2006

**Peroxynitrite Oxidation of Aromatic Thiols: Characterization of the Products Formed through Oxidation of Thionitrobenzoic Acid and Thionitropyridine by Peroxynitrite**

Catherine Balchunas Mall  
*College of William & Mary - Arts & Sciences*

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PEROXYNITRITE OXIDATION OF AROMATIC ThiOLS

Characterization of the Products Formed Through Oxidation of
Thionitrobenzoic Acid and Thionitropyridine by Peroxynitrite

A Thesis
Presented to
The Faculty of the Department of Chemistry
The College of William and Mary in Virginia

In Partial Fulfillment
Of the Requirements for the Degree of

Master of Science

by
Catherine Balchunas Mall
2006
APPROVAL SHEET

This thesis is submitted in partial fulfillment of
the requirements for the degree of

Master of Science

Catherine Balchunas Mall

Approved by the Committee, April 2006

Dr. Lisa M. Landino, Advisor and Chair

Dr. Chris J. Abelt

Dr. Gary W. Rice
DEDICATION

This thesis is dedicated to my husband, Matthew Mall, for being the guy I always dreamed I would someday marry. Your love, support, and constant encouragement have made me a better person in so many ways. Thank you for everything you do.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
<td>v</td>
</tr>
<tr>
<td>List of Tables</td>
<td>vi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vii</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>ix</td>
</tr>
<tr>
<td>List of Structures and Observed Maximum Absorbances</td>
<td>x</td>
</tr>
<tr>
<td>Abstract</td>
<td>xiii</td>
</tr>
<tr>
<td>Introduction</td>
<td>2</td>
</tr>
<tr>
<td>Experimental Procedures and Methods</td>
<td>13</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>26</td>
</tr>
<tr>
<td>I. Characterization of Peaks</td>
<td>26</td>
</tr>
<tr>
<td>II. NBD-CI Trapping of Sulfenic Acid</td>
<td>41</td>
</tr>
<tr>
<td>III. Dimedone Trapping of Sulfenic Acid</td>
<td>51</td>
</tr>
<tr>
<td>IV. Thiosulfinate Reactivity</td>
<td>60</td>
</tr>
<tr>
<td>Conclusions and Future Work</td>
<td>67</td>
</tr>
<tr>
<td>References</td>
<td>69</td>
</tr>
<tr>
<td>Vita</td>
<td>72</td>
</tr>
</tbody>
</table>
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LIST OF TABLES

Table                                      Page
1. Regular Method .......................................................... 15
2. Extended Method .......................................................... 15
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>2.</td>
<td>Oxidation States of Sulfur</td>
</tr>
<tr>
<td>3.</td>
<td>Reaction of Sulfenic Acid with Dimedone and NBD-Cl</td>
</tr>
<tr>
<td>4.</td>
<td>Peroxynitrite Oxidation of TNB</td>
</tr>
<tr>
<td>5.</td>
<td>Performic Acid Oxidation of DTNB</td>
</tr>
<tr>
<td>6.</td>
<td>Structure of 3-Nitrobenzenesulfonic Acid, sodium salt</td>
</tr>
<tr>
<td>7.</td>
<td>Performic acid oxidation of DTNP</td>
</tr>
<tr>
<td>8.</td>
<td>Peroxynitrite oxidation of TNB – Concentration Dependence</td>
</tr>
<tr>
<td>9.</td>
<td>TNB thiosulfinate treated with base</td>
</tr>
<tr>
<td>10.</td>
<td>Effect of pH on TNB thiosulfinate formation</td>
</tr>
<tr>
<td>11.</td>
<td>Peroxynitrite Oxidation of DTNB</td>
</tr>
<tr>
<td>12.</td>
<td>NBD-Cl trapping of TNP sulfenic acid</td>
</tr>
<tr>
<td>13.</td>
<td>NBD-Cl trapping of TNB sulfenic acid</td>
</tr>
<tr>
<td>14.</td>
<td>NBD-Cl trapping of TNB sulfenic acid – extended runtime</td>
</tr>
<tr>
<td>15.</td>
<td>NBD-Cl trapping of TNP sulfenic acid over time</td>
</tr>
<tr>
<td>16.</td>
<td>NBD-Cl trapping of TNP sulfenic acid – peroxynitrite concentration dependence</td>
</tr>
<tr>
<td>17.</td>
<td>14 minute Peak</td>
</tr>
<tr>
<td>18.</td>
<td>Dimedone trapping of TNB sulfenic acid – UV-Vis</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>19.</td>
<td>Dimedone trapping of TNB sulfenic acid – HPLC</td>
</tr>
<tr>
<td>20.</td>
<td>Dimedone purification</td>
</tr>
<tr>
<td>21.</td>
<td>Dimedone trapping of TNB sulfenic acid – temperature dependence</td>
</tr>
<tr>
<td>22.</td>
<td>Dimedone trapping of TNB sulfenic acid – peroxynitrite concentration dependence</td>
</tr>
<tr>
<td>23.</td>
<td>Dimedone trapping of TNB sulfenic acid – pH dependence</td>
</tr>
<tr>
<td>24.</td>
<td>Thiosulfinate reactivity with NBD-Cl</td>
</tr>
<tr>
<td>25.</td>
<td>Lipoic Acid</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

DTT – dithiothreitol
DTNB – 5,5’-Dithio-bis(2-nitrobenzoic acid)
DTNP – 2,2’-Dithiobis(5-nitropyridine)
TNB – 5-thio-2-nitrobenzoic acid
TNP – 2-thio-5-nitropyridine
NBD-C1 - 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole
Dimedone – 5,5’-Dimethyl-1,3-cyclohexanedione
RSO-NBD – sulfenic acid adduct – sulfoxide or sulenate ester – product of NBD bonding to protein sulfenic acid
RS-NBD – thiol adduct – thioether – product of NBD bonding to the protein sulfhydryl
LIST OF STRUCTURES AND OBSERVED MAXIMUM ABSORBANCES

DTNB Related Structures

DTNB
pH 7.4 - 328 nm
Acid - 328 nm

Thiosulfinate
pH 7.4 - 268 nm
Acid - 320 nm

Thiosulfonate
pH 7.4 - ?
Acid - ?

TNB
pH 7.4 - 409 nm
Acid - 332 nm

Sulfenic Acid
pH 7.4 - 405 nm
Acid - 319 nm

Sulfinic Acid
pH 7.4 - ?
Acid - ?

Sulfonic Acid
pH 7.4 - 260 nm
Acid - 263 nm
DTNP Related Structures

DTNP
pH 7.4 - 316 nm
Acid - 316 nm

Thiosulfinate
pH 7.4 - 340 nm
Acid - 340 nm

Thiosulfonate
pH 7.4 - ?
Acid - ?

TNP
pH 7.4 - 392 nm
Acid - 374 nm

Sulfenic Acid
pH 7.4 - 402 nm
Acid - 380 nm

Sulfinic Acid
pH 7.4 - 276 nm
Acid - 276 nm
Other Structures

NBD-Cl
pH 7.4 - 342 nm
Acid - 342 nm

Dimedone
pH 7.4 - 260 nm
Acid - 260 nm

3-Nitrobenzenesulfonic acid
pH 7.4 - 260 nm
Acid - 260 nm
ABSTRACT

The oxidation of thionitrobenzoic acid (TNB) yielded multiple unexpected oxidation products. As this prevented its use in a competition assay to calculate the rate constants for oxidation of protein thiols, the oxidation of this compound was studied.

The expected product was dithionitrobenzoic acid (DTNB), and this was found to be the major product formed when TNB was oxidized using peroxynitrite. HPLC separation of oxidation products implied the presence of two other products. The first was identified as the sulfonic acid of TNB, also created through performic acid oxidation. The second was identified as the thiosulfinate product, based on its polarity and susceptibility to base.

Thiosulfinates are thought to be the product of two sulfenic acid molecules combining through a hydrolysis reaction. The presence of thiosulfinate implies sulfenic acid intermediates, collaborated by the transient red color of the reaction mixture when yellow TNP is oxidized to the colorless DTNP. Attempts to trap the sulfenic acid intermediate using 5,5-dimethyl-1,3-cyclohexanedione (dimedone) and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) were made difficult by the formation of multiple reaction products.

NBD-Cl was also found to be reactive when mixed with a solution containing only the thiosulfinate product. This indicates that either the NBD-Cl reacts directly with the thiosulfinate, or the thiosulfinate is in equilibrium with sulfenic acid molecules, which can then react with NBD-Cl. Further experiments must be done utilizing mass spectrometry to confirm the NBD-sulfenic acid adduct.
PEROXYNITRITE OXIDATION OF AROMATIC THIOLS
INTRODUCTION

Reactive oxygen species such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (•OH) are formed in vivo when the mechanisms that control concentration and activity of oxygen are not properly regulated (Figure 1) [1,2]. Cells also encounter these reactive oxygen species when organisms release them as a defense against microbial pathogens [1]. Reactive oxygen species do significant damage to proteins, nucleic acids, and lipids, resulting in cumulative tissue injury responsible for many diseases in humans [1]. In proteins, reactive oxygen species can cause oxidation of amino acid side chains, protein-protein cross linking, and oxidation of the protein backbone causing fragmentation [3]. Those amino acids that are most susceptible to oxidation are the sulfur containing residues of cysteine and methionine, and aromatic residues like tryptophan, phenylalanine, tyrosine, and histidine [3].

Figure 1: Reactive Oxygen Species [1]

Under normal conditions, these oxidized proteins are degraded by proteases, and do not accumulate. When the rate of protein oxidation exceeds the rate at which the body
can correct the oxidation through antioxidants like superoxide dismutase and vitamin E or
degrade the protein with proteases, the concentration of oxidized proteins increases [3].
This accumulation leads to loss of biological function as more and more proteins become
damaged. The accumulated effects of reactive oxygen species are known to be involved
in the aging process, heart disease, cancer, and neurodegenerative diseases such as
Alzheimer’s disease [1,4].

The body has mechanisms for removing superoxide before it can convert to other
reactive oxygen species. Superoxide, the precursor to the hydroxyl radical through the
Fenton reaction, is converted to hydrogen peroxide and oxygen in vivo by superoxide
dismutase [5]. Hydrogen peroxide is then removed by catalase and glutathione
peroxidase [5]. Three times faster than the superoxide dismutase reaction is the reaction
between nitric oxide (a product of arginine metabolism) and superoxide to form the
peroxynitrite anion in vivo [3,5].

\[ \text{O}_2^- + \cdot\text{NO} \rightarrow \text{ONOO}^- \]

One of the main targets of oxidation by reactive oxygen species including
peroxynitrite is the thiol of cysteinyll residues in proteins. The thiol side chain of cysteine
is important biologically through its role in quaternary structure due to disulfide bridge
formation, signal transduction, DNA binding, as a redox center involved in catalysis, and
as a nucleophile [2]. The thiol side chain can be easily oxidized to several different
forms including the sulfenic acid, sulfinic acid, sulfonic acid, disulfide bond, and
glutathione conjugation (Figure 2) [1]. These modifications affect the structure of the
protein, as well as its functionality[1]. While disulfide bond formation and glutathione
conjugation can be reversed by reduction by thioredoxin and sometimes glutaredoxin, the sulfinic and sulfonic acid states cause irreversible damage to the protein [1].

Figure 2: Oxidation States of Sulfur

Roles of Protein Thiols in Proteins

The reversibility of disulfide bonds allows thiol modifications to serve a regulatory function and be involved in signal transduction [1]. A good example of this is the OxyR transcription factor found in E. coli, Salmonella enterica, and several other bacteria, which activates more than twenty antioxidant genes in response to hydrogen peroxide [1,6]. The Storz group found that when cells encounter peroxide stress, two cysteines are oxidized to form an intramolecular disulfide bond [1]. This results in a conformational change that alters OxyR binding to DNA so that it can activate the transcription of genes encoding antioxidant enzymes [1]. OxyR activation is regulated through a feedback loop in which a target gene of OxyR activation, grxA, encodes glutaredoxin 1, responsible for reducing oxidized OxyR [6]. A decrease in hydrogen peroxide concentration allows OxyR to be reduced and the disulfide bond to be broken with the cysteine residues returning to the thiol form [1]. The Stamler group has found that one of the cysteine residues on OxyR can be converted to the sulfenic acid, s-
nitrosylated, or converted to a mixed disulfide with glutathione [6]. They propose that each of these modifications leads to varying degrees of activation of OxyR [6]. Much research is still being done on this protein to learn how the different modifications affect the activation and deactivation of this transcription factor.

Another group of protein thiols that have been well studied are the cysteine residues in tubulin. Microtubules are the rod-like polymeric form of tubulin that provides support for the cell, as well as serving as tracks for the movement of motor proteins [7]. Microtubule-associated proteins assist in the polymerization of tubulin, stabilize the polymeric form, and sever cytoplasmic microtubules, making them responsible for the diverse structural forms of tubulin [7]. Each tubulin molecule is composed of two monomer units, α-tubulin and β-tubulin, with a total of twenty cysteine residues in each heterodimer [7,8]. While the role of the cysteine residues in tubulin is not definitively known, it is thought that they may be involved in the folding through transient disulfide bond formation [8]. The Landino group has found that tubulin is oxidized by peroxynitrite, and that this oxidation correlates with inhibition of polymerization of tubulin into microtubules [9]. They also found that this damage could be repaired by the glutaredoxin reductase system and thioredoxin reductase system, demonstrating that tubulin may serve as a redox buffer [10]. Because tubulin is easily oxidized to the disulfide form by oxidants or oxidized glutathione, and can then be repaired, it protects other susceptible proteins from oxidation [10]. The Landino group also found that two specific microtubule-associated proteins, MAP2 and tau, are oxidized in vitro by peroxynitrite to form disulfide-linked species, and that this oxidation impairs the ability of these proteins to aid microtubule assembly [11]. They found that this oxidative
damage is also reversible by the thioredoxin reductase system or by small molecule reductants [11].

**Sulfenic Acids in Proteins**

In addition to forming disulfides, protein thiols can also form sulfenic acids when treated with oxidants. They have traditionally been viewed as reactive intermediates, on the way to becoming thiosulfinates, disulfides, sulfinic acids, or sulfonic acids. It has recently been discovered that there are several cases in which stable sulfenic acids are important in redox signaling. Limited solvent access and an apolar micro-environment, absence of proximal thiol groups, hydrogen bonding, and ionization of the sulfenic acid to the sulfenate form have all been reported as factors that contribute to sulfenic acid stabilization in proteins [12]. Protein sulfenic acids can be formed by the hydrolysis of S-nitrosothiols, hydrolysis of disulfide bonds to form one thiol and one sulfenic acid, and through the oxidation of disulfide bonds to form a thiolsulfinate that reacts with a thiol to form a disulfide and a sulfenic acid [13]. Once made, the sulfenic acids can go on to become other forms of modified cysteine residues, or return back to the thiol state [13].

A well-characterized example of a stable sulfenic acid is the flavoprotein NADH peroxidase from *Enterococcus faecalis*. This protein has a single cysteine residue (Cys42), which serves as a redox center and undergoes the following reversible two-electron reduction and oxidation by H$_2$O$_2$ [14]:

\[
E(FAD, \text{Cys}42-\text{SOH}) + \text{NADH} + H^+ \rightarrow EH_2(FAD, \text{Cys}42-\text{SH}) + \text{NAD}^+ + H_2O
\]
It has been shown by $^{13}$C NMR and X-ray diffractometry at 2.8 Å resolution that it is the sulfenic acid derivative of Cys42 that serves as the non-flavin redox center of this protein [14,15]. These experiments showed that the sulfenic acid of NADH peroxidase is stabilized by all four of the stabilizing factors described above; hydrogen bonding, ionization of sulfenic acid to sulfenate, absence of vicinal protein thiols, and limited solvent access [14,15].

Poole has shown that the AhpC peroxidase protein from *Salmonella typhimurium* catalyzes the reduction of peroxide substrates through a cysteine mediated mechanism in which one of the cysteine residues is converted to a sulfenic acid [16]. The oxidized cysteine residue then condenses with a nearby cysteine to regenerate the active-site disulfide bond and prevent further oxidation of the sulfenic acid [16]. In order to demonstrate this, Poole used single and double AhpC mutants to test the importance of each of the two involved cysteine residues [16]. Only one mutant had full peroxidatic activity when compared to the wild-type, demonstrating that this was the residue responsible for reduction of alkyl hydroperoxides [16]. Poole used several methods to confirm that the cysteine residue had been converted to the sulfenic acid when oxidized by H$_2$O$_2$. The original conclusion was drawn from the ability of the oxidized form to be reduced by one equivalent of NADH in the presence of AhpF, the formation of a mixed disulfide when treated with TNB, and the sensitivity to further oxidation when exposed to air of excess H$_2$O$_2$ [16]. Poole went on to develop a new method for identifying sulfenic acids in proteins by using 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) to trap the sulfenic acid form. NBD-Cl is an electrophilic reagent that demonstrates characteristic absorbances when free, bound to a sulfenic acid, and bound to a protein thiol [16]. This
makes it easy to identify cysteine sulenic acids in proteins using spectroscopic methods.

In Poole's work, the proteins studied were the mutant forms, with only one cysteine residue. This prevented the self-condensation of the sulenic acids into the thiosulfinate form, ensuring that only the sulenic acid form of the protein was present for NBD-Cl bonding.

Poole's use of NBD-Cl for trapping of sulenic acids is an improvement on the traditional method of using 5,5-dimethyl-1,3-cyclohexanedione (dimedone) to trap sulenic acids. Dimedone is a nucleophilic reagent that attacks the sulenate sulfur, displacing the hydroxide (Figure 3) [16]. Dimedone is not spectroscopically active, so mass spectrometry is the common method of detecting its incorporation into compounds [16]. Poole wished to develop a trapping agent that left the oxygen of the sulenic acid intact, and was spectroscopically active. NBD-Cl exhibits characteristic peak absorbances at 343 nm for the free compound, 347 nm when bound to sulenic acids, and 420 nm when bound to the thiol form of a protein [16]. While NBD-Cl also binds to tyrosyl and amino groups of proteins, it exhibits different absorbances when bound to these groups. Due to the similarity in peak absorbances between free NBD-Cl and the sulenic acid adduct, it is necessary to separate free NBD-Cl from the solution before studying spectroscopically.
Figure 3: Reactions of Sulfenic Acid with Dimedone and NBD-Cl

\[
\begin{align*}
R-S-OH + \text{dimedone} & \rightarrow R-S-CO-CO + H_2O \\
R-S-OH + \text{NBD-Cl} & \rightarrow \text{sulfoxide} + H^+ + Cl^- \\
& \rightarrow \text{sulfanate ester} + H^+ + Cl^-
\end{align*}
\]
Background

As there are many proteins in the body that are oxidized by peroxynitrite, it became of interest to measure rate constants for peroxynitrite with varying proteins. The rate of the reaction is equal to the product of the concentrations of the reactants and the rate constant: \( \text{rate} = k[A]^m[B]^n \). If another reactant with a known rate constant is introduced into the equation, the concentrations of all substances can be calculated. By varying the concentration of the new substrate in this competition assay, the rate constant, \( k \), can be determined.

\[
\begin{align*}
\text{ONOO}^- & \xrightleftharpoons[k_2]{k_1} \text{protein} \\
\text{TNB} & \rightarrow \text{DTNB}
\end{align*}
\]

In 2003, Moynihan used stopped flow studies to measure a rate constant for the reaction between the yellow thiol compound 2-nitro-5-thiobenzoic acid (TNB) and peroxynitrite. When oxidized, TNB is converted to colorless 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The goal was to then be able to add a protein like tubulin to this reaction, and use the competition assay to determine the rate constant for the reaction between peroxynitrite and the protein. By doing this with multiple proteins, it would be possible to predict which proteins are preferentially oxidized in vivo.

Because the kinetics of the reaction between peroxynitrite and other reactants are so rapid, it is necessary to use stopped flow to calculate the rate constant. In stopped flow, small volumes of reactants are pushed through high performance syringes into a mixer, and then into a cell, where absorbance or fluorescence spectrometry is used to
determine the concentration of the substance. By the time the mixture has reached the
cell and stopped, it is only milliseconds old. By recording the absorbance or fluorescence
data at times useful for the specific reaction (every 0.1 ms to every 1 s), the reaction rate
can be calculated.

While it would be possible to use stopped flow to directly measure the reaction
rate between proteins and peroxynitrite, Moynihan found that this method required huge
quantities of protein and was therefore not useful. It was then decided that the rate
constant between TNB and peroxynitrite would be found, and then a competition assay
would be used to determine the rate constant for the protein.

When Moynihan used stopped flow to evaluate the rate constant for the reaction
between TNB and peroxynitrite, it became clear that the reaction could not be fitted to a
single exponential. This would imply that more than one product was forming from the
reaction.

In 2004, Josh Nicklay began to analyze the products of the reaction between
peroxynitrite and TNB using HPLC. In addition to the expected DTNB product peak,
Nicklay observed an unidentified peak at 17 minutes which was more polar than DNTB.
It was thought that this product could be the thiosulfinate form of DTNB. It was also
observed that immediately after mixing the yellow TNB and peroxynitrite, a short lived
red color developed, before fading to the light yellow DTNB. Nicklay then replaced
TNB with thionitropyridine (TNP) hoping to produce only the DTNP product, but with
no such success.

The goal of this project was first to try altering the reaction conditions to prevent
formation of the thiosulfinate. When it became apparent that this was impossible, efforts
went to studying the various reaction products, including the transient red-colored product. We suspected, based on literature reports, that the red intermediate was a sulfinic acid intermediate to the thiosulfinate. As stable sulfinic acids are not common, it became of interest to try to trap this relatively stable sulfinic acid. It is hoped that through the study of the reaction products of TNB and TNP oxidized by peroxynitrite, a better view of thiol oxidation can be achieved.
EXPERIMENTAL PROCEDURES and METHODS

Reagents

All DTNB solutions were made at 10 mM by dissolving DTNB (Sigma) in pH 7.4 sodium phosphate buffer. The solution was stored in 1.5 mL eppendorf tubes in a -20°C freezer until use. DTNP was made by dissolving DTNP (Fluka) in acetonitrile to make a 10 mM solution. Fresh DTNP solutions were prepared in acetonitrile every few days. To make TNB and TNP solution, DTNB or DTNP solution (10 mM, 50 uL) was mixed with sodium phosphate buffer (pH 7.4, 190 uL) and DTT (Sigma). Originally 10 uL of 50 mM DTT was used so that a 1:1 DTT:DTNB/DTNP ratio was created, allowing all disulfide bonds of DTNB and DTNP to be broken. Nicklay extracted TNB from a solution in which DTNB had been reduced by DTT in order to remove excess DTT, and repeated experimentation using this purified TNB. The product profile was not changed by extraction, showing that excess DTT was not affecting the product profile. This was changed for the sulfenic acid trapping experiments to 10 uL of 40 mM DTT, to further ensure that no reducing agents were left in the solution before subsequent experiments. When 50 mM DTT was used, all TNB/TNP solutions were 4 mM, when 40 mM DTT was used, TNB/TNP solutions were 3.2 mM.

Peroxynitrite was synthesized by LML from acidified H$_2$O$_2$ and nitrite. The solution was passed over a solid MnO$_2$ column to remove residual H$_2$O$_2$. Peroxynitrite was stored at -80°C.
All buffers were sodium phosphate buffers made by LML and stored at 4°C. A pH 7.4 buffer was used unless otherwise noted. Phosphoric acid and acetic acid (Fisher) were diluted to 10% before use.

\( \text{H}_2\text{O}_2 \), TFA, acetonitrile, methanol, and formic acid were all purchased from Fisher Scientific. 3-Nitrobenzenesulfonic acid and NBD-Cl were purchased from Acros. Dimedone was purchased from Aldrich.

**Instrumentation**

**HPLC**

All HPLC was performed on a Hewlett Packard 1100 Series HPLC with a variable wavelength detector. The column used was a Hewlett Packard Eclipse XDB-C8 column (C8, 4.6 x 150 mm, 5 µm particle size). Unless otherwise noted, 25 µL of the sample to be analyzed was mixed with phosphoric acid (10%, 75 µL) and manually injected into the HPLC and detected using a wavelength of 320 nm. For separations involving DTNB, Solvent A was 80% \( \text{H}_2\text{O} \) / 20% Acetonitrile / 0.1% TFA, and solvent B was 30% \( \text{H}_2\text{O} \) / 70% Acetonitrile / 0.1% TFA. For separations involving DTNP, solvent A remained the same but it was necessary to change solvent B to 5% \( \text{H}_2\text{O} \) / 95% Acetonitrile / 0.085% TFA. The column was washed weekly with solvent C, 90% acetonitrile / 10% \( \text{H}_2\text{O} \). A 25 minute method was usually used, though at times (especially with DTNP solutions) it was necessary to use an extended 32 minute method to separate all peaks (Tables 1, 2). Both methods were followed by a 1 minute post run of 100% solvent A during which time no data was collected.
UV-Vis

All UV-Vis scans were performed on a Lambda 35 UV/Vis Spectrometer by Perkin Elmer Instruments using 50 uL quartz Starna cells with a 10 mm pathlength. Scans were generally run from 700 nm to 200 nm.

Table 1. Regular Method

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>% Solvent B</th>
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<tr>
<td>0</td>
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</tr>
<tr>
<td>2</td>
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<tr>
<td>17</td>
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<td>20</td>
<td>0</td>
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<tr>
<td>25</td>
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Table 2. Extended Method

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>% Solvent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
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Methods

I. Characterization of Peaks

Performic Acid Oxidation

To make performic acid, formic acid (88%, 2.25 mL) and hydrogen peroxide (30%, 0.25 mL) were vortexed in a glass test tube. The mixture was allowed to react at room temperature for one hour, then placed in an ice bath and used immediately. DTNB (10 mM, 25 uL) in buffer (pH 7.4 phosphate buffer) was aliquoted into each of four test tubes. Performic acid was then added to each test tube, in the amounts 10, 20, 30, and 40 uL performic acid. The solution was then vortexed to mix, and placed into an ice bath. Samples were stored in the refrigerator for five days before being dried in a Speedvac on low drying rate.

H₂O (50 uL) and acetic acid (10%, 50 uL) were added to sample 1 (25 uL DTNB, 10 uL performic acid) to dissolve and protonate the sample. The sample was then injected into the HPLC and the absorbance was monitored at 320 nm wavelength. The first peak was collected and dried overnight in a Speedvac. The sample was dissolved in solvent A (1,000 uL) and UV-Vis was used to find the maximum absorbance of 260 nm.

Samples 2 and 4 (20 and 40 uL performic acid) were dissolved in H₂O (50 uL) and acetic acid (10%, 50 uL) and analyzed on HPLC at 260 nm.

The performic acid oxidation of DTNB was repeated using 10% phosphoric acid instead of acetic acid to protonate. It was thought that the acetic acid might have been visible on the HPLC chromatogram.

The performic acid oxidation was repeated using DTNP instead of DTNB. DTNP was not soluble in buffer, requiring it to be first dissolved in acetonitrile. DTNP was
dissolved in acetonitrile to make 10 mM DTNP, then treated as described for DTNB. HPLC was performed at 280 nm, where the sulfonic acid form of DTNP absorbs maximally.

3-Nitrobenzenesulfonic acid (10 mM) was dissolved in buffer. 3-Nitrobenzenesulfonic acid (10 mM, 25 uL), H₂O (25 uL), and acetic acid (10%, 50 uL) were mixed, separated on HPLC and monitored at 260 nm.

**Peroxynitrite Oxidation**

To make 4 mM TNB²⁻, buffer (190 uL), DTNB in buffer (10 mM, 50 uL), and DTT (50 mM, 10 uL) in H₂O to reduce disulfide bonds were mixed.

Peroxynitrite solutions stored at -80°C degraded slowly over time from 95-90 mM. Peroxynitrite concentrations were confirmed by UV-Vis spectroscopy at 302 nm (ε₃₀₂=1670 M⁻¹cm⁻¹). Dilutions of 0 mM, 10.5 mM, 21 mM, 42 mM, and 90 mM were made by mixing peroxynitrite from the freezer with NaOH (0.2 M). These samples were stored on ice while being used, and in the -80°C freezer for long term storage.

TNB²⁻ (4 mM, 100 uL) was mixed with 5 uL of each concentration of peroxynitrite. 25 uL of each was mixed with phosphoric acid (10%, 75 uL), separated on HPLC, and observed at 260 nm. Peaks 1 and 2 (2.4, 17.8 minutes) were collected, dried in a Speedvac, resuspended in 10% phosphoric acid, and injected in HPLC.

TNB²⁻ (4 mM, 100 uL) was mixed with peroxynitrite (90 mM, 5 uL), and allowed to react at room temperature. 25 uL was mixed with phosphoric acid (10%, 75 uL) and separated on HPLC after 30 minutes of reaction time, then again 6 and 10 days later.
Treatment of TNB with peroxynitrite was repeated so that the 17 minute product could be collected. This was scanned on UV-Vis to find the peak absorbance. It was then treated with NaOH, and rescanned to see if a shift in peak absorbance was visible.

**pH Dependence**

DTNB (10 mM) was made in 0.1 M phosphate buffer of pH 6.0, 6.4, 6.9, 7.4, and 8.0. TNB²⁻ was made by mixing 50 uL DTNB of varying pH, 190 uL buffer of same pH, and DTT (50 mM, 10 uL). TNB²⁻ (4 mM, 100 uL) of each pH and peroxynitrite (90 mM, 2.5 uL) were mixed. 25 uL of the product was mixed with phosphoric acid (10%, 75 uL), separated on HPLC, and observed at 320 nm. This was repeated at 260 nm to better view the 17 minute peak.

**Peroxynitrite Oxidation of DTNB**

DTNB was dissolved in pH 7.4 buffer to make 2 mM DTNB. Peroxynitrite (90 mM, 2.5 uL) was added to DTNB (2 mM, 100 uL) and mixed. This solution was immediately analyzed on HPLC at 320 nm, then repeated after 5 hours of reaction time at 320 nm and 380 nm. Samples of DTNB without peroxynitrite were also analyzed at 320 nm immediately after being mixed with buffer and again after 5 hours of reaction time at room temperature.

**II. NBD-Cl Trapping of Sulfenic Acid**

The sulfenic acid form of DTNB and DTNP is something that has not been extensively studied. The fact that these intermediates survive for seconds to hours
depending on the pH of solution made them of interest. Very few stable sulfenic acids have been well-characterized, and it was thought that a small molecule sulfenic acid may be of interest, especially to organic chemists. In order to show that the red color of the solution was caused by the sulfenic acid intermediate, it became of interest to “trap” this compound using NBD-Cl and dimedone.

Poole et al have reported a novel use for the compound NBD-Cl as a sulfenic acid trapping reagent. Their method requires all reactions to be performed under anaerobic conditions. Because the sulfenic acid containing compounds being studied by other groups have been proteins, it is possible to remove excess NBD-Cl from the solution by gel filtration. With free NBD-Cl out of the solution, a simple UV spectra with a peak at 347 nm would indicate a trapped sulfenic acid. This method was not possible for our uses, as DTNP and NBD-Cl are not easily separated by gel filtration. Therefore, the goal was to use HPLC to separate the products and reagents and identify the sulfenic acid adduct (RSO-NBD).

TNP (4 mM, 100 uL) was mixed with peroxynitrite (90 mM, 5 uL) and allowed to react for two minutes. NBD-Cl (16 mM, 25 uL) was added to the solution so that the final ratio of TNP and NBD-Cl was 1:1. This solution was allowed to react for 30 minutes before being analyzed on HPLC. Many small peaks were visible, making it impossible to determine which peak was from the desired RSO-NBD complex.

The reaction was repeated, using the exact concentrations of all reagents as described by Caballel et al, so that the final concentrations of NBD-Cl and TNP were in a 2:1 ratio [17]. TNP (4 mM, 100 uL) and peroxynitrite (90 mM, 3.55 uL) were mixed, and allowed to react for 5 minutes. NBD-Cl (16 mM, 50 uL) in acetonitrile was added.
After 30 minutes, 25 uL of the reaction mixture and 75 uL 10% phosphoric acid were mixed, separated on HPLC, and observed at 343 and 420 nm. This was repeated using 20x and 4x excess of NBD-Cl. As a control to identify the RS-NBD, TNP (4 mM, 50 uL) was mixed with NBD-Cl in acetonitrile (16 mM, 25 uL) and allowed to react for 30 minutes. The solution was then mixed with 20% phosphoric acid (75 uL), and 100 uL was separated on HPLC and observed at 420 nm.

New TNP was made using DTNP (10 mM, 50 uL), DTT (40 mM, 10 uL), and buffer (190 uL), so that the final concentration of DTT was 4/5 that of DTNP, to ensure that no DTT was left in solution. TNP (3.2 mM, 50 uL), peroxynitrite (90 mM, 2.5 uL), and buffer (1000 uL) were mixed. NBD-Cl (20 mM, 5 uL) was added and the mixture was immediately scanned on UV-Vis against a buffer blank and repeated after 1, 2, 4, 7, 12, 18, and 30 minutes.

TNP (3.2 mM, 50 uL) and peroxynitrite (90 mM, 2.5 uL) were mixed, NBD-Cl (20 mM, 20 uL) was added, and the mixture was allowed to react for 30 minutes. Reaction was analyzed using HPLC, then again after 4 hours of reaction time.

The above procedure was repeated using varying peroxynitrite concentration (0 mM, 10.5 mM, 21 mM, 42 mM), and injecting after 30 minute reaction time.

Four controls were performed under the same conditions using concentrations identical to those above: TNP/DTNP mixture, NBD-Cl, NBD-Cl with peroxynitrite, and NBD-Cl with TNP and NaOH. The three peaks at 9, 14, and 17 minutes that were unaccounted for by controls and also increased with increasing peroxynitrite concentration were collected. These were dried in a Speedvac and resuspended in 70% acetonitrile solvent B (60 ul), and scanned on UV-Vis.
The mixing of TNP, peroxynitrite, and NBD-Cl as described above was repeated, varying the pH of solution (pH 6.0, 6.4, 6.9, 8.0). This was allowed to react for 30 minutes and injected into HPLC as described above, monitoring at 332 nm to observe the 14 minute peak.

As a control, TNB (4 mM, 100 uL) was mixed with NBD-Cl (16 mM, 50 uL). 25 uL of the product was mixed with 75 uL 10% phosphoric acid, separated on HPLC, and monitored at 420 nm to observe the thiol adduct (RS-NBD).

An attempt was made to trap the sulfenic acid intermediate of TNB using the same method as above for TNP. The peroxynitrite and NBD-Cl were mixed, then TNB was added, as well as a trial in which all three were added at the same time. As it was thought that the RSO-NBD peak was overlapping with NBD-Cl, the normal method was extended to a total of 32 minutes to separate the peaks. At this point, it became clear that the peak originally thought to be RSO-NBD was simply DTNB, and no other peaks that could have been the desired RSO-NBD were visible. Poole reports that NBD-Cl reacts only with cysteinyl residues at neutral pH, rather than with thiol, amino, or tyrosine residues. The trial was repeated using pH 6.9 and 7.4 in order to try to prevent side reactions, and the samples were injected after 30 minute incubation and several hour incubations. There were still no peaks that could be from the RSO-NBD complex.

III. Dimedone Trapping of Sulphenic Acid

Dimedone is a well-characterized sulphenic acid trapping reagent, that unlike NBD-Cl, reportedly does not react with thiol derivatives. For this reason, it was explored as a trapping reagent. Dimedone was not soluble in H₂O, ethanol, or acetonitrile, but was
soluble in methanol. Dimedone (0.8M) was made in methanol. TNB (4 mM, 100 uL) and dimedone (0.8 M, 25 uL) were mixed and allowed to react overnight. The product (12.5 uL) was mixed with phosphoric acid (10 %, 200 uL) and the maximum absorbance was found on UV-Vis to be 340 nm. The product (25 uL) was mixed with 75 uL phosphoric acid, separated on HPLC, and monitored at 340 nm. This was repeated, adding 3.55 uL peroxynitrite to the TNB-dimedone solution, with a maximum absorbance at 380 nm.

TNB (4 mM, 100 uL) was mixed with dimedone (0.8 M, 25 uL) to create a 50:1 dimedone:TNB ratio. Peroxynitrite (90 mM, 3.55 uL) was then added, and the mixture was allowed to react for 21.5 hours. The product was separated on HPLC, and observed at 380 nm and 320 nm. This was repeated, allowing the mixture to react only 30 minutes. Reactants of the same volumes and concentrations were mixed again, this time mixing TNB and peroxynitrite first, then adding dimedone. This solution was also allowed to react 21.5 hours before being separated and monitored on HPLC at 380 nm.

**Time Dependence of Dimedone Trapping**

TNB (200 uL) was mixed with dimedone (0.8 M, 50 uL), and diluted 10 uL in 200 uL phosphoric acid to scan on UV-Vis. Peroxynitrite (7.1 uL) was added to the TNB-dimedone mixture, diluted 10 uL in 200 uL phosphoric acid, and scanned on UV-Vis. The scan was repeated after 30 minutes, 1 hour, 2 hours, 3.5 hours, and 5 hours. The samples were injected into HPLC immediately after mixing, and then again after 30 minutes, 1 hour, 2 hours, 3.5 hours, and 5 hours and monitored at 380 nm.
The UV-Vis scans were repeated using TNP instead of TNB immediately after mixing and at times 30 minutes, and 1, 2, and 3 hours.

**Dimedone Purification**

When dimedone was dissolved in methanol, and separated on HPLC with no other reactants, several peaks were visible. This implied that there were contaminants in the dimedone. As some of these peaks overlapped with reactant peaks, it was necessary to remove them. Dimedone (2.10 g) was dissolved in 2-propanol (17.5 mL) and water (75 mL). Ammonium acetate (60 g) was then added to the solution, and the solution was stirred overnight. A 3 mL capacity C18 PrepSep separation column was conditioned using methanol (2 mL) followed by water (2 mL). Approximately 5 mL of the dimedone solution was then passed through the column, with the eluent containing the purified dimedone.

**Temperature Dependence for Dimedone Trapping**

A solution of TNB (4 mM, 100 uL), dimedone (100 mM, 200 uL), and peroxynitrite (90 mM, 3.55 uL) was mixed and divided into 4 tubes. Each was incubated overnight at 4°C, 22°C, 37°C, or 62°C. Each sample (10 uL) was then mixed with 10% phosphoric acid (100 uL), separated on HPLC, and monitored at 380 nm.

**Peroxynitrite Concentration Dependence**

Solutions of TNB (4 mM, 100 uL) and dimedone (100 mM, 200 uL) were mixed with 5 uL of peroxynitrite of 0 mM, 10.5 mM, 21 mM, 42 mM, and 90 mM
concentrations. These samples were allowed to react overnight at room temperature. 10 uL of each sample was mixed with phosphoric acid (10%, 100 uL), injected into HPLC, separated and monitored at 380 nm.

**pH Dependence**

Solutions were made using TNB of the desired pH (4 mM, 200 uL), dimedone in methanol (0.2 M, 200 uL), and peroxynitrite (90 mM, 7.1 uL). Each sample was allowed to react overnight at room temperature. 10 uL of each sample was mixed with phosphoric acid (10%, 100 uL), injected into HPLC, and observed at 380 nm.

**IV. Thiosulfinate Reactivity**

In order to test to whether the trapping agents were reacting with the sulenic acid or the thiosulfinate, the thiosulfinate form of DTNP was collected after a separation on the HPLC. To do this, TNP (3.2 mM, 500 uL) was oxidized with peroxynitrite (90 mM, 12.5 uL) and allowed to react overnight. This solution was then mixed 50:50 with 10% H₃PO₄ and separated over several runs on HPLC, monitoring at 340 nm. The peak that eluted at approximately 17 minutes was collected each time. The total collected thiosulfinate solution was divided between three eppendorf tubes and dried overnight in a speedvac. The following day, NBD-Cl (12.8 mM, 25 uL) in methanol and 25 uL pH 7.4 buffer were added to one tube to create a 2:1 NBD-Cl to TNP ratio, based on the amount of TNP originally injected into the HPLC. The resulting ratio of NBD-Cl:thiosulfinate was probably much higher. To a second tube, dimedone (640 mM, 25 uL) in methanol and 25 uL buffer was added to create a 100:1 dimedone to TNP ratio, based on the
original TNP. To the third tube was added 25 uL methanol and 25 ul buffer. All three tubes were allowed to react overnight.

The products were diluted in 10% H$_3$PO$_4$ and scanned on UV-Vis. Of the remaining product, 20 uL of each was diluted in 80 uL 10% H$_3$PO$_4$ and separated on HPLC, viewing at 347 nm and 420 nm.

**Lipoic Acid**

Oxidized α-Lipoic acid was dissolved in pH 7.4 buffer to make a 10 mM solution. This was then reduced using DTT by mixing lipoic acid (10 mM, 50 uL), DTT (50 mM, 10 uL), and pH 7.4 buffer (190 uL) to make 2 mM reduced α-lipoic acid. Reduced α-lipoic acid was then mixed 1:1 with HOCl to oxidize it to β-Lipoic acid, the thiosulfinate form. Both oxidized and reduced α-Lipoic acid, and β-Lipoic acid were scanned on UV-Vis. α-Lipoic acid was analyzed on HPLC at 330 nm, and β-lipoic acid was analyzed at 240 nm. The oxidation of α-lipoic acid using bleach was repeated, using 2:1, 1:1, and 1:2 lipoic acid to bleach ratios.
RESULTS and DISCUSSION

I. Characterization of Peaks

Performic Acid Oxidation

It was originally thought that oxidation of TNB using peroxynitrite should yield a full conversion of TNB to DTNB, resulting in one peak on the chromatogram at 20 minutes when separated using HPLC. Instead, this reaction resulted in four visible peaks (Figure 4). It was hypothesized that the large peak that eluted at approximately 2.3 minutes was the sulfonic acid product of TNB, based on the elution time. Of the potential oxidation products of TNB treated with peroxynitrite, the sulfonic acid product is the most polar.

In order to test this hypothesis, performic acid was used to oxidize the disulfide bond of DTNB to form two sulfonic acid molecules. As described in *Guide to Protein Purification*, performic acid oxidizes cysteine and cystine to cysteic acid, the sulfonic acid form of cysteine which should be stable to acid hydrolysis [18]. When the reaction mixture was protonated using 10% phosphoric acid and separated using HPLC, the sulfonic acid was found to elute at approximately 2.3 minutes, aligning with the large peak seen when TNB was oxidized with peroxynitrite (Figure 5). The sulfonic acid product clearly increased with increasing concentration of performic acid. When
Peroxynitrite oxidation of TNB was expected to produce DTNB. In this figure, 5 uL of 42 mM peroxynitrite was used to oxidize 100 uL of 4 mM TNB, then analyzed on HPLC at 260 nm. The peak visible at 12 minutes is excess TNB and the peak at 20 minutes is DTNB. The peaks at 2.5 and 18 minutes were unknown.
FIGURE 5

PERFORMIC ACID OXIDATION OF DTNB

HPLC separation of performic acid oxidation product and 3-Nitrobenzenesulfonic acid at 260 nm. The large peak visible at ~1.7 minutes when 20 μL and 40 μL of performic acid were mixed with DTNB was removed by using phosphoric acid instead of acetic acid to protonate the samples, as shown by the absence of this peak in the orange and blue plots.
collected, dried down, and resuspended, this product was found to absorb maximally at 260 nm, like the product obtained from the reaction of TNB with peroxynitrite.

The standard 3-nitrobenzenesulfonic acid was purchased from Acros and analyzed on HPLC as a comparison, as the structure of this sulfonic acid is similar to the sulfonic acid form of TNB (Figure 6). As can be seen when comparing the two molecules, the TNB sulfonic acid has a carboxylic acid group that is not present on 3-nitrobenzenesulfonic acid. As this is a polar group, its presence should cause the TNB sulfonic acid to elute earlier than the standard. 3-Nitrobenzenesulfonic acid eluted at approximately 2.4 minutes, corroborating the previous conclusion that the peak seen at 2.3 minutes was the sulfonic acid form of DTNB.

The performic acid oxidation was repeated, using DTNP instead of DTNB. The sulfonic acid form of DTNP eluted at approximately 1.9 minutes. As the performic acid concentration increased, the sulfonic acid product also increased and DTNP decreased in concentration. This can be seen in UV-Vis scans as a decrease in absorbance at 320 nm and an increase in absorbance at 280 nm when the concentration of performic acid is increased (Figure 7). It is also clear in HPLC chromatograms as an increase in the peak at 1.9 minutes (sulfonic acid) and a decrease in the peak at 29 minutes (DTNP). When collected and scanned on UV-Vis, the sulfonic acid product was found to absorb maximally at 280 nm.

**Peroxynitrite Oxidation**

Treatment of DTNB with DTT in a 1:1 ratio reduced the disulfide bond of DTNB to form two TNB\(^2^-\) molecules. Addition of peroxynitrite to this solution should oxidize
FIGURE 6

STRUCTURE OF 3- NITROBENZENESULFONIC ACID, SODIUM SALT

3-Nitrobenzenesulfonic acid, sodium salt

TNB sulfonic acid
The maximum absorbance was approximately 320 nm when only 10 uL performic acid was used to oxidize the DTNP (blue line), indicating that DTNP was still the dominating species. As the amount of performic acid added was increased from 30 uL to 40 uL and then to 50 uL (green, pink, and teal lines, respectively), the absorbance at 320 decreased signaling a decrease in DTNP, and increased at 240 nm and 280 nm. When the sulfonic acid form of DTNP was later collected and scanned, the maximum absorbance was found to be 280 nm.
the two TNB$^{2-}$ molecules so that they form one DTNB molecule.

\[
\text{O}_2\text{N}-\text{S-S-NO}_2\text{H}_2\text{C} + \text{HS-C-CH-CH-C-SH} \rightarrow 2 \text{O}_2\text{N}-\text{S-S-N}_2\text{H}_2\text{C} + \text{HO}_2\text{C}\text{H}_2\text{S-S-S-Ar}
\]

Immediately after addition of peroxynitrite to the yellow TNB$^{2-}$ solution, the mixture turned a deep orange color. This faded to the lighter yellow of DTNB within several seconds.

Blakeley describes the alkaline hydrolysis of DTNB in 3.0 M NaOH [19]. In his studies, he attributes the “intense red color” to the sulfenate ion, an intermediate in the reaction to form the thiosulfinate as shown by the reaction scheme below. In his studies, the red color faded to a light yellow, which he attributed to the thiophenoxide ion (TNB).

Scheme 1. As described by Blakeley

\[
\begin{align*}
\text{HO}^- + \text{Ar-S-S-Ar} & \rightarrow \text{Ar-SO}^- + \text{Ar-S}^- + \text{H}^+ \\
\text{Ar-SO}^- + \text{Ar-S-S-Ar} & \rightarrow \text{O}^- \cdot \text{Ar-S}^+ - \text{S-Ar} + \text{Ar-S}^- \\
\text{Ar} = \text{O}_2\text{N}-\text{S-S-Ar}-\text{H}_2\text{C}
\end{align*}
\]

We also believe that the short-lived red color seen in our experiments using peroxynitrite to oxidize TNB is the sulfenate ion (also called the sulfenic acid when in its
protonated form). It was found that the red color persisted for a much longer period of
time in more basic buffers, several seconds in pH 8.0 buffer versus barely perceptible in
pH 6.0 or 6.4 buffer. While the pKa of the sulfenic form of DTNB is not known, the red
color is probably longer lived in basic pH due to more of the sulfenate ion remaining
unprotonated, allowing for the intense red color to remain.

While peroxynitrite is stable, its conjugate acid peroxynitrous acid (ONOOH)
rearranges very rapidly into nitrate with a half-life of less than 1 second [20]. The pKa of
peroxynitrous acid is 6.8 at 37°C, so at pHs of 6.0 and 6.4 the peroxynitrite will convert
to peroxynitrous acid very quickly. This leaves a much lower concentration of
peroxynitrite in solution to oxidize TNB to the sulfenic acid form. As described by
Quijano, there are two possible mechanisms for the oxidation of thiols by peroxynitrite
(Scheme 2) [20]. Direct oxidation by peroxynitrite follows a two-electron oxidation
pathway to produce the sulfenic acid, which can then react with a reduced thiol to form
the disulfide. Alternatively, peroxynitrite can be protonated to form peroxynitrous acid.
Peroxynitrous acid then follows a one-electron pathway and oxidizes the thiol to the thyl
radical which can then convert to the disulfide, the peroxyl radical, or the disulfide
radical which converts to the disulfide.
Scheme 2. As described by Quijano

\[
\text{ONO}^-- \xrightarrow{\cdot \text{NO}_2} \text{RS}^* \xrightarrow{+ \text{O}_2} \text{RSSR}^- \xrightarrow{+ \text{RS}^-} \text{RSSR} \\
\text{RSOH} \xrightarrow{\text{H}_2\text{O}} \text{RSSR} \\
\text{ONOOH} \xrightarrow{\cdot \text{NO}_2} \text{RS}^* \xrightarrow{+ \text{O}_2} \text{RSOO}^- \xrightarrow{+ \text{RS}^-} \text{RSSR}^--
\]

This could be another explanation as to why the red color does not last as long in more acidic solutions, but the disulfide (DTNB) still forms. In lower pH solutions, the one-electron pathway is the most likely, as the peroxynitrite will be quickly protonated. This pathway does not include the sulfenic acid as a product. At higher pH, the two-electron pathway can be followed, allowing for the formation of the sulfenic acid form of TNB. If the reason for the lack of visible red color was due to less sulfenic acid being formed as opposed to protonation status, it would also be assumed that less thiosulfinate would form as a result. As will be seen later, the thiosulfinate product formation was unaffected by pH. This leads to the conclusion that the red color was present for longer times due to the protonation status of the sulfenic acid, and not to the reaction mechanism.

When TNP was oxidized by peroxynitrite, the red color was visible for much longer than with TNB. At pH 8.0, TNP treated with peroxynitrite stayed red for several hours, as opposed to TNB, which stayed red for only seconds even at pH 8.0. The
nitrogen atom in the ring of pyridine is electronegative, and pulls electron density from the ring making the ring carbons slightly electron deficient as compared to the benzene ring in TNB. Because of this, the sulfenate ion is more stable in TNP than in TNB, and remains in the sulfenate ion form for a longer time. This also implies that the TNP sulfenate ion would be slower to form the thiosulfinate than would the TNB sulfenate ion.

When the reaction mixture of TNB and peroxynitrite was protonated using 10% phosphoric acid, separated on HPLC, and observed at 260 nm, 4 peaks were observed at 2.4, 12, 17.8, and 20 minutes (Figure 8). The peaks at 2.4, 17.8, and 20.0 minutes all increased with increasing peroxynitrite concentration, and the peak at 12.0 minutes decreased with increasing peroxynitrite concentration. The peaks at 12 and 20 minutes were confirmed to be TNB and DTNB, respectively, by injecting pure samples of these. The peak at 2.4 minutes was shown to be the sulfinic acid form of DTNB by the performic acid oxidation described previously. The remaining peak at 17.8 minutes was suspected to be the thiosulfinate form of DTNB, based on its polarity.

The sulfinic acid and suspected thiosulfinate peaks were collected and dried down. These were then resuspended in 10% phosphoric acid and analyzed by HPLC several days later. The peak from the sulfinic acid eluted at 2.4 minutes, showing that it did not change and was stable. The peak that was suspected to be the thiosulfinate eluted at 20 minutes, showing that it was unstable and converted to DTNB.

As the sulfinic acid form of TNB is the most oxidized form, it was not susceptible to further oxidation. Further, the sulfinic acid products are stable to acid hydrolysis. In Wang's studies of disulfides, he also found that the thiosulfinate form of
TNB was oxidized using varying concentrations of peroxynitrite, then analyzed on HPLC at 260 nm. With increasing concentration of peroxynitrite, the peaks at 2.4, 17.5, and 20 (corresponding to the sulfonic acid, thiosulfinate, and DTNB) all increased, while the peak at 12 minutes (TNB) decreased. It is not clear why the peak at 17.5 minutes was split into two peaks at the highest concentration of peroxynitrite, but this occurred in all trials and was probably due to over-oxidation by the slight excess of peroxynitrite.
these compounds was generally unstable. He found that the thiosulfinates tended to 
further react to form thiosulfonates and disulfides through a disproportionation process. 
As there was no extra peak visible when the thiosulfinate was injected after several days, 
it is not likely that our product was converting to the thiosulfonate product. It is more 
likely, based on the HPLC product profiles, that the thiosulfinate of TNB converted to 
DTNB.

TNB\(^2\) was treated with peroxynitrite and was separated on HPLC after 30 
minutes, 6 days, and 10 days. When observed at 260 nm, the thiosulfinate peak 
decreased substantially throughout the time period. The sulfonic acid remained at the 
same concentration throughout, and DTNB increased slightly, probably from 
decomposition of the thiosulfinate into DTNB.

Blakeley describes the chemistry of the thiosulfinate, and shows the following 
mechanism for the reaction of the thiosulfinate form of DTNB with NaOH [19]:

\[
\text{HO}^- \quad \text{Ar}^{-\text{SO}_2^-} \quad + \quad \text{Ar}^{-\text{S}^-} \quad + \quad \text{H}^+ \\
\text{Ar}^{-\text{S}^-} \quad + \quad \text{Ar}^{-\text{S}^-} \\
\]

If the peak that eluted at 17 minutes was the thiosulfinate product, the maximum 
absorbance should change after treatment with NaOH. The maximum absorbance of the 
17 minute peak was found to be about 325 nm (Figure 9). This did not change 
immediately upon addition of peroxynitrite, or four days after addition of peroxynitrite. 
After addition of NaOH, the maximum absorbance shifted to approximately 425 nm. 
This provided further evidence that the peak eluting at 17 minutes was the thiosulfinate 
product. If the 17 minute peak had been the thiosulfonate form, there would have been 
no change in absorbance after addition of NaOH.
FIGURE 9

TNB THIOSULFINATE PEAK TREATED WITH BASE

The suspected thiosulfinate was collected as it eluted from the HPLC, then scanned using UV-Vis (blue line). It was then treated with NaOH and rescanned (red line), showing a change in maximum absorbance from \(~315\) nm to \(~425\) nm.
**pH Dependence**

It was thought that changing the pH of the solutions might influence the product profile, based on the pKa of TNB of 4.53. The oxidation of TNB$^{2-}$ by peroxynitrite was repeated at varying pH so that a relationship between the product profile and pH could be found. Each TNB solution was made using phosphate buffer of a different pH: 6.0, 6.4, 6.9, 7.4, 8.0. Peroxynitrite in NaOH was added in such a small quantity that it did not affect the pH of solution. Many of the commonly used buffers in the physiological pH range, like Tris, are oxidizable by peroxynitrite. This not only complicates product profiles, by also allows for less peroxynitrite in solution to oxidize the compounds being studied. Phosphate buffer was used because it does not react with peroxynitrite. While none of the buffers were at a low enough pH to ensure full protonation of TNB, pH 6.0 buffer should have given about 3.3% of the TNB in the protonated state. No clear relationship between pH of solution and relative concentrations of products could be identified, most likely due to there being too little of the protonated TNB present to see a difference in the product profile (Figure 10).

The pH of the solution also could have affected the thiosulfinate yield through the peroxynitrite oxidation mechanism preferred at varying pH. At alkaline pH, Quijano reports that the preferred mechanism for thiol oxidation by peroxynitrite is the two-electron pathway. This pathway gives higher yields of thiol oxidation than the competing one-electron pathway preferred at neutral and acidic pH. It would seem, based on this report, that lower pHs would give less thiosulfinate and disulfide products. In her studies, however, she used cysteine and glutathione which both have significantly higher pKas (8 and 9) than TNB. This means that in her studies at the lower pHs studied these
TNB at varying pH was oxidized with peroxynitrite in amounts so that TNB was in 1.8-fold excess of peroxynitrite, then separated on HPLC and monitored at 260 nm to best view the thiosulfinate (enlarged in lower graph). No significant change in the thiosulfinate yield was observed.
compounds were both in the protonated thiol form, and peroxynitrite was in the peroxynitrous acid form. In our studies, at low pH peroxynitrite would quickly convert to peroxynitrous acid, but TNB was still unprotonated.

**Peroxynitrite Oxidation of DTNB**

Oxidation of DTNB using peroxynitrite did not yield any thiosulfinate, but did yield a very small amount of the sulfonic acid product (Figure 11). After five hours of reaction time, the solution had changed from colorless to light yellow. As TNB$^{2-}$ is yellow in color, HPLC was repeated and monitored at 380 nm to better view any TNB present, but none was observed.

Comparatively, the amount of sulfonic acid formed from oxidation of DTNB was significantly less than was formed by the oxidation of TNB by peroxynitrite.

**II. NBD-Cl Trapping of Sulfenic Acid**

The first of the sulfenic acid intermediates that was studied was the TNP sulfenic acid, because this compound was present for a much longer time period based on the color of the solution. In pH 7.4 or 8.0 buffer solutions, the red color of the TNP sulfenic acid was visible for upwards of 2 hours, as opposed to 2 seconds for the TNB sulfenic acid.

Mixing of TNP and NBD-Cl without peroxynitrite in solution formed the RS-NBD product. This product was easily observable at 420 nm and eluted at approximately 18 minutes.
Very little sulfonic acid formed by the oxidation of DTNB with a slight excess of peroxynitrite, as shown in the enlarged view of the peak eluting at 2.3 minutes. The final ratio of peroxynitrite to DTNB was 1.125.
All attempts to identify the product of the TNP sulfenic acid and NBD-Cl failed, as too many product peaks appeared to identify which one was the desired product (Figure 12). It was thought that the NBD-Cl could be reacting with the ring nitrogen of the pyridine, so TNB (which has no nitrogen in the ring) was tried next.

Mixing of TNB and NBD-Cl without peroxynitrite in solution formed the thioether product, visible at 420 nm. This product eluted at approximately 17.5 minutes.

It was originally thought that the peak being formed by the TNB sulfenic acid-NBD complex was overlapping with the NBD-Cl peak (Figure 13). For this reason, the separation was expanded from 25 minutes to 32 minutes to better separate the peaks. At this point it became clear that the peak that was believed to be the trapped sulfenic acid was actually just DTNB that had formed from treatment of TNB with peroxynitrite (Figure 14). No other peaks were formed that seemed to be from the trapped sulfenic acid. Based on the assumption that the red color visible immediately after mixing TNB with peroxynitrite was due to the sulfenic acid intermediate, the sulfenic acid form of TNB was present for only one or two seconds. It is likely that the sulfenic acid intermediates reacted to form the thiosulfinate product before NBD-Cl could trap them. The red color persisted much longer with the TNP sulfenic acid, showing that this compound was much more stable than the TNB sulfenic acid. While there was sufficient time for the NBD-Cl to react with the TNP sulfenic acid, too many side reactions occurred to make it possible to identify the trapped sulfenic acid product.

An attempt to distinguish the TNP-NBD product peak was attempted again, as the TNB sulfenic acid was too short-lived to be trapped. When UV-Vis scans were taken of the TNP, peroxynitrite, NBD-Cl mixture over half an hour, the absorbance decreased
FIGURE 12
NBD-CL TRAPPING OF TNP SULFENIC ACID

The products of TNP, peroxynitrite, and NBD-Cl were analyzed on HPLC at 343 nm to view the sulfenic acid adduct, and 420 nm to view the thiol adduct. As can be seen in the graph above, many products formed from the reaction of oxidized TNP (including the sulfenic acid) and NBD-Cl. The peaks corresponding to TNP, NBD-Cl, RS-NBD, and DTNP were identified at 6, 12, 16, and 18 minutes, respectively. The other small peaks were unidentifiable.
Products were analyzed on HPLC at 343 nm and 420 nm. It was originally thought that the peak shown at 15.5 minutes (overlapping with the 15 minute peak corresponding to DTNB) was the desired RSO-NBD. The RS-NBD is clearly visible at 17.5 minutes.
When the method was extended to separate the peaks that had been occurring at 15 minutes, it became clear that the second peak was due to DTNB. The peak at 11 minutes was then identified as TNB, 17 minutes was NBD-CI, 18 minutes was DTNB, and 20 minutes was RS-NBD. This left no unidentified peaks that could be the desired RSO-NBD product.
over time at all wavelengths (Figure 15). This was due to the products and excess reagents precipitating over the half hour. In the UV-Vis scans at time=0, 1, and 2 minutes, there is a slight shift of peak absorbance from about 335 nm to 330 nm. Because this was present on multiple trials of this reaction, using both 50 mM and 40 mM DTT to reduce the disulfide bonds, it is possible that this shift signals a change in product. Poole reports that free NBD-Cl absorbs maximally at 343 nm, and at 347 nm when bound to a protein sulfenic acid. Because Poole was using NBD-Cl to trap a sulfenic acid on a protein, the products formed in her studies were not aromatic rings like TNB and TNP. As all of the compounds being trapped in our studies were aromatic and had their own characteristic absorbances, it is likely that the peak absorbance of NBD-Cl bound to the sulfenic acid of TNB or TNP would be shifted from the peak observed by Poole.

HPLC separations of the reaction mixture yielded many unidentifiable peaks. Peaks corresponding to NBD-Cl, the product formed by reaction of TNP with NBD-Cl, and DTNP were all easily identified. Of the peaks unaccounted for by controls, there were three that increased with increasing peroxynitrite concentration and were not present in the absence of peroxynitrite. (Figure 16). The peak that eluted at 17 minutes, immediately before NBD-Cl was collected, dried down, and resuspended in 70% Acetonitrile Solvent B. As there was no absorbance peak at 347 nm, it was assumed that this peak was not an NBD-Cl complex. This was repeated with the peaks eluting at 9 minutes and 14 minutes. The 9 minute peak absorbed at both 292 nm and 385 nm, causing it to be discarded as a possibility as well. The remaining peak, that at 14 minutes, yielded a maximum at 332 nm (Figure 17). This peak appeared to be created by
TNP was oxidized with peroxynitrite, NBD-Cl was added, and the solution was immediately scanned on UV-Vis (top blue line). The scan was then repeated after 1, 2, 4, 7, 12, 18, and 30 minutes showing a decrease in absorbance at all wavelengths. This was most likely due to the reagents precipitating out of solution. There is a slight shift in peak absorbance from minute=0 to minute=1, as visible from the shift from the blue line to the red line.
The relevant section of the HPLC chromatogram (343 nm) is enlarged below, with the concentrations labeled to show the order of peak amplitude from highest to lowest. Only the peak at 14 minutes followed the expected pattern with the highest amplitude (and therefore amount of product) corresponding to the trial using the highest concentration of peroxynitrite.
The peak that eluted at 14 minutes (shown in Figure 11) was collected as it eluted from the HPLC, then scanned on UV-Vis. The peak appears to be an overlay of two peaks, based on the shape. This could be explained by an average of the maximum absorbance of NBD-Cl bound to a protein sulfenic acid at 347 nm, and the maximum absorbance of DTNP in solvent B at 316 nm. A compound made up of NBD-Cl bound to the sulfenic acid form of DTNP might have the absorbance average of the two components.
overlapping absorbance maximums. As NBD-Cl bound to a protein sulfenic acid absorbs maximally at 347 nm, and DTNP in solvent B absorbs maximally at 316.8 nm, it seemed possible that this could be the peak of interest.

The reaction was repeated, varying the pH of solution. Samples were separated on HPLC and observed at 332 nm. The peak at 14 minutes varied little at pH 6.0, 6.4, and 6.9, but dropped significantly at pH 8.0, contrary to expected. The quantity of thiosulfinate product was not affected by pH. As this product is thought to be formed by sulfenic acid in solution, it was thought that if the yield of thiosulfinate was not affected by pH, then the sulfenic acid would not be either, and the amount of trapped sulfenic acid would also be unaffected by pH. As this experiment was not repeated, it is possible that the decrease in absorbance of the peak at 14 minutes was simply an experimental error.

III. Dimedone Trapping of Sulfenic Acid

It was hoped that the use of dimedone as a trapping agent would eliminate the side-reaction problems experienced when using NBD-Cl, as dimedone is nucleophilic and does not react with reduced thiols.

When dimedone was initially used to trap the sulfenic acid form of TNB, the separations yielded peaks at 6, 7, 11, 12, 18, and 20 minutes. Only those provided by dimedone (6 minutes), TNB (12 minutes), and DTNB (18 minutes) could be easily identified.
Time Dependence of Dimedone Trapping

A mixture of TNB and dimedone that had been diluted in phosphoric acid absorbed maximally at 330 nm. When this was repeated adding peroxynitrite before dilution in phosphoric acid and scanned immediately after mixing, the absorbance decreased slightly. When scanned again at 0.5, 1, 2, and 5 hours, the absorbance at 330 nm fell away, and the absorbance at 380 rose significantly (Figure 18). Identical samples were separated on HPLC immediately after mixing then again after 0.5, 1, 2, 3.5, and 5 hours and observed at 320 nm (Figure 19). Overlay of these plots showed an increase in the peaks at 12 and 20 minutes with time, and a decrease in the peak at 18 minutes.

Dimedone Purification

Dimedone was purified to remove impurities that were visible on HPLC, and were interfering with the identification of peaks. Purification of dimedone using a C18 column removed the impurity that was causing the dimedone solution to be yellow. It also removed the peak from the HPLC plot that eluted immediately before TNB at 11 minutes, making it easier to see the TNB peak (Figure 20). Purification using a C18 column required a high concentration of ammonium acetate in solution to keep dimedone in the enol form so that it would not stick to the column. Dimedone passed through the column and was collected, while the impurity remained bound to the C18 column. Ammonium acetate is a neutral salt with a pH of approximately 7, which made it impossible to change the pH of solution when using purified dimedone. This, combined with concern that the high concentration of ammonium acetate could interfere with the desired reaction, caused purified dimedone solution to be used in only some experiments.
TNB was mixed with dinedone so that dinedone was in 50-fold excess of TNB, and the solution was scanned on UV-Vis yielding an absorbance maximum at 330 nm as shown by the blue line. Once peroxynitrite was added, the absorbance at 330 nm began to decrease as seen in the red line. The scan was repeated on the same solution after 30 minutes, 1 hour, 2 hours, and 5 hours, yielding the green, pink, teal, and navy lines. This demonstrates a decrease in absorbance at 330 nm, and an increase at 380 nm.
TNB was oxidized with peroxynitrite, then mixed with a 50-fold excess of dimedone to trap the sulfenic acid. This was separated on HPLC and monitored at 320 nm immediately after mixing, then repeated after 0.5 hour, 1 hour, 2 hours, 3.5 hours, and 5 hours. The peaks are enlarged in the lower plots, showing an increase in the peaks at 12 and 20 minutes, and a decrease in the peak at 18 minutes.
Dimedone was taken directly from the bottle and dissolved in methanol, separated on HPLC, and monitored at 260 nm. Dimedone was then purified using ammonium acetate and a C18 column, and analyzed on HPLC under the same conditions. The peak at 12 minutes that had been interfering with product peaks with the unpurified dimedone was successfully removed by the C18 column. However, a new peak appeared at 3.5 minutes, probably due to the ammonium acetate.
Temperature Dependence for Dimedone Trapping

Though there was not much variation in the product profile when the temperature was varied, there were slight differences in relative amounts of products (Figure 21). The peaks corresponding to TNB, thiosulfinate, and DTNB all decreased in amplitude with increasing temperature. The unexplained peak at 12 minutes increased with increasing temperature, and the peak at 21 minutes was highest at 37C.

Peroxynitrite Concentration Dependence

When the concentration of peroxynitrite in solution was increased, the peak that eluted at approximately 21 minutes also increased in amplitude, as expected for the RSO-dimedone peak (Figure 22).

pH Dependence

When the pH of the solution was changed, the change in amplitude of the 21 minute peak was not affected in a clear pattern (Figure 23). It was expected that the pH 8.0 solution would yield the highest amount of RSO-dimedone, as this pH gave the solution that stayed red the longest, implying a more stable sulfenate ion. For the peak
TNB was mixed with peroxynitrite, then treated with a 50-fold excess of purified dimedone. The samples were incubated at 4, 22, 37, and 62°C, then analyzed on HPLC at 380 nm. The unidentified peak at 12 minutes (1) increased with increasing temperature, and the peak at 21 minutes (3) was found to be the highest at 37°C. DTNB (2) decreased with increasing temperature.
TNB was oxidized using varying concentrations of peroxynitrite, then mixed with dimedone to trap the sulfenic acid and analyzed on HPLC at 380 nm. The peaks eluting at 12 and 20 minutes both increased with increasing peroxynitrite, while the peak at 11 minutes (TNB) decreased. It was originally thought that the peak that eluted at 12 minutes was the desired RSO-dimedone peak, but this was shown to be wrong by its presence even with 0 mM peroxynitrite.
TNB at varying pH was oxidized with peroxynitrite then mixed with a 50-fold excess of dimedone and analyzed by HPLC at 380 nm. As can be seen above, the peak at 20 minutes, which is suspected to be the trapped sulfenic acid, did not follow a specific pattern, with the highest amount of product being formed at pH 7.4, followed by 8.0 and 6.9.
eluting at 21 minutes, the highest absorbance was found with pH 7.4 solution, followed by pH 8.0 and pH 6.9.

IV. Thiosulfinate Reactivity

All of the procedures used by other research groups to trap the sulfenic acid form of proteins using NBD-Cl and dimedone required the solutions to react for 30 minutes with NBD-Cl and for twelve hours with dimedone. In their studies, they were trapping sulfenic acids in proteins that either had only one cysteine residue total, or they used mutated versions that did not contain other cysteines. This was important because it made it impossible for the sulfenic acid to react with other thiols to form the thiosulfinate.

In our studies, it was impossible to prevent the sulfenic acid from reacting with other thiols in solution, thus thiosulfinate forms and can react with the trapping agents NBD-Cl and dimedone. As the HPLC product profiles and UV-Vis absorbance spectra all changed over several hours, even after the sulfenic acid was suspected to be gone from solution, it was likely that some of the products seen were formed by reactions between the trapping agents and the thiosulfinate. This was suspected to be a problem, especially with dimedone, as it is a nucleophile and can react with thiosulfinates by the following mechanism:

\[
\begin{align*}
\text{Ph—S—S—Ph} & \quad + \quad \text{Nu—S—Ph} \\
\quad + \quad \text{Nu—S—Ph} & \quad \quad \quad \text{Ph—S—Ph} \\
\quad + \quad \text{Nu—S—Ph} & \quad \quad \quad \text{Ph—S—S—Ph}
\end{align*}
\]
In order to test the possibility that these trapping agents were reacting with the thiosulfinate, it was collected after an HPLC separation, dried overnight, then reacted with the trapping agents.

The thiosulfinate did not decay into DTNP overnight while stored in methanol and buffer, as determined by UV-Vis scans. There was a characteristic thiosulfinate peak at approximately 340 nm, but no peak at 320 nm to signal DTNP. HPLC separations also showed thiosulfinate present, but no clear DTNP peak. It was also found by HPLC that dimedone did not react with the thiosulfinate, contrary to expected.

From the scans, it was clear that some of the NBD-Cl reacted with the thiosulfinate as seen by the slight increase in absorption at approximately 420 nm. The RS-NBD product was described by Poole to absorb at 420 nm, so this is probably the product seen.

Once separated on HPLC, the NBD-Cl thiosulfinate solution showed three distinct peaks (Figure 24). While one of these was clearly due to NBD-Cl and the other to unreacted thiosulfinate, there was still a third peak present that was unidentified. Interestingly, the third product peak was not from the RS-NBD product, as shown by a control experiment in which TNP was allowed to react with NBD-Cl, then separated on HPLC.

As NBD-Cl is an electrophilic reagent, it is unlikely that it would react directly with the thiosulfinate sulfur atoms. One possibility would involve a breakdown of the thiosulfinate into the sulfinic acid and TNP as shown:

\[
\text{OH}^- \quad \overset{\text{O}^-}{\text{Ar}} \quad \overset{\text{S}+}{\text{S}} \quad \overset{\text{S}^-}{\text{Ar}} \quad \rightarrow \quad \text{Ar} \quad \overset{\text{SO}_2^-}{\text{Ar}} \quad + \quad \text{Ar} \quad \overset{\text{S}^-}{\text{S}} \quad + \quad \text{H}^+ 
\]
The thiosulfinate form of DTNP was collected as it eluted it from an HPLC separation, then dried down and mixed with NBD-Cl in methanol. It can be seen that the thiosulfinate did not decay into DTNP, and that the thiosulfinate and NBD-Cl did react. While one of the products can be identified as the RS-NBD, the other is unknown.
If this reaction were to occur, TNP in solution would react with NBD-Cl to form the RS-NBD. As there was no peak at 19 minutes where the RS-NBD characteristically elutes, this was probably not the process occurring.

The most likely scenario is that the thiosulfinate decomposed into two sulfenic acid molecules once dissolved in the pH 7.4 buffer solution as shown:

\[
\text{Ar} \quad \text{S}^+ \quad \text{S} \quad \text{Ar} + \text{OH}^- \quad \rightarrow \quad 2 \quad \text{Ar} \quad \text{SO}^- \quad + \quad \text{H}^+
\]

While these sulfenic acids could quickly react to reform the thiosulfinate, there was probably an equilibrium established in which a small amount of sulfenic acid was present to react with the NBD-Cl. As the NBD-Cl trapped the sulfenic acid molecules, more thiosulfinate would break down to create more sulfenic acid molecules. Following Le Chatelier’s Principle, NBD-Cl binding the sulfenic acids would shift the equilibrium to the products side (more sulfenic acid), allowing for a significant amount of sulfenic acid to be trapped.

**Lipoic Acid**

Another way to demonstrate whether or not dimedone and NBD-Cl react with the thiosulfinate forms of DTNB and DTNP is to test their reactivity with another thiosulfinate. α-Lipoic acid is an antioxidant containing two sulfur groups which can bond to form a disulfide bond. When mixed with hypochlorous acid (bleach), the reduced form of α-Lipoic acid is oxidized to the thiosulfinate form, β-Lipoic acid [22,23].
When scanned on UV-Vis, the oxidized form of $\alpha$-Lipoic acid had a maximum absorbance at 330 nm, and the reduced form had a maximum around 280 nm, though the total absorbance for the reduced form was relatively low. Once the reduced form was mixed 1:1 with bleach, the absorbance shifted to 240 nm. This was suspected to be due to formation of $\beta$-lipoic acid. When separated on HPLC, there were several product peaks visible in the $\beta$-lipoic acid sample (Figure 25). While the peak at 16.5 minutes was relatively nonpolar and could easily be identified as oxidized $\alpha$-lipoic acid by controls, the other two were much more polar and unidentified. The peak that eluted at 7 minutes (the more nonpolar) was highest in amplitude when a 1:1 ratio of lipoic acid to bleach was used. The most polar of the three peaks, eluting at 4.5 minutes, was highest when 1:2 lipoic acid to bleach was used. It is most likely that the peak eluting at 7 minutes is the desired thiosulfinate, and the other peak is the more oxidized thiosulfonate:
α-Lipoic acid was reduced using DTT, then mixed with varying concentrations of hypochlorous acid (bleach) to oxidize it to the thiosulfinate form (β-lipoic acid) and analyzed on HPLC at 240 nm. The peak eluting at 17 minutes is α-lipoic acid, and the two at 4 and 6 minutes are thought to be the thiosulfonate and thiosulfinate, respectively. The suspected thiosulfinate was in greatest yield when a 1:1 ratio of lipoic acid to bleach was used, while the suspected thiosulfonate was in greatest yield with 1:2 lipoic acid to bleach, as expected.
In order to confirm that the peak at 7 minutes is β-lipoic acid, GC-MS could be used, but the carboxyl group would need to be derivatized using N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) to silylate the carboxyl group.
CONCLUSIONS and FUTURE WORK

The original goal of this project was to find conditions under which TNB could be oxidized using peroxynitrite to yield a single oxidation product, DTNB. It quickly became clear that this was impossible, due to the high oxidative power of peroxynitrite and the ease with which the small TNB and TNP molecules were able to react to form multiple products. While these compounds are probably not useful for calculating rate constants for the oxidation of protein thiols, they are of great interest due to the products that formed.

The oxidation of thiols has been of great interest to biochemists in the last several years, due to the medical relevance of protein oxidation. While biochemists have been discovering multiple proteins that contain stable cysteine sulfenic acids, most of these are largely stabilized by the absence of other cysteine residues with which the sulfenic acid can react. The relatively high stability of the TNP sulfenic acid in basic solution is very unexpected, due to the ease by which this small molecule can react with other thiols in solution. In experiments in which TNP was oxidized using peroxynitrite, there was always excess TNP in solution, providing a source of unreacted thiols with which the sulfenic acid could react. Furthermore, the sulfenic acid groups were not at all sterically hindered from reacting, as they often are in proteins. In this molecule, the stability must
come from the stabilization of the negative charge of the sulfenate ion by the electron withdrawing ring structure.

The reactivity of the thiosulfinate form of TNP with NBD-Cl is also of interest, as this molecule has just recently begun to be used as a sulfenic acid trapping agent. The presence of a product formed by the mixing of the TNP thiosulfinate with NBD-Cl demonstrates the importance of there being only a single cysteine residue on the proteins being studied using this compound. If there were more than one cysteine residue, two cysteine sulfenic acids could react to form a thiosulfinate. While it is not clear from this study whether NBD-Cl reacted directly with the thiosulfinate or with sulfenic acid molecules that were in equilibrium with the thiosulfinate, it is clear that in situations where thiosulfinates can occur the formation of a sulfenic acid adduct with NBD-Cl is not necessarily indicative of a stable sulfenic acid.

Future work on this project would necessitate the use of mass spectrometry to confirm the formation of a dimedone-sulfenic acid adduct, or an NBD-Cl-sulfenic acid adduct by collecting the suspected peaks as they eluted from HPLC. After confirmation of adduct formation, the characteristic elution time of the adduct on HPLC would be known. This could then be used to find the experimental conditions which maximize adduct formation, and likewise sulfenic acid formation.
REFERENCES


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