-MS could be due to the protonation of the sulfoxide adduct during the MS ionization process. Unlike the AhpC-SOH/NEM adduct, the SOH/bio-PEO-IAA adduct forms rapidly through the formation of a covalent sulfoxide product (Scheme 20).

To rule out the presence of methionine oxidation as a cause for the mass increase, AhpC-SH (100 μ M) and bio-PEO-IAA (8 mM) were incubated for 20 min at rt in HEN buffer (200 mM) followed by the rapid removal of bio-PEO-IAA and buffer exchange to AB (50 mM) using BioGel P6 spin filtration. The whole protein ESI-MS shows no evidence of the +18 amu adduct (data not shown). All of the digest peaks visualized in the MALDI were in accordance with the expected fragmentation for AhpC (Figure 36). Unlike the AhpC-SH/NEM adduct, there was no evidence of air-oxidation on the Cys-46 peptide. Additionally, we also saw no Met oxidation on any of the met-containing peptides (data not shown), Cys-SOH residue on AhpC-SOH, resulting in a new adduct at

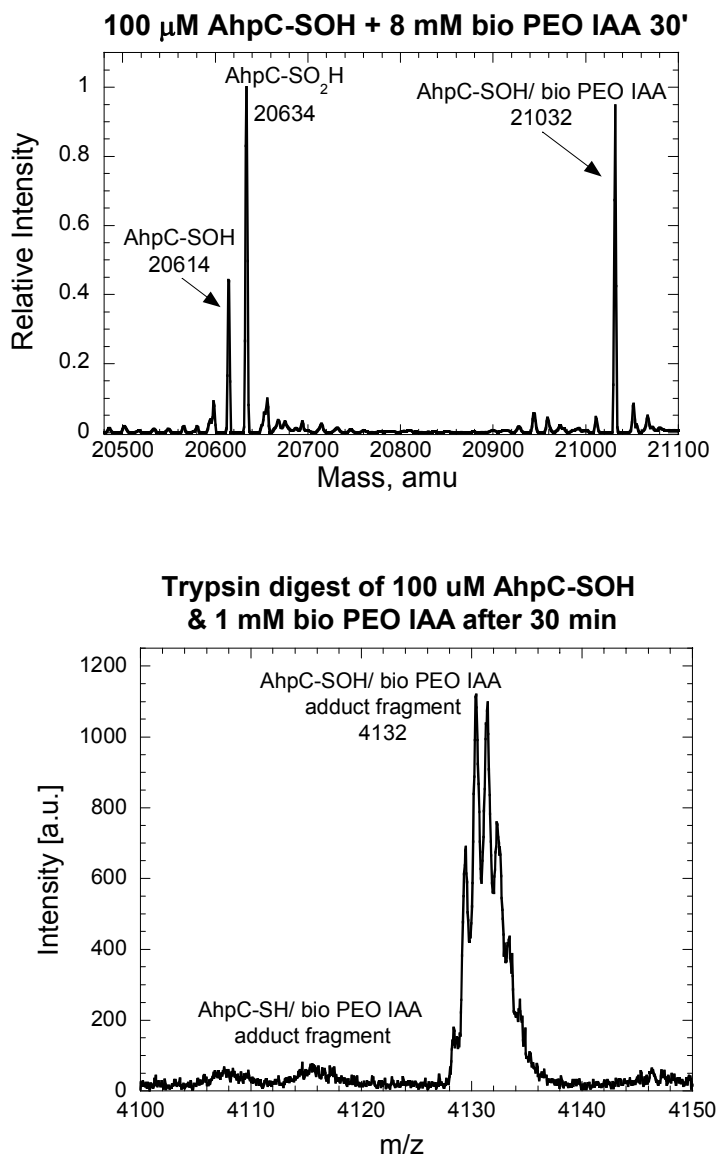


Figure 35. The reaction of AhpC-SOH with bio-PEO-IAA (**Top**) Whole protein ESI-TOF MS of 100 μ M AhpC-SOH + 1 mM bio-PEO-IAA after 30 min. After 30 min the bio-PEO-IAA was removed using a BioGel P6 spin column. The sulfoxide-containing AhpC-SOH/bio-PEO-IAA adduct (21032 amu) is clearly visible. (**Bottom**) MALDI-TOF MS of the trypsin digest of this sample. Bio-PEO-IAA is covalently bound to the is 16 amu larger than the SH/bio-PEO-IAA adduct.

The Reaction of MMTS with AhpC-SOH

MMTS (1 mM diluted from a 500 mM stock in DMSO, MW = 126.20 amu) was incubated with AhpC-SOH (100 μ M) for 20 min. Based on whole protein ESI-TOF MS (Figure 37) there is clear evidence of an AhpC-SOH/ MMTS adduct that can be differentiated from the AhpC-SH/ MMTS adduct by +16 amu. This result suggests the nucleophilic addition of AhpC-SOH to MMTS, forming the predicted sulfoxide product (Scheme 21). The whole protein ESI-MS also shows some AhpC-SH/MMTS adduct due to a fraction of unreacted thiol present at the time of MMTS incubation as well as AhpC-SO₂H due to air over-oxidation of the AhpC-SOH during the experiment.

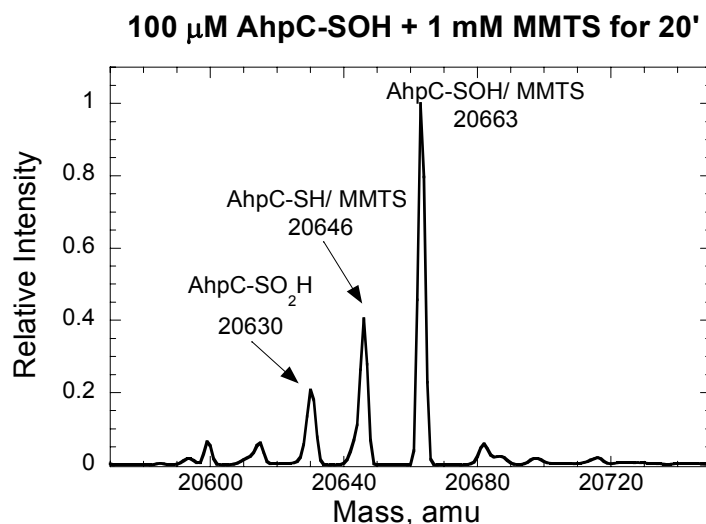
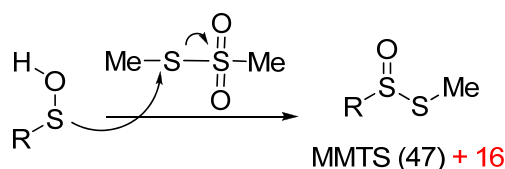


Figure 37. Whole protein ESI-TOF MS for the reaction of AhpC-SOH (100 μ M) with MMTS (1 mM). After the 20 min incubation, the MMTS was removed using a BioGel P6 spin column. There is evidence of a +16 amu AhpC-SOH/ MMTS adduct at 20663 amu.



Scheme 21. The reaction of MMTS with protein sulfenic acids.

The Reaction of Biotin-HPDP with AhpC-SOH

Biotin-HPDP (1mM, diluted from a 50 mM stock in DMSO, MW = 539.78 amu) was incubated with AhpC-SOH (100 μ M) for 60 min (Figure 38). Unlike the other alkylating agents, this reaction with AhpC-SOH did not provide strong evidence of an SOH/ biotin-HPDP adduct. For comparison, biotin HPDP (1 mM) labeled AhpC-SH >90% after 60 min based on the MALDI-TOF MS (data not shown). An unexpected

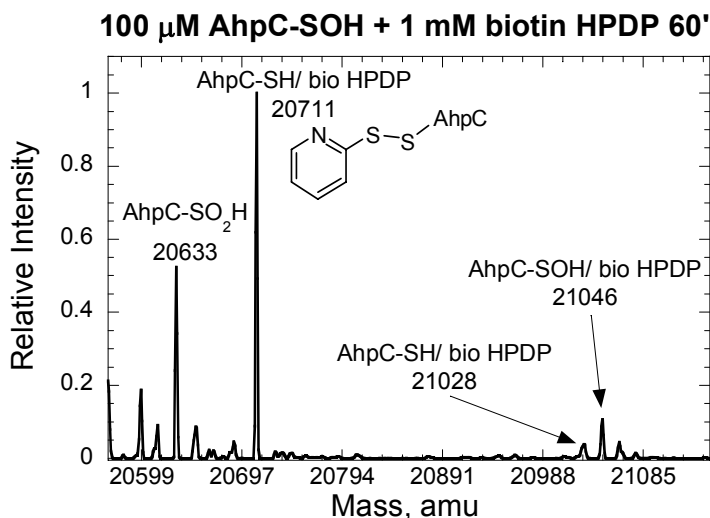
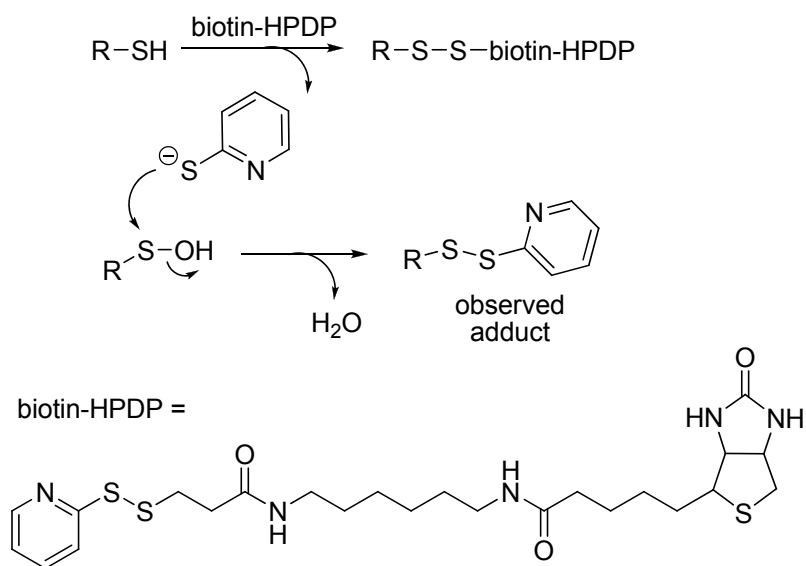


Figure 38. Whole protein ESI-TOF MS of AhpC-SOH (100 μ M) and biotin-HPDP (1 mM). After 60 min, the biotin-HPDP was removed using a BioGel P6 spin column. While AhpC-SOH did react with biotin-HPDP to form the mixed disulfide adduct (20711 amu), the expected biotinylated adduct did not form to any appreciable degree.

adduct was observed with the pyridyl-substituted mixed disulfide (20711 amu), which was not seen when AhpC-SH and biotin-HPDP were incubated. The failure to detect a biotinylated mixed disulfide adduct with AhpC-SOH remains unclear. If trace AhpC-SH was present at the beginning of the experiment, the pyridyl-containing thiolate by-product could immediately displace water from an AhpC-SOH, forming the observed adduct (Scheme 22). However, if large concentrations of AhpC-SH were present in solution at the beginning of the experiment, it is unclear why only trace AhpC-SH/biotin-HPDP adduct was observed in the whole protein ESI MS. While these results are confusing and warrant further investigation, the present result is that no biotinylated adduct was observed between AhpC-SOH and biotin-HPDP.



Scheme 22. The reaction of biotin-HPDP with protein sulfenic acids

Verifying protein sulfenic acid formation

For all AhpC-SOH experiments, NBD-Cl was used to determine the extent of sulfenic acid formation each time. As seen in Table 1, AhpC-SH/ SOH concentrations were compared before and after H₂O₂ treatment respectively. The percentage of free thiol (AhpC-SH) after DTT reduction was compared to the percentage of AhpC-SOH formation after treatment with 1 eq. H₂O₂ to gauge the efficiency of oxidation. Percentages are based on UV/vis absorbance at 280 nm ($\epsilon_{\text{AhpC}} = 24300 \text{ M}^{-1}\text{cm}^{-1}$), 347 nm ($\epsilon_{\text{SOH}} = 13,000 \text{ M}^{-1}\text{cm}^{-1}$), and 420 nm ($\epsilon_{\text{SH}} = 13,000 \text{ M}^{-1}\text{cm}^{-1}$). In all cases there was close to 100 % conversion to the RSOH, but the slight differences may reflect trace sulfinic acid formation or variable NBD-Cl incubation times.

Table 1. Percent conversion of AhpC-SH to sulfenic acid after H₂O₂ treatment, but before alkylation

Experiment	% thiol	% sulfenic acid
100 μM AhpC-SOH + 1 mM NEM	84 %	86 %
100 μM AhpC-SOH + 8 mM bio-PEO-IAA	94 %	83 %
100 μM AhpC-SOH + 1mM bio HPDP	100 %	94 %

The reaction of AhpC-SOH with Sodium Ascorbate

The effect of ascorbate on protein sulfenic acid content was investigated using a combination of NBD-Cl and gel-based techniques with AhpC-SOH and AhpC-SNO. For the NBD-Cl experiments, AhpC (200 μM) was added to H₂O₂ (200 μM) for 1 min. to

form AhpC-SOH. The samples were incubated with or without ascorbate (100mM) in HEN buffer. The ascorbate was removed from samples using a spin column containing Sephadex G-25 resin (1 mL) and the reaction was quenched with NBD-Cl (20 eq.). In all cases, NBD-Cl was incubated for 30 min. in the dark. Excess NBD-Cl was removed through a spin column, and samples were diluted to a total volume of 1mL. Using UV/vis to compare the total protein and SOH/SH concentrations, we confirmed a

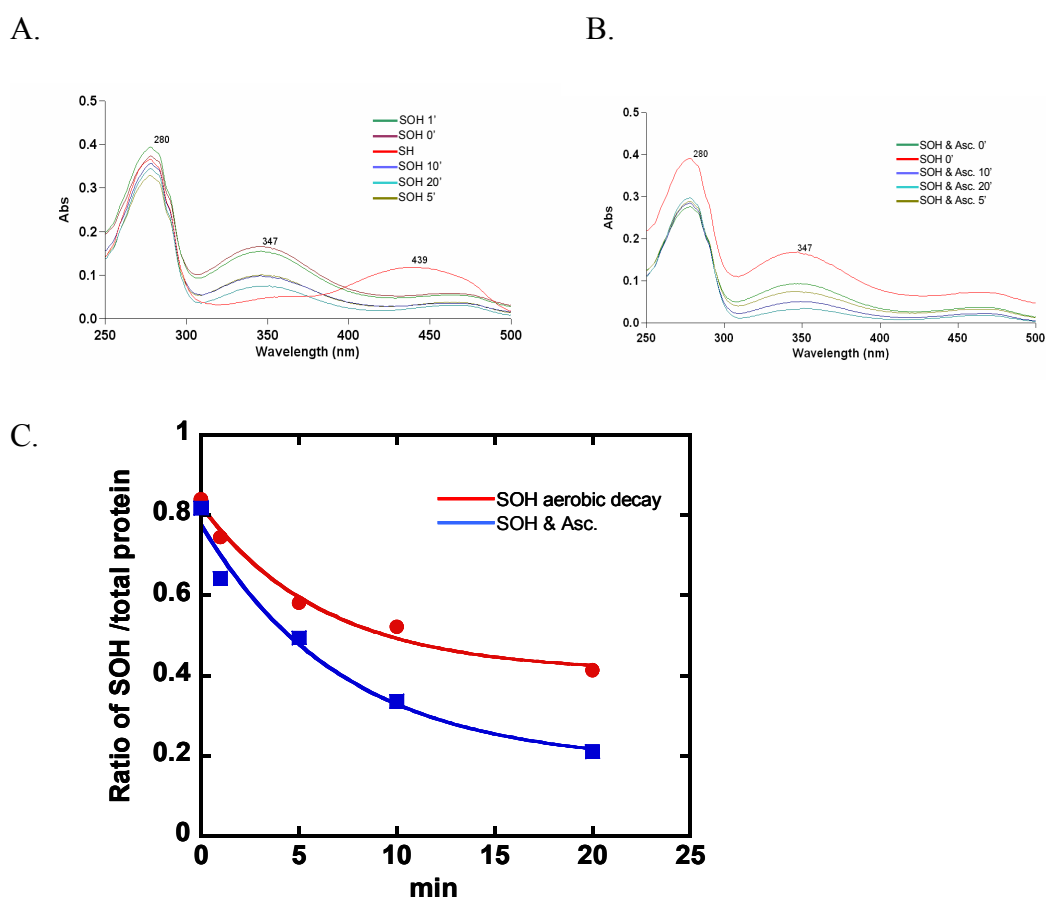


Figure 39. The reaction of sodium ascorbate with AhpC-SOH. **(A)** Normal aerobic over-oxidation of AhpC-SOH. **(B)** The loss of an AhpC-SOH/NBD-Cl adduct upon incubation with 100 mM ascorbate. **(C)** A comparison of the % AhpC-SOH at various timepoints in the presence and absence of ascorbate.

measurable loss of AhpC-SOH upon incubation with ascorbate however the re-emergence of AhpC-SH (reduced form) was not observed as expected (Figure 39A). Additionally, the presence of ascorbate enhanced the loss of AhpC-SOH as opposed to normal aerobic oxidation (Figure 39B-C).

In a similar experiment, AhpC-SOH and ascorbate (25 mM) were incubated and at various time points (5 – 30 min), the excess ascorbate was removed and samples were run on 10% SDS-PAGE gels under non-reducing conditions. Surprisingly, AhpC-SOH formed covalent dimers upon exposure to ascorbate (Figure 40A), but we could not conclude whether this dimerization occurs through the formation of a disulfide (-S-S-) or a thiol sulfinate

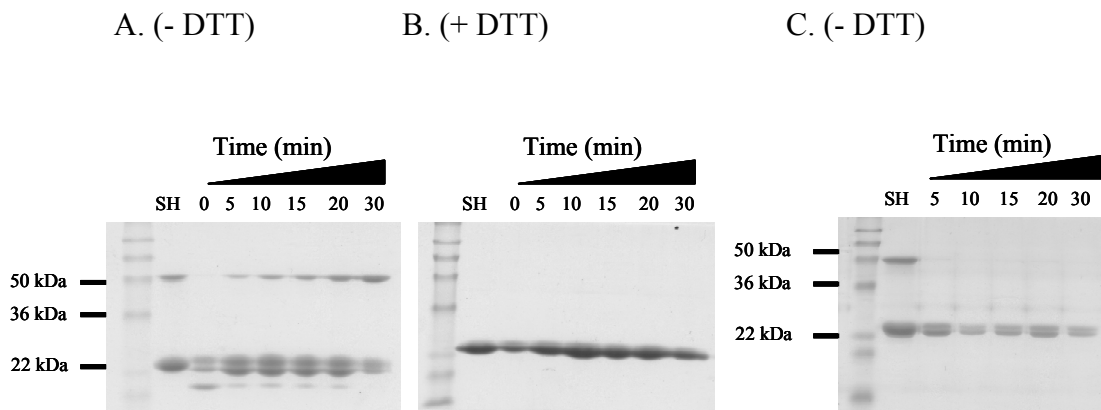


Figure 40. The reaction of AhpC-SOH and AhpC-SNO with sodium ascorbate. **(A)** Non-reducing SDS-PAGE gel of AhpC-SOH + 25 mM Asc. at various time points. The multiple bands observed around 21 kDa are characteristic of SOH formation. **(B)** Reducing SDS-PAGE gel of AhpC-SOH + 25 mM Asc. **(C)** Non-reducing SDS-PAGE gel of AhpC-SNO + 25 mM Asc. at various time points.

bond (-SO-S-). When the same samples were run under reducing conditions on an SDS-PAGE gel, only reduced AhpC was visible (Figure 40B). To ensure that this dimerization was ascorbate-dependent and not a result of gel preparation, AhpC-SOH and ascorbate (25 mM) were combined again but the reaction was quenched through the addition of NEM and dithionite (1 mM each) instead of the removal of ascorbate. Under these conditions, dimerization is also observed, indicating that dimerization is an ascorbate-dependent phenomenon (data not shown). The dimerization of AhpC-SOH also explains why the re-generation of AhpC-SH did not occur in the NBD-Cl experiments.

AhpC-SNO and ascorbate (25 mM) were incubated under the same experimental conditions. The lack of covalent dimer formation (Figure 40C) suggests the rapid reduction of the RS-NO bond to free thiol. UV/vis experiments measuring the RS-NO bond decay at 336 nm show a rapid loss of the RS-NO within 30 min after ascorbate incubation (data not shown). Using NBD-Cl, we also observe a re-generation of AhpC-SH (data not shown) indicating that ascorbate is reducing the RS-NO bond to RSH as reported.

Tryptophan (Trp) fluorescence experiments suggest that ascorbate is acting as a denaturant of AhpC-SOH (Figure 41), which may account for the observed dimerization, by making the RSOH residues more accessible. A marked decrease in Trp fluorescence emission intensity at 355 nm was observed as ascorbate concentration increases from 10 μ M to 100 mM. Trp residues are typically buried within the protein core and since the excited-state dipole moment of Trp is quite large, the emission energy is highly sensitive to the polarity of the environment. Following large changes in the tertiary structure of the

protein, decreases in fluorescence may indicate unfolding¹⁷⁴. As seen in Figure 41, Trp underwent a 95 % decrease in fluorescence emission from 10 μ M – 1 mM ascorbate and an even more dramatic reduction at higher concentrations.

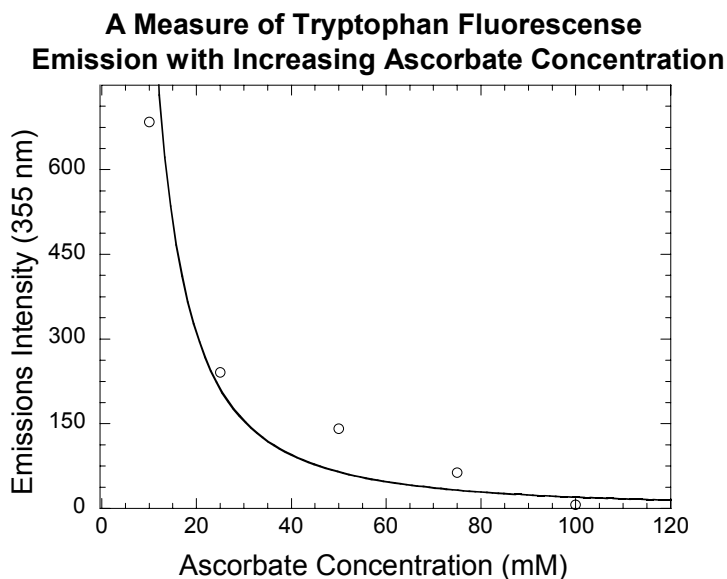


Figure 41. The denaturation of AhpC-SOH in the presence of ascorbate (25 mM). A loss of tryptophan emission intensity at 355 nm may indicate denaturation of the protein upon exposure to ascorbate.

The Biotin Switch Assay for AhpC-SOH and AhpC-SNO

To examine whether the putative non-specificity of the ascorbate reduction reaction would impact the outcome of a full Biotin Switch assay, purified AhpC-SH, AhpC-SOH, and AhpC-SNO were subjected to the BST and biotinylation was quantified based on Western blot analysis (Figure 42). To ensure no additional RSOH formation following the assay, an identical experiment was run pre-treating the buffers with DTPA

and catalase. As seen below, only biotinylation of the AhpC-SNO sample was observed, indicating that the BST selectively detected our RSNO under these conditions.

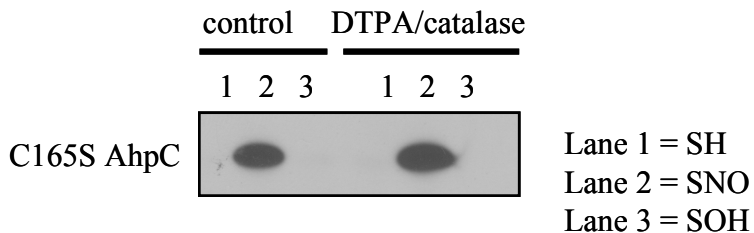
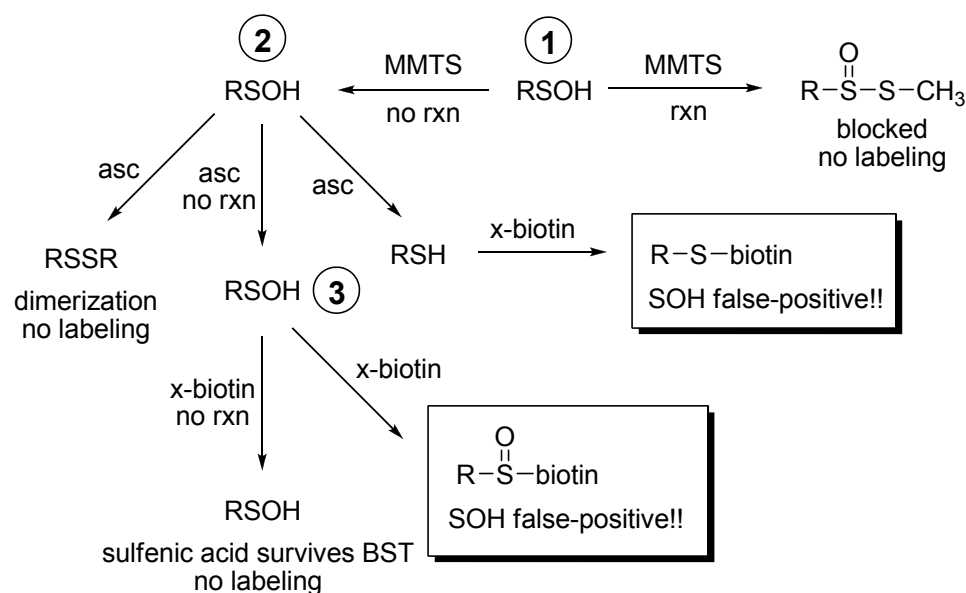


Figure 42. Western blot showing biotinylation following the BST. AhpC-SH, -SOH, and -SNO were subjected to a full Biotin Switch assay under two buffered conditions. The left 3 lanes represent the assay run in standard HEN buffer (pH 7.6) and the right 3 lanes represent the assay run after the buffer was pretreated with DTPA and catalase to prevent additional RSOH formation.

4.3 Sulfenic Acids Detection in the Context of the Biotin Switch Technique

The Biotin Switch assay represents one of the most widely used techniques for the semi-quantitative cellular quantification of protein S-nitrosothiols⁸⁶. As mentioned in the introduction, this assay, while useful, is controversial due to reports that ascorbate can reduce sulfenic acids and disulfides^{88, 89}. Since the Biotin Switch Technique is usually preformed on cell lysates, it is likely that both sulfenic acids and S-nitrosothiols, in addition to other oxidative modifications, would be present. A critical investigation of all aspects of this assay, including the specificity of thiol alkylating agents as well as the ascorbate reduction, was therefore performed.

Western blot analyses following a full BST in the presence of RSOH and RSNO show that only our S-nitrosated protein was biotinylated. This result is supported by our



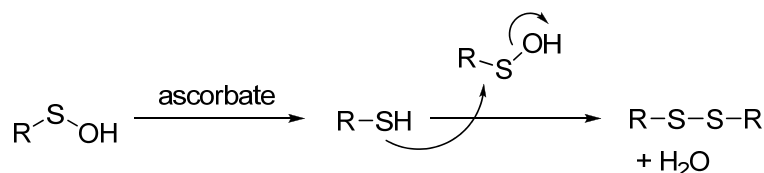
Scheme 23. The consequences of SOH cross-reactivity in the BST

data and has multiple explanations. Scheme 23 summarizes all of the possible outcomes for a sulfenic acid subjected to the BST, and how false positives would be generated. First, we report that the reaction of MMTS with AhpC-SOH produces a covalent adduct. Since MMTS is typically the blocking agent of choice for the first step of the BST, both free thiols and sulfenic acids would be expected to be “blocked” at the beginning of the assay. Alternative thiol alkylating agents have been used in place of MMTS, however, in such cases an RSOH may or may not become alkylated prior to the ascorbate incubation. Even in the case of inefficient blocking of RSOH, our data suggests that ascorbate would induce dimerization of AhpC-SOH, and the resulting dimer would not be biotinylated in the final step of this assay. This explains why we see no evidence of biotinylation in our Western blot (Figure 42). However, these reactivities may change depending on the

nature of the RSOH. It is possible that in some instances, ascorbate will reduce the RSOH bond to RSH which would be biotinylated in the last step of the BST. Additionally, if the RSOH does not dimerize in the presence of ascorbate, our data suggest that biotinylation could occur in the last step of the BST forming a sulfoxide-containing adduct which would be detected in the Western blot.

Despite the conclusion that the BST can selectively detect AhpC-SNO in the presence of AhpC-SOH, these data highlight a deeper concern that the reagents used for this technique, while effective in our system, do not react as described in the literature. The alkylation of protein sulfenic acids by thiol-specific reagents could vary from protein to protein and will depend on the stability and location of the sulfenic acid. This reactivity has implications in the context of the BST, but also in similar labeling experiments which aim to quantify cellular sulfenic acids. Blocking free thiols is typically thought to enhance the specificity of these assays, and prevent dimerization and additional RSOH formation upon lysis⁹⁸. However, depending on the alkylating agent used, a reaction with RSOH could occur to block their detection.

The ascorbate-dependent dimerization of AhpC-SOH may be due to a rapid reduction to AhpC-SH and dimerization with a neighboring AhpC-SOH to release water (Scheme 24). Sulfenic acids are highly unstable intermediates and would be expected to dimerize rapidly in the presence of free thiols. The denaturation of AhpC-SOH by



Scheme 24. The ascorbate-induced dimerization of AhpC-SOH

ascorbate would enhance this dimerization. These results highlight the importance of a more thorough investigation of the reaction between protein sulfenic acids and sodium ascorbate.

A few minor changes can be made to improve the specificity of the BST and other labeling assays. Our results suggest that protein sulfenic acids *could* generate false-positive results in the BST on a protein-by-protein basis. To ensure consistency in this assay, sulfenic acids should be “blocked” in the same manner as protein thiols. To prevent any cross-reactivity with thiol alkylating agents, prior to the first step of the BST (where samples are treated with MMTS), dimedone could be added to the samples. Since dimedone is known to be highly specific for RSOH, we expect it to “block” all sulfenic acid intermediates present in the lysate. The assay would proceed as usual, eliminating any possibility of biotinylating sulfenic acid residues.

4.4 Conclusions

Using a stable protein sulfenic acid (C165S AhpC-SOH), common thiol alkylating agents were examined and shown to have cross-reactivity with this model RSOH. To our knowledge, this is the first time that thiol alkylating agents were clearly shown to react with cysteine sulfenic acids to give sulfoxide-containing products. NEM, bio-PEO-IAA, and MMTS reacted with AhpC-SOH to form covalent adducts which retained the sulfenic acid oxygen. These products could be differentiated from the AhpC-SH adducts using MS-based methods. Sodium ascorbate, an S-nitrosothiol-specific reducing agent, was shown to denature AhpC-SOH and induce dimerization of the

protein. These results highlight the importance of controls and understanding both the utility and limitations of the Biotin Switch assay.

4.5 Materials and Methods

General: 5,5-Dimethyl-1,3-cyclohexanedione (dimedone), 4-Chloro-7-nitrobenzofurazan (NBD-Cl), Neocuproine, biotin maleimide (bio mal), biotin polyethylene oxide iodoacetamide (biotin PEO IAA), DL-Methionine sulfoxide (Met-O), methyl methanethiolsulfonate (MMTS), and N-ethyl maleimide (NEM) were purchased from Sigma. A hydrogen peroxide solution (10.4 M in water) was purchased from Fluka. EZ link biotin HPDP was purchased from Pierce (Thermo Scientific), HEPES free acid, was purchased from Omnipur (EM Science), and 1,4-dithiothreitol (DTT) from Anatrace. Ethylenediamine tetraacetic acid (EDTA), and ammonium bicarbonate were purchased from Research Organics. BioGel P6 gel and spin columns were purchased from BioRad.

Protein expression and purification

The mutant form of *Salmonella typhimurium* AhpC, C165S, was overexpressed and purified from *Escherichia coli* as previously described^{146, 175}. AhpC concentrations were determined by absorbance¹⁴⁶ at 280 nm with ϵ 24,300M⁻¹cm⁻¹.

Fluorescence Measurements

Fluorescence spectroscopy was performed on a Tecan SAFIRE II ® spectrometer. Tryptophan (Trp) emissions spectra were recorded at 355 nm upon excitation at 280 nm. Temperature was held at 30 °C for the duration of the experiment¹⁷⁴.

MALDI-TOF mass spectrometry

Reflectron mass spectrometry was performed on an Bruker Autoflex MALDI-TOF instrument with a 337 nm nitrogen laser source.

ESI-TOF whole protein mass spectrometry

Protein samples (50 µM) in ammonium bicarbonate (50 mM) were submitted to the Wake Forest School of Medicine Mass Spectrometry facility for ESI-TOF analysis.

Preparation of AhpC sulfenic acids

Unless otherwise stated, all experiments were run in a HEPES (250 mM), EDTA (100 µM), neocuproine (10 µM) buffered solution (HEN buffer, pH 7.6). AhpC was reduced for 30 min. at 4°C in DTT (20 mM), then reisolated using BioGel P6 spin filtration columns before all experiments. AhpC (100 µM) was combined with H₂O₂ (100 µM) for 30 sec. at room temperature to form the sulfenic acid (AhpC-SOH). Successful synthesis of AhpC-SOH was confirmed each time by NBD-Cl absorbance measurements⁶¹ at 347 nm with $\epsilon_{\text{SOH}} = 13000 \text{ M}^{-1}\text{cm}^{-1}$.

General procedure for reacting AhpC-SOH with thiol alkylating agents

Stock solutions of NEM (100 mM in DMSO), bio-PEO-IAA (18 mM in H₂O), biotin-HPDP (50 mM in DMSO), and MMTS (500 mM in DMSO) were made fresh prior to use. In all cases, freshly prepared AhpC-SOH (as described above) was incubated with various concentrations of each reagent. Care was taken to ensure that no more than 2% DMSO was present in the reaction mixture, as AhpC precipitates at concentrations >2%. After incubation, AhpC-SOH was reisolated and buffer exchanged into ammonium bicarbonate (50 mM) using BioGel P6 spin filtration columns at a variety of time points between 1 min. – 60 min. Samples were analyzed using MALDI TOF and ESI TOF MS for SOH-derived sulfoxide adducts.

Preparation of AhpC S-nitrosothiol

AhpC-SNO was made according to the same protocol summarized in Ch. 2.

MALDI-TOF protein digest analysis

Trypsin digest followed by MS analysis was used to verify the covalent attachment of NEM and bio-PEO-IAA to the Cys₄₆-SOH on AhpC and rule out methionine oxidation (Met-O) as the source of the +16 amu peak in the whole protein MS. AhpC samples (19 µL in 50 µM in ammonium bicarbonate) were incubated with a Trypsin solution (1 µL of 2.5 µg/ µL) for 24 h. at 37°C. A dihydroxy benzoic acid (DHB) matrix was used and all mass spectra were internally calibrated using the masses of known peptide fragments with an α -Cyano-4-hydroxycinnamic acid (CHCA) matrix.

CONCLUSIONS

This dissertation focuses on the development of new chemical probes to covalently modify and label S-nitrosothiols and sulfenic acids, two cysteine-based oxidative signaling modifications. Chapter 1 extensively reviews their unique roles as post-translational modifications involved in cellular signal transduction. Additionally, we provide data in Chapter 4 which suggests that a current method for detecting S-nitrosation (the Biotin Switch Technique) could detect sulfenic acid formation within the proteome as well. MS-based methods demonstrated that many of the reagents used during the Biotin Switch Technique are non-specific and had an unexpected cross-reactivity with sulfenic acids. Specifically, the thiol alkylating agents NEM, bio-PEO-IAA, and MMTS reacted with a model protein sulfenic acid to form sulfoxide-based covalent products. These data suggests that in the presense of sulfenic acids false-positive results could arise using the Biotin Switch Assay. We also present data that ascorbate, used to reduce RSNOs during the assay, acts as a denaturant and initiates dimerization in our sulfenic acid model.

After questioning the specificity of the Biotin Switch Technique, we sought to develop highly specific chemical-based probes for the detection of RSNO and RSOH modifications on proteins. Chapter 2 reports the use of a water-soluble triaryl phosphine (TXPTS) to covalently label Cys-SNO, GSNO, and C165S AhpC-SNO. In all cases, the phosphine reacted to form an S-alkyl phosphonium product with phosphine oxide and an SNO-derived aza-ylide as by-products of the reaction. TXPTS did not react with the free thiol or disulfide forms of cysteine, glutathione, or C165S AhpC and is therefore a

promising candidate for labeling cellular S-nitrosation. The use of phosphines to detect RSNOs would also circumvent the need to use the Biotin Switch Technique.

We have also expanded on previous work in our lab and synthesized novel dimedone-based chemical probes for the quantification of protein sulfenic acids. In Chapter 3 we report the synthesis of a DABCYL-based probe (**14**), which can act as a universal FRET acceptor. This reagent provides a platform for future FRET-based RSOH experiments. We also report the synthesis of an alkyne-containing probe (**20**), with which click-type reactions can conjugate a variety of fluorescent and biotinylated derivatives to RSOHs. In Chapter 3 we also discuss our advancements towards the synthesis of an ICAT-capable biotin-azide for the quantification of RSOHs within complex proteomes at a variety of time-points.

In the future, we hope to develop a comprehensive assay for the simultaneous detection of RSNO and RSOH modifications. Detecting both of these reactive intermediates in a single experiment would provide meaningful information on their roles in signal transduction, and would also highlight the differences between the two types of cysteine oxidation in order to differentiate their *in vivo* roles.

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“WATER-SOLUBLE TRIARYLPHOSPHINES AS BIOMARKERS FOR PROTEIN S-NITROSATION”

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CRYSTAL STRUCTURE ANALYSIS FOR COMPOUND (1)

Colorless crystals of $C_{37}H_{30}NPS - 0.5 C_7H_8$ are, at 193(2) K, monoclinic, space group $P2_1/c - C_{2h}^5$ (No. 14) with $a = 17.631(2) \text{ \AA}$, $b = 10.525(1) \text{ \AA}$, $c = 17.177(2) \text{ \AA}$, $\beta = 101.859(2)^\circ$, $V = 3119.5(7) \text{ \AA}^3$, and $Z = 4$ $\{d_{\text{calcd}} = 1.273 \text{ g cm}^{-3}$; $\mu_a(\text{MoK}\alpha) = 0.186 \text{ mm}^{-1}\}$. A full hemisphere of diffracted intensities (1868 30-second frames with an ω scan width of 0.30°) was measured for a single-domain specimen using graphite-monochromated MoK α radiation ($\lambda = 0.71073 \text{ \AA}$) on a Bruker SMART APEX CCD Single Crystal Diffraction System. X-rays were provided by a fine-focus sealed x-ray tube operated at 50kV and 30mA.

Lattice constants were determined with the Bruker SAINT software package using peak centers for 3979 reflections having $7.72^\circ \leq 2\theta \leq 52.08^\circ$. A total of 25048 integrated reflection intensities having $2\theta(\text{MoK}\alpha) \leq 53.00^\circ$ were produced using the Bruker program SAINT; 6427 of these were unique and gave $R_{\text{int}} = 0.054$ with a coverage which was 99.4% complete. The data were corrected for scaling and variable absorption effects using SADABS; the ratio of minimum and maximum transmission values was 0.81618.

The Bruker software package SHELXTL was used to solve the structure using “direct methods” techniques. All stages of weighted full-matrix least-squares refinement were conducted using F_o^2 data with the SHELXTL Version 6.14 software package. The resulting structural parameters have been refined to convergence $\{R_1$ (unweighted, based on F) = 0.0591 for 5070 independent reflections having $2\theta(\text{MoK}\alpha) < 53.00^\circ$ and $F^2 > 2\sigma(F^2)\}$ $\{R_1$ (unweighted, based on F) = 0.0761 and wR_2 (weighted, based on F^2) = 0.1476 for all 6427 reflections} using counter-weighted full-matrix least-squares techniques and a structural model which incorporated anisotropic thermal parameters for all nonhydrogen atoms. The toluene solvent molecule of crystallization lies on a crystallographic inversion center at $\frac{1}{2}, \frac{1}{2}, 0$ in the unit cell and is therefore disordered with two orientations in the crystal. Restraints were imposed on the positional and displacement parameters of the seven (half-occupancy) carbon atoms. The hydrogen atoms were included in the structure

factor calculations as idealized atoms (assuming sp^2 - hybridization of the carbon atoms and C-H bond lengths of 0.95Å) "riding" on their respective carbon atoms. The isotropic thermal parameters of the hydrogen atoms were fixed at values 1.2(non-methyl) times the equivalent isotropic thermal parameters of the carbon atoms to which they are covalently bonded. Hydrogen atoms on the methyl group of the disordered toluene molecule were not included in the structural model. A total of 409 parameters were refined using 61 restraints and 6427 data. The largest shift/s.u. was 0.000 in the final refinement cycle. The final difference map had maxima and minima of 0.482 and -0.585 $e^-/\text{\AA}^3$, respectively.

Acknowledgment

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Table 1. Crystal data and structure refinement for C₃₇H₃₀NPS – 0.5 C₇H₈

Identification code	a13m
Empirical formula	C _{40.50} H ₃₄ N P S
Formula weight	597.72
Temperature	193(2) K
Wavelength	0.71073 Å
Crystal system	Monoclinic
Space group	P2(1)/c – C _{2h} ⁵ (No. 14)
Unit cell dimensions	a = 17.631(2) Å b = 10.5248(13) Å, β = 101.859(2)° c = 17.177(2) Å
Volume	3119.5(7) Å ³
Z	4
Density (calculated)	1.273 g/cm ³
Absorption coefficient	0.186 mm ⁻¹
F(000)	1260
Crystal size	0.32 x 0.08 x 0.04 mm ³
Theta range for data collection	3.86 to 26.50°
Index ranges	-22 ≤ h ≤ 22, -13 ≤ k ≤ 13, -21 ≤ l ≤ 21
Reflections collected	25048
Independent reflections	6427 [R(int) = 0.0539]
Completeness to theta = 26.50°	99.4 %
Absorption correction	Multi-scan (SADABS)
Ratio of Min./Max. transmission	0.8162
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	6427 / 61 / 409
Goodness-of-fit on F ²	1.114
Final R indices [5070 I > 2σ(I) data]	R ₁ = 0.0591, wR ₂ = 0.1386
R indices (all data)	R ₁ = 0.0761, wR ₂ = 0.1476
Largest diff. peak and hole	0.482 and -0.585 -/Å ³

$$R_1 = \sum ||F_o| - |F_c|| / \sum |F_o|$$

$$wR_2 = \{ \sum [w(F_o^2 - F_c^2)^2] / \sum [w(F_o^2)^2] \}^{1/2}$$

Table 2. Atomic coordinates ($\times 10^4$) and equivalent isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for $\text{C}_{37}\text{H}_{30}\text{NPS} - 0.5 \text{ C}_7\text{H}_8$. $U(\text{eq})$ is defined as one third of the trace of the orthogonalized U_{ij} tensor.

	x	y	z	$U(\text{eq})$
S(1)	2531(1)	3970(1)	1802(1)	25(1)
P(1)	3438(1)	1916(1)	1357(1)	25(1)
N(1)	3092(1)	3312(2)	1231(1)	30(1)
C(1)	1619(1)	4444(2)	1099(1)	22(1)
C(1A)	1084(1)	4768(2)	1671(1)	25(1)
C(2A)	1320(2)	5700(2)	2254(2)	32(1)
C(3A)	841(2)	6079(3)	2752(2)	41(1)
C(4A)	114(2)	5541(3)	2680(2)	44(1)
C(5A)	-121(2)	4619(3)	2120(2)	45(1)
C(6A)	359(1)	4231(2)	1618(2)	35(1)
C(1B)	1319(1)	3336(2)	545(1)	24(1)
C(2B)	1082(1)	3477(2)	-270(1)	31(1)
C(3B)	799(2)	2453(3)	-756(2)	38(1)
C(4B)	748(2)	1272(3)	-428(2)	40(1)
C(5B)	980(2)	1108(2)	383(2)	36(1)
C(6B)	1265(1)	2126(2)	865(2)	29(1)
C(1C)	1736(1)	5646(2)	627(1)	24(1)
C(2C)	1123(2)	6478(2)	378(2)	33(1)
C(3C)	1216(2)	7560(2)	-56(2)	39(1)
C(4C)	1915(2)	7828(3)	-248(2)	43(1)
C(5C)	2525(2)	7003(3)	-13(2)	45(1)
C(6C)	2442(2)	5917(2)	422(2)	36(1)
C(1D)	2979(1)	815(2)	1923(2)	28(1)
C(2D)	2601(2)	-260(2)	1580(2)	39(1)
C(3D)	2221(2)	-1057(3)	2014(2)	52(1)
C(4D)	2221(2)	-784(3)	2794(2)	54(1)
C(5D)	2586(2)	297(3)	3142(2)	50(1)
C(6D)	2968(2)	1101(3)	2710(2)	37(1)
C(1E)	4457(1)	1907(2)	1832(1)	29(1)
C(2E)	4793(2)	921(3)	2311(2)	48(1)

C(3E)	5583(2)	927(3)	2637(2)	54(1)
C(4E)	6036(2)	1922(3)	2487(2)	48(1)
C(5E)	5718(2)	2861(3)	1970(2)	51(1)
C(6E)	4928(2)	2861(3)	1643(2)	41(1)
C(1F)	3403(1)	1208(2)	392(1)	29(1)
C(2F)	2825(2)	1601(3)	-239(2)	34(1)
C(3F)	2763(2)	1055(3)	-984(2)	41(1)
C(4F)	3274(2)	127(3)	-1100(2)	44(1)
C(5F)	3846(2)	-276(3)	-477(2)	48(1)
C(6F)	3914(2)	250(3)	268(2)	41(1)

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C(1G)	4903(4)	4180(8)	-257(4)	114(3)
C(2G)	4366(4)	4975(8)	-36(4)	121(4)
C(3G)	4563(5)	6110(9)	358(5)	142(4)
C(4G)	5315(6)	6465(9)	556(5)	138(4)
C(5G)	5884(5)	5682(11)	335(6)	144(4)
C(6G)	5688(4)	4568(10)	-58(6)	155(5)
C(7G)	4677(5)	2973(8)	-681(5)	137(5)

^a The numbers in parentheses are the estimated standard deviations in the last significant digit.

^b Atoms are labeled in agreement with Figures 1 - 4.

^c $U(eq)$ is defined as one third of the trace of the orthogonalized U^{ij} tensor.

^d The toluene solvent molecule of crystallization lies on a crystallographic inversion center at $\frac{1}{2}, \frac{1}{2}, 0$ in the unit cell and is therefore disordered with two orientations in the crystal. Restraints were imposed on the positional and displacement parameters of the seven (half-occupancy) carbon atoms.

Table 3. Bond lengths [$\text{\AA}$] and angles [$^\circ$] for $\text{C}_{37}\text{H}_{30}\text{NPS} - 0.5 \text{ C}_7\text{H}_8$

S(1)-N(1)	1.679(2)	S(1)-C(1)	1.870(2)
P(1)-N(1)	1.589(2)		
P(1)-C(1D)	1.807(2)	C(1)-C(1B)	1.530(3)
P(1)-C(1F)	1.806(2)	C(1)-C(1A)	1.533(3)
P(1)-C(1E)	1.813(2)	C(1)-C(1C)	1.539(3)
C(1A)-C(6A)	1.384(3)	C(4D)-C(5D)	1.382(5)
C(1A)-C(2A)	1.402(3)	C(5D)-C(6D)	1.388(4)
C(2A)-C(3A)	1.379(4)	C(1E)-C(2E)	1.380(4)
C(3A)-C(4A)	1.384(4)	C(1E)-C(6E)	1.384(4)
C(4A)-C(5A)	1.370(4)	C(2E)-C(3E)	1.390(4)
C(5A)-C(6A)	1.387(4)	C(3E)-C(4E)	1.373(4)
C(1B)-C(2B)	1.384(3)	C(4E)-C(5E)	1.370(4)
C(1B)-C(6B)	1.398(3)	C(5E)-C(6E)	1.391(4)
C(2B)-C(3B)	1.392(3)	C(1F)-C(2F)	1.390(3)
C(3B)-C(4B)	1.376(4)	C(1F)-C(6F)	1.397(3)
C(4B)-C(5B)	1.379(4)	C(2F)-C(3F)	1.386(4)
C(5B)-C(6B)	1.384(3)	C(3F)-C(4F)	1.371(4)
C(1C)-C(2C)	1.389(3)	C(4F)-C(5F)	1.379(4)
C(1C)-C(6C)	1.391(3)	C(5F)-C(6F)	1.378(4)
C(2C)-C(3C)	1.390(4)	C(1G)-C(2G)	1.3739
C(3C)-C(4C)	1.368(4)	C(1G)-C(6G)	1.4165
C(4C)-C(5C)	1.378(4)	C(1G)-C(7G)	1.4769
C(5C)-C(6C)	1.390(4)	C(2G)-C(3G)	1.3812
C(1D)-C(2D)	1.382(4)	C(3G)-C(4G)	1.3519
C(1D)-C(6D)	1.389(4)	C(4G)-C(5G)	1.4084
C(2D)-C(3D)	1.384(4)	C(5G)-C(6G)	1.3625
C(3D)-C(4D)	1.370(5)		
N(1)-S(1)-C(1)	105.34(10)	P(1)-N(1)-S(1)	123.91(12)
N(1)-P(1)-C(1D)	117.62(11)	N(1)-P(1)-C(1F)	108.43(11)

C(1D)-P(1)-C(1F)	107.14(11)	C(1D)-P(1)-C(1E)	105.45(11)
N(1)-P(1)-C(1E)	112.56(11)	C(1F)-P(1)-C(1E)	104.78(11)
		C(1B)-C(1)-S(1)	109.86(14)
C(1B)-C(1)-C(1A)	112.85(18)	C(1A)-C(1)-S(1)	101.91(14)
C(1B)-C(1)-C(1C)	111.44(18)	C(1C)-C(1)-S(1)	111.64(15)
C(1A)-C(1)-C(1C)	108.79(17)		
		C(2D)-C(1D)-C(6D)	119.4(2)
C(6A)-C(1A)-C(2A)	117.9(2)	C(2D)-C(1D)-P(1)	121.7(2)
C(6A)-C(1A)-C(1)	123.3(2)	C(6D)-C(1D)-P(1)	118.83(19)
C(2A)-C(1A)-C(1)	118.8(2)	C(1D)-C(2D)-C(3D)	120.7(3)
C(3A)-C(2A)-C(1A)	121.0(2)	C(4D)-C(3D)-C(2D)	119.9(3)
C(2A)-C(3A)-C(4A)	120.1(3)	C(3D)-C(4D)-C(5D)	120.1(3)
C(5A)-C(4A)-C(3A)	119.5(2)	C(4D)-C(5D)-C(6D)	120.4(3)
C(4A)-C(5A)-C(6A)	120.7(3)	C(1D)-C(6D)-C(5D)	119.6(3)
C(1A)-C(6A)-C(5A)	120.8(3)	C(2E)-C(1E)-C(6E)	118.7(2)
C(2B)-C(1B)-C(6B)	117.7(2)	C(2E)-C(1E)-P(1)	122.5(2)
C(2B)-C(1B)-C(1)	122.8(2)	C(6E)-C(1E)-P(1)	118.48(19)
C(6B)-C(1B)-C(1)	119.5(2)	C(1E)-C(2E)-C(3E)	120.6(3)
C(1B)-C(2B)-C(3B)	121.3(2)	C(4E)-C(3E)-C(2E)	120.1(3)
C(4B)-C(3B)-C(2B)	119.9(2)	C(5E)-C(4E)-C(3E)	119.7(3)
C(3B)-C(4B)-C(5B)	119.8(2)	C(4E)-C(5E)-C(6E)	120.3(3)
C(4B)-C(5B)-C(6B)	120.2(2)	C(1E)-C(6E)-C(5E)	120.3(3)
C(5B)-C(6B)-C(1B)	121.0(2)	C(2F)-C(1F)-C(6F)	119.2(2)
C(2C)-C(1C)-C(6C)	118.2(2)	C(2F)-C(1F)-P(1)	118.34(18)
C(2C)-C(1C)-C(1)	120.0(2)	C(6F)-C(1F)-P(1)	122.4(2)
C(6C)-C(1C)-C(1)	121.7(2)	C(3F)-C(2F)-C(1F)	120.2(2)
C(1C)-C(2C)-C(3C)	120.8(2)	C(4F)-C(3F)-C(2F)	120.0(3)
C(4C)-C(3C)-C(2C)	120.6(2)	C(3F)-C(4F)-C(5F)	120.3(3)
C(3C)-C(4C)-C(5C)	119.2(2)	C(6F)-C(5F)-C(4F)	120.5(3)
C(4C)-C(5C)-C(6C)	120.9(3)	C(5F)-C(6F)-C(1F)	119.8(3)
C(5C)-C(6C)-C(1C)	120.2(2)		

^a The numbers in parentheses are the estimated standard deviations in the last significant digit.

^b Atoms are labeled in agreement with Figures 1 -4.

Table 4. Anisotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for $\text{C}_{37}\text{H}_{30}\text{NPS} - 0.5 \text{C}_7\text{H}_8$.
The anisotropic displacement factor exponent takes the form: $-2\pi^2 [h^2 a^{*2} U^{11} + \dots + 2 h k a^* b^* U^{12}]$

	U ¹¹	U ²²	U ³³	U ²³	U ¹³	U ¹²
S(1)	23(1)	24(1)	25(1)	-1(1)	1(1)	2(1)
P(1)	22(1)	23(1)	29(1)	3(1)	5(1)	3(1)
N(1)	28(1)	25(1)	39(1)	6(1)	11(1)	6(1)
C(1)	18(1)	21(1)	26(1)	-1(1)	2(1)	0(1)
C(1A)	25(1)	22(1)	27(1)	3(1)	4(1)	4(1)
C(2A)	34(1)	29(1)	33(1)	-3(1)	5(1)	2(1)
C(3A)	54(2)	37(2)	32(1)	-5(1)	11(1)	11(1)
C(4A)	50(2)	49(2)	41(2)	9(1)	25(1)	18(1)
C(5A)	33(1)	50(2)	60(2)	2(2)	22(1)	1(1)
C(6A)	29(1)	33(1)	45(2)	-4(1)	9(1)	1(1)
C(1B)	17(1)	24(1)	31(1)	-4(1)	3(1)	1(1)
C(2B)	28(1)	30(1)	33(1)	-2(1)	1(1)	3(1)
C(3B)	35(1)	44(2)	31(1)	-8(1)	-3(1)	3(1)
C(4B)	36(1)	35(2)	47(2)	-18(1)	1(1)	-4(1)
C(5B)	32(1)	25(1)	50(2)	-5(1)	7(1)	-3(1)
C(6B)	25(1)	27(1)	34(1)	-2(1)	4(1)	-2(1)
C(1C)	29(1)	22(1)	21(1)	-1(1)	1(1)	0(1)
C(2C)	32(1)	29(1)	36(1)	3(1)	4(1)	3(1)
C(3C)	50(2)	25(1)	36(1)	4(1)	-1(1)	7(1)
C(4C)	63(2)	26(1)	38(2)	7(1)	6(1)	-5(1)
C(5C)	46(2)	42(2)	50(2)	13(1)	15(1)	-6(1)
C(6C)	33(1)	33(1)	43(2)	7(1)	9(1)	0(1)
C(1D)	23(1)	26(1)	36(1)	7(1)	5(1)	5(1)
C(2D)	36(1)	30(1)	49(2)	7(1)	2(1)	-1(1)
C(3D)	39(2)	37(2)	74(2)	16(2)	0(2)	-8(1)
C(4D)	31(2)	53(2)	80(2)	31(2)	15(2)	-2(1)
C(5D)	43(2)	63(2)	49(2)	19(2)	20(1)	10(2)
C(6D)	33(1)	39(2)	41(2)	6(1)	10(1)	4(1)
C(1E)	27(1)	27(1)	33(1)	-1(1)	6(1)	4(1)
C(2E)	30(1)	55(2)	59(2)	23(2)	8(1)	7(1)

C(3E)	33(2)	68(2)	58(2)	21(2)	2(1)	14(2)
C(4E)	26(1)	52(2)	60(2)	-9(2)	-2(1)	6(1)
C(5E)	33(2)	34(2)	83(2)	-6(2)	4(2)	-7(1)
C(6E)	33(1)	29(1)	58(2)	5(1)	1(1)	-1(1)
C(1F)	28(1)	27(1)	33(1)	0(1)	9(1)	1(1)
C(2F)	33(1)	36(1)	34(1)	3(1)	10(1)	6(1)
C(3F)	40(2)	51(2)	31(1)	1(1)	6(1)	2(1)
C(4F)	46(2)	53(2)	34(1)	-9(1)	13(1)	-1(1)
C(5F)	47(2)	48(2)	52(2)	-10(1)	17(1)	13(1)
C(6F)	36(2)	45(2)	40(2)	-2(1)	4(1)	13(1)

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C(1G)	50(5)	202(10)	87(8)	60(7)	11(5)	-25(4)
C(2G)	52(5)	207(10)	101(8)	99(7)	10(5)	3(5)
C(3G)	63(6)	263(13)	98(8)	41(8)	6(6)	17(7)
C(4G)	82(7)	268(13)	55(6)	37(8)	-3(6)	-21(7)
C(5G)	46(5)	255(13)	119(9)	18(8)	-10(5)	-40(6)
C(6G)	52(5)	222(12)	185(13)	31(9)	11(8)	-28(6)
C(7G)	106(9)	175(10)	122(11)	82(7)	7(7)	-57(7)

^a The numbers in parentheses are the estimated standard deviations in the last significant digit.

^b The form of the anisotropic thermal parameter is: $\exp[-2\pi^2 (U_{11}h^2a^{*2} + U_{22}k^2b^{*2} + U_{33}l^2c^{*2} + 2U_{12}hka^*b^* + 2U_{13}hla^*c^* + 2U_{23}klb^*c^*)]$.

^c Atoms are labeled in agreement with Figures 1 - 4.

^d The toluene solvent molecule of crystallization lies on a crystallographic inversion center at $\frac{1}{2}, \frac{1}{2}, 0$ in the unit cell and is therefore disordered with two orientations in the crystal. Restraints were imposed on the positional and displacement parameters of the seven (half-occupancy) carbon atoms.

Table 5. Hydrogen coordinates ($\times 10^4$) and isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for $\text{C}_{37}\text{H}_{30}\text{NPS} - 0.5 \text{ C}_7\text{H}_8$

	x	y	z	U(eq)
H(2A)	1818	6076	2306	39
H(3A)	1011	6710	3145	49
H(4A)	-221	5810	3018	53
H(5A)	-617	4241	2074	55
H(6A)	188	3590	1233	42
H(2B)	1113	4290	-502	37
H(3B)	641	2570	-1315	46
H(4B)	554	571	-758	48
H(5B)	944	294	611	43
H(6B)	1426	2001	1422	35
H(2C)	634	6305	507	39
H(3C)	790	8119	-222	46
H(4C)	1978	8574	-539	52
H(5C)	3010	7178	-151	54
H(6C)	2869	5357	579	43
H(2D)	2603	-455	1040	47
H(3D)	1961	-1791	1772	62
H(4D)	1969	-1340	3096	65
H(5D)	2576	491	3679	60
H(6D)	3220	1843	2951	44
H(2E)	4482	233	2419	57
H(3E)	5809	243	2965	65
H(4E)	6568	1958	2740	57
H(5E)	6038	3516	1835	61
H(6E)	4711	3519	1287	49
H(2F)	2471	2245	-160	41
H(3F)	2366	1324	-1414	49
H(4F)	3233	-240	-1612	53
H(5F)	4197	-922	-563	57
H(6F)	4306	-38	697	49

Disordered Toluene of Crystallization

H(2G)	3836	4734	-159	145
H(3G)	4172	6639	489	171
H(4G)	5458	7232	841	165
H(5G)	6412	5936	462	173
H(6G)	6079	4049	-200	186

^a Hydrogen atoms were included in the structure factor calculations as idealized atoms (assuming sp^2 - hybridization of the carbon atoms and C-H bond lengths of 0.95Å) "riding" on their respective carbon atoms. The isotropic thermal parameters of the hydrogen atoms were fixed at values 1.2(non-methyl) times the equivalent isotropic thermal parameters of the carbon atoms to which they are covalently bonded. Hydrogen atoms on the methyl group of the disordered toluene molecule were not included in the structural model.

^b Hydrogen atoms bonded to carbon are labeled with the same numerical and literal subscripts as their respective carbon atoms.

Table 6. Torsion angles [°] for C₃₇H₃₀NPS – 0.5 C₇H₈

C(1D)-P(1)-N(1)-S(1)	19.9(2)
C(1F)-P(1)-N(1)-S(1)	141.56(15)
C(1E)-P(1)-N(1)-S(1)	-102.99(16)
C(1)-S(1)-N(1)-P(1)	-123.64(15)
N(1)-S(1)-C(1)-C(1B)	48.73(17)
N(1)-S(1)-C(1)-C(1A)	168.60(14)
N(1)-S(1)-C(1)-C(1C)	-75.43(17)
C(1B)-C(1)-C(1A)-C(6A)	-8.3(3)
C(1C)-C(1)-C(1A)-C(6A)	115.9(2)
S(1)-C(1)-C(1A)-C(6A)	-126.0(2)
C(1B)-C(1)-C(1A)-C(2A)	175.1(2)
C(1C)-C(1)-C(1A)-C(2A)	-60.7(3)
S(1)-C(1)-C(1A)-C(2A)	57.3(2)
C(6A)-C(1A)-C(2A)-C(3A)	-0.7(4)
C(1)-C(1A)-C(2A)-C(3A)	176.1(2)
C(1A)-C(2A)-C(3A)-C(4A)	-0.2(4)
C(2A)-C(3A)-C(4A)-C(5A)	0.9(4)
C(3A)-C(4A)-C(5A)-C(6A)	-0.8(4)
C(2A)-C(1A)-C(6A)-C(5A)	0.8(4)
C(1)-C(1A)-C(6A)-C(5A)	-175.9(2)
C(4A)-C(5A)-C(6A)-C(1A)	-0.1(4)
C(1A)-C(1)-C(1B)-C(2B)	114.1(2)
C(1C)-C(1)-C(1B)-C(2B)	-8.6(3)
S(1)-C(1)-C(1B)-C(2B)	-132.9(2)
C(1A)-C(1)-C(1B)-C(6B)	-64.8(3)
C(1C)-C(1)-C(1B)-C(6B)	172.46(19)
S(1)-C(1)-C(1B)-C(6B)	48.2(2)
C(6B)-C(1B)-C(2B)-C(3B)	0.0(3)
C(1)-C(1B)-C(2B)-C(3B)	-178.9(2)
C(1B)-C(2B)-C(3B)-C(4B)	0.2(4)
C(2B)-C(3B)-C(4B)-C(5B)	-0.2(4)
C(3B)-C(4B)-C(5B)-C(6B)	-0.2(4)
C(4B)-C(5B)-C(6B)-C(1B)	0.4(4)
C(2B)-C(1B)-C(6B)-C(5B)	-0.4(3)

C(1)-C(1B)-C(6B)-C(5B)	178.6(2)
C(1B)-C(1)-C(1C)-C(2C)	87.1(2)
C(1A)-C(1)-C(1C)-C(2C)	-38.0(3)
S(1)-C(1)-C(1C)-C(2C)	-149.67(19)
C(1B)-C(1)-C(1C)-C(6C)	-91.4(3)
C(1A)-C(1)-C(1C)-C(6C)	143.5(2)
S(1)-C(1)-C(1C)-C(6C)	31.8(3)
C(6C)-C(1C)-C(2C)-C(3C)	-0.7(4)
C(1)-C(1C)-C(2C)-C(3C)	-179.3(2)
C(1C)-C(2C)-C(3C)-C(4C)	0.0(4)
C(2C)-C(3C)-C(4C)-C(5C)	0.8(4)
C(3C)-C(4C)-C(5C)-C(6C)	-0.8(4)
C(4C)-C(5C)-C(6C)-C(1C)	0.0(4)
C(2C)-C(1C)-C(6C)-C(5C)	0.7(4)
C(1)-C(1C)-C(6C)-C(5C)	179.2(2)
N(1)-P(1)-C(1D)-C(2D)	114.9(2)
C(1F)-P(1)-C(1D)-C(2D)	-7.4(2)
C(1E)-P(1)-C(1D)-C(2D)	-118.7(2)
N(1)-P(1)-C(1D)-C(6D)	-61.3(2)
C(1F)-P(1)-C(1D)-C(6D)	176.39(19)
C(1E)-P(1)-C(1D)-C(6D)	65.2(2)
C(6D)-C(1D)-C(2D)-C(3D)	-0.6(4)
P(1)-C(1D)-C(2D)-C(3D)	-176.8(2)
C(1D)-C(2D)-C(3D)-C(4D)	-0.4(4)
C(2D)-C(3D)-C(4D)-C(5D)	1.3(4)
C(3D)-C(4D)-C(5D)-C(6D)	-1.2(4)
C(2D)-C(1D)-C(6D)-C(5D)	0.7(4)
P(1)-C(1D)-C(6D)-C(5D)	177.0(2)
C(4D)-C(5D)-C(6D)-C(1D)	0.2(4)
N(1)-P(1)-C(1E)-C(2E)	150.3(2)
C(1D)-P(1)-C(1E)-C(2E)	20.8(3)
C(1F)-P(1)-C(1E)-C(2E)	-92.1(2)
N(1)-P(1)-C(1E)-C(6E)	-36.4(2)
C(1D)-P(1)-C(1E)-C(6E)	-165.8(2)
C(1F)-P(1)-C(1E)-C(6E)	81.3(2)
C(6E)-C(1E)-C(2E)-C(3E)	4.0(4)

P(1)-C(1E)-C(2E)-C(3E)	177.3(2)
C(1E)-C(2E)-C(3E)-C(4E)	0.3(5)
C(2E)-C(3E)-C(4E)-C(5E)	-4.6(5)
C(3E)-C(4E)-C(5E)-C(6E)	4.7(5)
C(2E)-C(1E)-C(6E)-C(5E)	-3.9(4)
P(1)-C(1E)-C(6E)-C(5E)	-177.5(2)
C(4E)-C(5E)-C(6E)-C(1E)	-0.4(5)
N(1)-P(1)-C(1F)-C(2F)	-28.8(2)
C(1D)-P(1)-C(1F)-C(2F)	99.1(2)
C(1E)-P(1)-C(1F)-C(2F)	-149.2(2)
N(1)-P(1)-C(1F)-C(6F)	153.8(2)
C(1D)-P(1)-C(1F)-C(6F)	-78.3(2)
C(1E)-P(1)-C(1F)-C(6F)	33.4(2)
C(6F)-C(1F)-C(2F)-C(3F)	-0.7(4)
P(1)-C(1F)-C(2F)-C(3F)	-178.1(2)
C(1F)-C(2F)-C(3F)-C(4F)	-0.1(4)
C(2F)-C(3F)-C(4F)-C(5F)	0.5(4)
C(3F)-C(4F)-C(5F)-C(6F)	-0.2(5)
C(4F)-C(5F)-C(6F)-C(1F)	-0.7(5)
C(2F)-C(1F)-C(6F)-C(5F)	1.1(4)
P(1)-C(1F)-C(6F)-C(5F)	178.4(2)
C(6G)-C(1G)-C(2G)-C(3G)	0.4
C(7G)-C(1G)-C(2G)-C(3G)	-179.7
C(1G)-C(2G)-C(3G)-C(4G)	-1.4
C(2G)-C(3G)-C(4G)-C(5G)	1.8
C(3G)-C(4G)-C(5G)-C(6G)	-1.4
C(4G)-C(5G)-C(6G)-C(1G)	0.4
C(2G)-C(1G)-C(6G)-C(5G)	0.1
C(7G)-C(1G)-C(6G)-C(5G)	-179.8

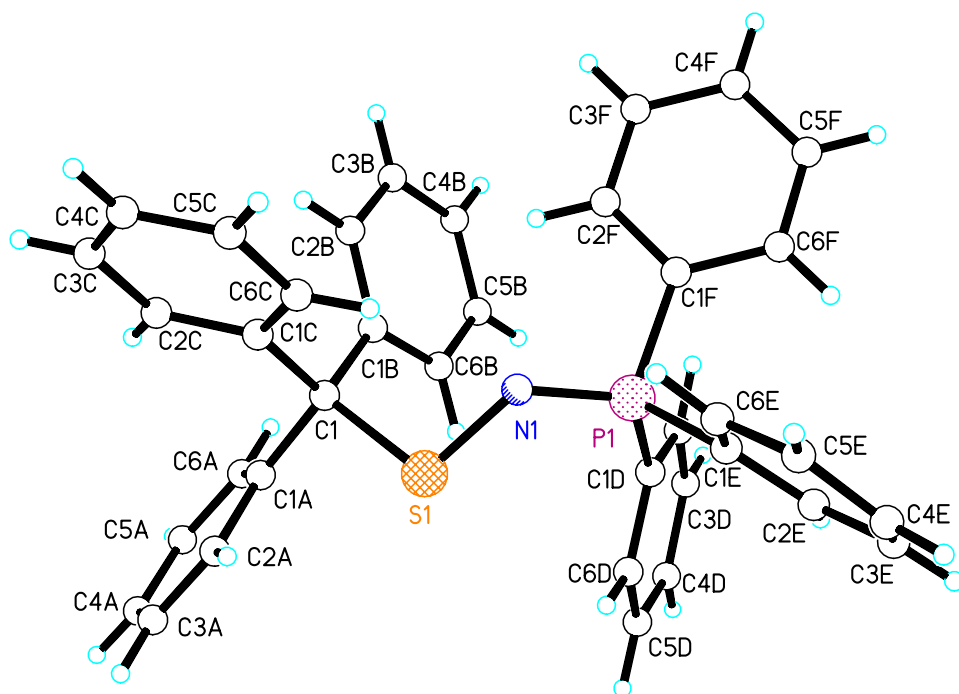


Figure 1.

shows a perspective drawing of the $C_{37}H_{30}NPS$ molecule present in the solid state structure of $C_{37}H_{30}NPS - 0.5 C_7H_8$. The sulfur and phosphorous atoms are represented by a large cross-hatched and dotted sphere, respectively. The nitrogen atom is represented by a medium-sized shaded sphere and carbon and hydrogen atoms are represented by medium and small open spheres, respectively.

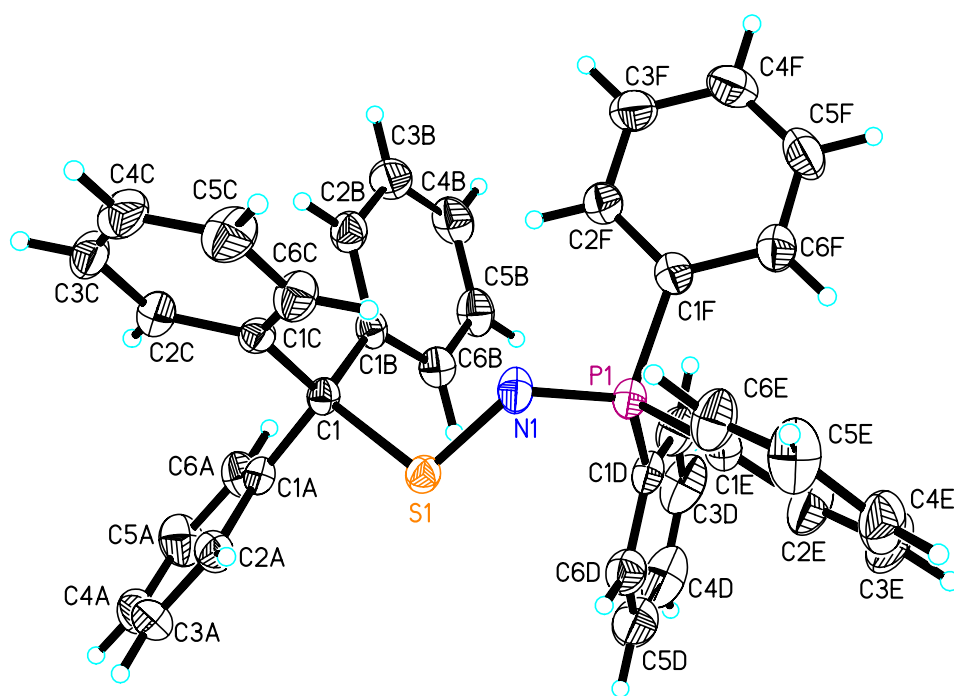


Figure 2.

shows a perspective drawing of the $C_{37}H_{30}NPS$ molecule present in the solid state structure of $C_{37}H_{30}NPS - 0.5 C_7H_8$. Nonhydrogen atoms are represented by thermal vibration ellipsoids drawn to encompass 50% of their electron density. Hydrogen atoms are represented by arbitrarily-small spheres, which are in no way representative of their true thermal motion.

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