BIOLOGICALLY RELEVANT CHEMISTRY OF SULFUR HETEROCYCLES: FROM REDOX REGULATION OF PTP1B TO THE BIOLOGICAL ACTIVITY OF S-DEOXY LEINAMYCIN

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by
SANTHOSH SIVARAMAKRISHNAN
Dr. Kent S. Gates, Dissertation Supervisor

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

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FROM REDOX REGULATION OF PTP1B TO THE BIOLOGICAL ACTIVITY OF S-
DEOXY LEINAMYCIN

presented by Santhosh Sivaramakrishnan,
a candidate for the degree of doctor of philosophy
and hereby certify that, in their opinion, it is worthy of acceptance.

________________________________________
Professor Kent S. Gates

________________________________________
Professor Timothy Glass

________________________________________
Professor Rainer Glaser

________________________________________
Professor Jason Cooley

________________________________________
Professor Frank Schmidt
Dedicated to the loving memory of my grandparents

Mrs. Karpagam Venkatraman & Mr. Venkatraman

--whose divine blessings made everything possible
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TABLE OF CONTENTS

ABSTRACT .................................................................................................................................................. ii
TABLE OF CONTENTS ................................................................................................................................. iv
LIST OF FIGURES ......................................................................................................................................... xii
LIST OF SCHEMES ....................................................................................................................................... xvi
LIST OF TABLES .......................................................................................................................................... xxii

CHAPTER 1. Chemical Model for the Redox Regulation of Protein tyrosine phosphatase 1B (PTP1B)

1.1 Introduction ........................................................................................................................................ 1

1.1.1 Background .................................................................................................................................. 1

1.1.2 Insulin-mediated signal transduction cascade .............................................................................. 3

1.1.3 Role of PTPs in insulin-mediated glucose uptake ........................................................................... 3

1.1.4 Endogenously produced reactive oxygen species can modulate the activity of PTPs ........ 4

1.1.5 Evidences for the role of PTP1B as a negative regulator of insulin signaling cascade... 6

1.1.6 Generation of sulfenyl amide during the oxidative inactivation of PTP1B ......................... 8

1.1.7 Proposed mechanisms for the formation of sulfenyl amide at the active site of PTP1B ........................................................... 9

1.2 Goals of this Chapter .......................................................................................................................... 10

1.3 Design of the PTP1B model system ...................................................................................................... 11
1.4 Synthesis of sulfenic acid precursors...............................................................12

1.4.1 Synthesis of sulfenic acid precursor 27.......................................................15

1.4.2 Synthesis of sulfenic acid precursor, 29......................................................16

1.4.3 Synthesis of sulfenic acid precursor, 32......................................................16

1.5 Generation of 1,2-benzisothiazolin-3-(2H)-one under aqueous buffer conditions.........17

1.6 Trapping the intermediate sulfenic acid under our reaction conditions.....................18

1.7 Experiments to rule out the intermediacy of thiosulfinate in the cyclization reaction........21

1.7.1 Background and set up.................................................................................21

1.7.2 Characterization of the authentic products stemming from the dimerization of sulfenic acid........................................................................................................22

1.7.3 Reaction of 36 under excess methyl iodide conditions that blocks the potential dimerization...........................................................................................................23

1.7.4 Reaction of 32 under excess methyl iodide conditions that completely block the dimerization of sulfenic acid........................................................................24

1.7.5 Reaction of thiosulfinate adjacent to secondary amide (49) under aqueous buffer conditions.............................................................................................................25

1.7.6 Monitoring the cyclization reaction under HPLC............................................26

1.8 Can our model mimic the oxidative inactivation chemistry of PTP1B?......................27
CHAPTER 2. Chemical Model Studies Suggests that Sulfenyl amide Protects Against
Hydrogen peroxide-mediated Over-oxidation of enzyme PTP1B

2.1 Introduction...........................................................................................................101

2.2 Goals of this Chapter............................................................................................103

2.3 Synthesis of model compounds...........................................................................104

2.3.1 Synthesis of 1,2-benzisothiazolin-3(2H)-one (34)..........................................104

2.3.2 Synthesis of mixed disulfide analog (55)........................................................104

2.3.3 Synthesis of sulfenic acid mimic (61)..............................................................104

2.4 Stability of analogs to hydrogen peroxide mediated over-oxidation in aqueous buffer

Solution.....................................................................................................................107

2.4.1 Oxidation of benzisothiazolin-3(2H)-one (34).................................................107
2.4.2 Oxidation of mixed disulfide compound (55)……………………………………...109

2.4.3 Oxidation of methyl benzenesulfenate (68)………………………………………..110

2.5 Stability of analogs to hydrogen peroxide mediated over-oxidation in aqueous acetonitrile mixture…………………………………………………………………………………………111

2.5.1 Oxidation of sulfenyl amide (34)……………………………………………………111

2.5.2 Oxidation of mixed disulfide analog (55)……………………………………………112

2.5.3 Oxidation of methyl benzenesulfenate (68)……………………………………….....113

2.6 PTP1B resists irreversible over-oxidation…………………………………………………113

2.7 Conclusion………………………………………………………………………………....116

2.8 Experimental Procedures…………………………………………………………………..117

References……………………………………………………………………………………..125

CHAPTER 3:Insights into the Properties of Sulfenyl amide

3.1 Introduction…………………………………………………………………………….....135

3.2 Goals of this Work………………………………………………………………………...135

3.3 Generation of sulfenyl amide by hydrogen peroxide mediated oxidation of PTP1B in aqueous buffer………………………………………………………………………………………136

3.3.1 Thiol reversible oxidative inactivation of PTP1B in presence of hydrogen peroxide136
3.3.2 Trapping the oxidatively inactivated intermediate using NBD-Cl .................. 140

3.4 Determining the rate constant for the cyclization reaction ($k_3$) .................. 143

3.5 Can glutathione trap the intermediate sulfenic acid? .............................. 145

3.6 Conclusion .................................................................................................... 148

3.7 Experimental procedures ............................................................................. 149

References .......................................................................................................... 156

CHAPTER 4. Chemical Model Studies Predict that “Over-oxidation” of the Active site Cysteine of PTP1B to the Sulfinyl Oxidation State Yields a Thiol Reversible but Hydrolytically Labile form of the enzyme

4.1 Introduction .................................................................................................... 158

4.1.1 Background ............................................................................................... 158

4.1.2 Over-oxidation to sulfinic acid is reversible in peroxiredoxins (Prxs) ....... 159

4.1.3 Hydrogen peroxide-mediated oxidation of PTP generates a novel sulfenic oxidation state ................................................................. 160

4.2 Goals of this chapter ................................................................................... 161

4.3 Properties of 1,2-benzisothiazolin-3(2H)-one 1-oxide ............................... 162

4.3.1 Synthesis of 1,2-benzisothiazolin-3(2H)-one 1-oxide (69) ..................... 162

4.3.2 Reaction of compound 69 with thiol ......................................................... 162

4.3.3 Possible mechanism for the thiolysis reaction of compound 69 ............... 163

4.3.4 Hydrolysis of 1,2-benzisothiazolin-3(2H)-one 1-oxide .......................... 165

4.4 Measuring rate constants for the reaction of 69 with water and thiol under aqueous buffer conditions ................................................................. 166
4.4.1 Rate constant for the reaction of 69 with water.........................................................166
4.4.2 Rate constant for the reaction of 69 with thiol..........................................................167
4.5 Properties of 1,2-benzisothiazolin-3(2H)-one 1,1-dioxide..............................................168
  4.5.1 Synthesis of 1,2-benzisothiazolin-3(2H)-one 1,1-dioxide (99).................................168
  4.5.2 Stability of 99 in presence of water and thiol.........................................................169
4.6 Discussion.......................................................................................................................169
4.7 Experimental methods.................................................................................................173
References.........................................................................................................................180

CHAPTER 5. Evidence for the Presence of a Thiol Reversible but Hydrolytically Labile Intermediate during the Oxidative Inactivation of PTP1B

5.1 Introduction....................................................................................................................190
5.2 Goals of this Chapter....................................................................................................191
5.3 Determining the generation of sulfinyl amide at the active site of enzyme PTP1B............192
  5.3.1 Experimental strategy to measure sulfinyl amide at the active site of PTP1B..............193
5.4 Evidence for a thiol reversible but hydrolytically labile intermediate during the oxidative inactivation of PTP1B.................................................................196
  5.4.1 Sulfinyl amide formed at the active site of PTP1B is hydrolytically stable at pH 7......198
5.5 Rate constant for the hydrolysis of sulfinyl amide (k9)...............................................199
5.6 Determining the rate constant for the over-oxidation of sulfinyl amide (k4)...............201
5.7 Conclusion......................................................................................................................204
CHAPTER 6. Possible Chemical Mechanisms Underlying the Biological Activity of S-deoxy Leinamycin

6.1 Introduction

6.1.1 Leinamycin – a novel structure with a potent biological activity

6.1.2 Chemical mechanisms of DNA damage by leinamycin

6.2 Goals of this Chapter

6.3 Synthesis of 1,2-dithiolan 3-one heterocycle

6.4 DNA damage by 1,2-dithiolan 3-one heterocycle

6.5 Investigation of the mechanism of thiol triggered DNA damage by 116

6.6 Other possible reasons for the SOD mediated increase in strand breaks

6.7 Generation of hydrogen sulfide in the reaction of 116 + thiol

6.7.1 Qualitative detection of hydrogen sulfide in our assays

6.8 Characterizing the products in the reaction of thiol with 116

6.9 Generation of polysulfides in the reaction of thiol with 116

6.10 Possible chemical mechanisms for the reaction of 116 with thiol
6.10.1 Attack on S1 sulfur of 1,2-dithiolan 3-one heterocycle………………………………………234

6.10.2 Attack on S2 sulfur of 1,2-dithiolan 3-one heterocycle………………………………………235

6.10.3 Attack of thiol at the carbonyl carbon of the 1,2-dithiolan 3-one heterocycle………237

6.11 Comparison of DNA damage by 1,2-dithiolan 3-one analogs (102, 116 and 133)………237

6.12 Conclusions………………………………………………………………………………………………………240

6.13 Experimental procedures…………………………………………………………………………241

References……………………………………………………………………………………………………………...249
LIST OF FIGURES

Chapter 1

Figure 1.1. Role of PTP1B in insulin mediated signal transduction cascade…………………..3

Figure 1.2. Insulin triggered production of hydrogen peroxide inactivates PTP1B………………5

Figure 1.3. Formation of sulfenyl amide during the oxidative inactivation of PTP1B………………8

Figure 1.4. Basic chemical model to mimic the redox sensing assembly of PTP1B’s active site………………………………………………………………………………………………………………………………………12

Figure 1.5. HPLC monitoring of the reaction of 32 under aqueous buffer conditions……………26

Figure 1.6. NMR spectrum of 34 under various conditions of oxidative stress………………34

Figure 1.7. A plot of HPLC peak area vs time for the disappearance of 55………………………53

Figure 1.8. HPLC monitoring of the reaction of 34 with 2-mercaptoethanol…………………56

Figure 1.9. A plot for determining pK_a of 54………………………………………………….57

Chapter 2

Figure 2.1. Chemical models for the protective intermediate forms of enzyme PTP1B………103

Figure 2.2. Chemical model for sulfenic acid mimic…………………………………………105

Figure 2.3. HPLC chromatogram of 61 in acetonitrile and aqueous buffer conditions………..106

Figure 2.4. A plot of ln(a/a_0) vs time for the hydrogen peroxide mediated oxidation of 34 108
Figure 2.5. A plot of ln(a/a_0) vs time for the hydrogen peroxide mediated oxidation of 55 …109

Figure 2.6. HPLC chromatogram of 68 in acetonitrile and aqueous buffer conditions………110

Figure 2.7. A plot of ln(a/a_0) vs time for the hydrogen peroxide mediated oxidation of 34 …112

Figure 2.8. A plot of ln(a/a_0) vs time for the hydrogen peroxide mediated oxidation of 55 …112

Figure 2.9. A representative plot of ln(a/a_0) vs time for the oxidation of 68 .....................113

Figure 2.10. Comparison of % recoverable activities after the oxidation of Cdc25-(C426S) and PTP1B in presence of 1 mM hydrogen peroxide.........................................................114

Chapter 3

Figure 3.1. A plot of remaining activity vs time for the oxidation of PTP1B with 50 µM H_2O_2 for 40 min .................................................................139

Figure 3.2. UV spectrum of the NBD modified PTP1B.................................................142

Figure 3.3. A representative plot of [H_2O_2] vs unrecoverable/recoverable activity towards measuring k_3 .................................................................145

Figure 3.4. A plot of [GSH] vs recoverable activity during the oxidation of PTP1B........148

Figure 3.5. Oxidation of PTP1B with increase in concentration of hydrogen peroxide………153

Figure 3.6. Oxidation of sulfenyl amide under the oxidation conditions used to measure k_3 ......................................................................................................................154

Figure 3.7. Oxidation of PTP1B in presence of varying concentrations of GSH..............155
Chapter 4

**Figure 4.1.** A representative plot of ln(a/a₀) vs time for the hydrolysis of 69 ..................167

**Figure 4.2.** A representative plot of ln(a/a₀) vs time for the thiolysis of 69 ..................168

**Figure 4.3.** A plot of HPLC peak area vs time for the hydrolytic decomposition of 99 ......178

**Figure 4.4.** A plot of HPLC peak area vs time for the reaction of 99 in presence of thiol.....179

Chapter 5

**Figure 5.1.** Electron density map of RPTPα during oxidation.................................191

**Figure 5.2.** Experimental strategy towards identifying sulfinyl amide (85) ....................195

**Figure 5.3.** Strategy to measure the thiol reversible and hydrolytically labile intermediate upon oxidation of PTP1B ..........................................................196

**Figure 5.4.** A plot showing the remaining activity of oxidized PTP1B after reactivation and hydrolysis .................................................................197

**Figure 5.5.** Decrease in recoverable activity upon hydrolysis of the oxidized PTP1B........197

**Figure 5.6.** Hydrolytic stability of sulfenyl amide......................................................199

**Figure 5.7.** Experimental strategy to measure the rate constant for hydrolysis of sulfinyl amide (kₒ).................................................................200

**Figure 5.8.** Plot of ln(a/a₀) vs time towards measuring the rate constant for hydrolysis of sulfinyl amide (kₒ)......................................................201
Figure 5.9. An experimental strategy for oxidation of sulfenyl amide towards measuring k4...202

Figure 5.10. A representative plot of ln(a/a0) vs time for the oxidation of sulfenyl amide……203

Figure 5.11. A representative plot of decrease in [sulfinyl amide] over time.........................207

Figure 5.12. A representative plot of decrease in [sulfenyl amide] vs time.........................209

Chapter 6

Figure 6.1. Leinamycin.................................................................211

Figure 6.2. Analogs of leinamycin’s active dithiolanone heterocycle.................................213

Figure 6.3. S-deoxy leinamycin having a 1,2-dithiolan 3-one moiety (with no sulfoxide).....216

Figure 6.4. Thiol dependent DNA cleavage by various concentrations of 1,2-dithiolan 3-one (116)......................................................................................................................221

Figure 6.5. Thiol dependent DNA cleavage by 116 in presence of various additives.......222

Figure 6.6. Thiol dependent DNA cleavage by 116, 102 and 133 .....................................238

Figure 6.7. HPLC chromatogram of polysulfides in the reaction of 2-ME with 116 ..........248
LIST OF SCHEMES

Chapter 1

**Scheme 1.1.** Redox regulation of protein tyrosine phosphatases (PTPs)..............................6

**Scheme 1.2.** Catalytic mechanism for dephosphorylation of tyrosine residues by PTPs ........7

**Scheme 1.3.** Novel sulfenyl amide intermediate during the redox regulation of PTP1B.........9

**Scheme 1.4.** Different mechanisms for the formation of sulfenyl amide intermediate.........10

**Scheme 1.5.** Generation of sulfenic acid via $\beta$ elimination of 4-pyridyl ethyl sulfoxide......13

**Scheme 1.6.** Generation of isothiazolidinone via $\beta$ elimination of 4-pyridyl ethyl protecting group........................................................................................................................................14

**Scheme 1.7.** Sulfenic acid precursors 23 and 24.................................................................15

**Scheme 1.8.** Synthesis of sulfenic acid precursor 27.........................................................15

**Scheme 1.9.** Synthesis of sulfenic acid precursor 29.........................................................16

**Scheme 1.10.** Synthesis of sulfenic acid precursor 32....................................................17

**Scheme 1.11.** Generation of 1,2-benzisothiazolin-3-(2H)-one via the sulfenic acid intermediate ........................................................................................................................................18

**Scheme 1.12.** Synthesis of diethyl amide protected sulfenic acid precursor 36..............19

**Scheme 1.13.** Trapping the intermediate sulfenic acid using methyl iodide.....................20

**Scheme 1.14.** Reactions of sulfenic acid and its derivatives..............................................21
Scheme 1.15. Potential dimerization pathway leading to the generation of compound 34……21

Scheme 1.16. Isolation of products resulting from the dimerization of sulfenic acid………23

Scheme 1.17. Blocking the self-dimerization of sulfenic acid through reaction with excess methyl iodide……………………………………………………………………………………23

Scheme 1.18. Generation of 34 under excess methyl iodide conditions where the dimerization is blocked…………………………………………………………………………………………24

Scheme 1.19. Reaction of authentic thiosulfinate in aqueous buffer conditions………………25

Scheme 1.20. Direct oxidation of 52 in presence of hydrogen peroxide to generate 22………28

Scheme 1.21. Reactivation of 34 in presence of thiol……………………………………………28

Scheme 1.22. Thiol mediated reactivation of the 1,2-benzisothiazolin-3-(2H)-one………………29

Scheme 1.23. Potential pathway for isothiazolidinone generation via a mixed disulfide………30

Scheme 1.24. Synthesis of mixed disulfide with 2-mercapto ethanol…………………………31

Scheme 1.25. Products of decomposition of 55 in aqueous buffer solution…………………31

Scheme 1.26. Structures of products stemming from the reaction of thiol with 1,2-
benzisothiazolin-3-(2H)-one………………………………………………………………33

Scheme 1.27. Reaction of thiol with benzisothiazolidinone heterocycle (34)…………………34
Chapter 2

**Scheme 2.1.** Reversible and irreversible oxidation of cysteine containing enzymes........102

**Scheme 2.2.** Formation of sulfenyl amide during the redox regulation of PTP1B.............102

**Scheme 2.3.** Synthesis of sulfenyl amide mimic.......................................................104

**Scheme 2.4.** Synthesis of a mixed disulfide mimic (55) of enzyme PTP1B......................104

**Scheme 2.5.** Synthesis of sulfenate methyl ester (61)................................................105

**Scheme 2.6.** Synthesis of benzene methylsulfenate ester 68........................................107

**Scheme 2.7.** Hydrogen peroxide mediated over-oxidation of 34 to generate 69...........108

**Scheme 2.8.** Hydrogen peroxide mediated oxidation of Cdc25 phosphatase enzyme........114

Chapter 3

**Scheme 3.1.** Formation of sulfenyl amide during oxidative inactivation of PTP1B........135

**Scheme 3.2.** Partitioning protocol for separating the reversibly oxidized forms of enzyme from other forms.................................................................138

**Scheme 3.3.** Generation of sulfenyl amide through mild oxidation of PTP1B .................139

**Scheme 3.4.** Reactions of protein thiolate and sulfenic acid groups with NBD-Cl.............141

**Scheme 3.5.** Partitioning of the oxidized PTP1B to sulfenyl amide and sulfinic acid........143

**Scheme 3.6.** Trapping intermediate sulfenic acid by a physiological thiol glutathione........146
Chapter 4

Scheme 4.1. Hydrogen peroxide mediated redox switch of cysteine containing enzymes......159

Scheme 4.2. Reversible over-oxidation of sulfinic acid in presence of sulfiredoxin (Srx1)…160

Scheme 4.3. Projected over-oxidation of the novel sulfenyl amide during redox regulation..161

Scheme 4.4. Synthesis of 1,2-benzisothiazolin-3(2H)-one 1-oxide………………………….162

Scheme 4.5. Thiol mediated conversion of compound 69 to the active form of the enzyme..162

Scheme 4.6. Derivatization of product 54 using methyl iodide……………………………..163

Scheme 4.7. Proposed mechanism for the thiol mediated reactivation of 69…………………164

Scheme 4.8. Reaction of thiol with sulfinamide……………………………………………….165

Scheme 4.9. Hydrolysis of 69 in an aqueous buffer solution…………………………………166

Scheme 4.10. Synthesis of 1,2-benzisothiazolin-3(2H)-one 1,1-dioxide………………….168

Scheme 4.11. Reaction of 99 with water and thiol………………………………………….169

Scheme 4.12. Chemical model studies suggesting a “reversible over-oxidation” of the sulfenyl amide……………………………………………………………………………….170

Scheme 4.13. A sequence of oxidation followed by hydrolysis towards generation of compound 97 & 100……………………………………………………………………………….171

Scheme 4.14. Mechanism proposed by Barford et al. for the generation of sulfinic acid from sulfenyl amide……………………………………………………………………………….171
Scheme 4.15. Oxidation of 34 under aqueous buffer conditions………………………………………172

Scheme 4.16. Chemical model studies revealed a novel redox regulation pathway………………173

Chapter 5

Scheme 5.1. Generation of irreversibly over-oxidized forms of the enzyme from
sulfinyl amide……………………………………………………………………………………………191

Scheme 5.2. The difference in reactivity of sulfinyl amide towards water and thiol………..193

Scheme 5.3. Hydrolysis of sulfinyl amide to generate thiol unrecoverable sulfinic acid…..199

Scheme 5.4. Hydrogen peroxide mediated oxidation of sulfenyl amide to sulfinyl amide....201

Chapter 6

Scheme 6.1. Reaction of 102 in presence of thiol………………………………………………..213

Scheme 6.2. Hydrodisulfide mediated production of ROS………………………………………..214

Scheme 6.3. Reactions involved in the production of reactive oxygen species (ROS)……214

Scheme 6.4. Mechanism of thiol mediated DNA alkylation by leinamycin…………………..215

Scheme 6.5. Sodium glycidate approach for the synthesis of 1,2-dithiolan 3-one moiety..218

Scheme 6.6. Synthesis of 1,2-dithiolan 3-one heterocycle………………………………………..219
Scheme 6.7. Generation of hydroxyl radical in the reaction of 116 with thiol.............223

Scheme 6.8. Oxidation of hydrogen sulfide by superoxide dismutase.................227

Scheme 6.9. Proposed mechanism for the reaction of thiol with compound 116.........230

Scheme 6.10. Alkylation of the thioester to obtain the methylated thioester..........231

Scheme 6.11. Persulfide mediated production of ROS......................................232

Scheme 6.12. Reactions of persulfide leading to the production of ROS...............232

Scheme 6.13. Initial attack of thiolate on S1 sulfur of 1,2-dithiolan 3-one heterocycle...234

Scheme 6.14. Thiolate attack on mixed disulfide to generate the product 124.........235

Scheme 6.15. Attack of thiolate on S2 sulfur of the 1,2-dithiolan 3-one heterocycle......236

Scheme 6.16. Intramolecular thiol attack towards generating the expected products.....236

Scheme 6.17. Attack of thiol at the carbonyl carbon of compound 116...............237

Scheme 6.18. Reaction of thiol with 3H-1,2-benzodithiolan 3-one.......................239

Scheme 6.19. Dimerization of a benzothietone (135)......................................239
LIST OF TABLES

Chapter 2

Table 2.1. Rate constants for the hydrogen peroxide mediated oxidation of active site mimics

.................................................................116

Chapter 6

Table 6.1. Effect of additives on thiol mediated DNA damage by compound 116.................224
Chapter 1

A Chemical Model for the Redox Regulation of Protein Tyrosine Phosphatase 1B (PTP 1B)

1.1 Introduction

1.1.1 Background

Diabetes mellitus, a condition of high blood glucose levels, has evolved into a global epidemic over the years.\(^1\) It poses a great challenge to human health in the current 21\(^\text{st}\) century as the world witnessed a huge increase in number of people affected by diabetes over the past decade.\(^2\) About 150 million people are reported to be diabetic worldwide and the number is estimated to increase to 220 million by the year 2010.\(^3\) Westernization and change to a sedentary lifestyle have both contributed to an increase in obesity rates among human population, and these things are blamed for the growing percentage of diabetes cases.\(^4\) Hence, there is a pressing need to address this issue with a deeper understanding of the disease and the mechanisms associated with it.
There are two types of diabetes, Type I and Type II. Type I diabetes occurs primarily due to an autoimmune disorder, wherein the insulin producing pancreatic β cells are destroyed by the autoreactive T cells. This result in an insulin deficiency and the paucity of insulin must often be compensated through an exogenous intake of the peptide hormone in patients affected by this type of diabetes. On the other hand, type II diabetes results from abnormal insulin secretion and/or insulin resistance. The type II diabetes accounts for almost 90-95% of all diabetes cases. Hereditary and obesity are expected to contribute to the insulin resistance which results in a condition called impaired glucose tolerance (IGT). IGT or hyperglycemia is defined as the state in which the blood glucose level varies between normal and diabetic levels. People with IGT run a greater risk of developing diseases like type II diabetes, macrovascular disorders, hypertension and dyslipidaemia. Additionally, it has been shown that controlling the condition of IGT in patients can also help reduce the risks of retinopathy and nephropathy.

Insulin is a potent anabolic hormone that is known to regulate glucose levels in the human body. The concentration of glucose in normal human blood is estimated to be in the range of 4 to 7 mM. This is tightly regulated by insulin, which controls the balance between glucose absorption from the intestine, production by the liver and uptake and metabolism in muscle and fat tissues. Insulin is also known to stimulate cell growth and differentiation, induce protein and glycogen synthesis and promote synthesis and storage of carbohydrates, lipids and proteins. Thus insulin is considered to be the primary regulator of the glucose concentration in blood.
1.1.2 Insulin-mediated signal transduction cascade

Insulin-mediated increase in uptake of glucose in peripheral tissues occurs through a series of steps often referred as signal transduction. When insulin binds to the insulin receptor on the surface of the cell, it stimulates the autophosphorylation of the tyrosine residues of the receptor in the inside of the cell.\textsuperscript{10} Phosphorylation of the insulin receptor leads to the recruitment of several insulin receptor substrate proteins (IRS) that get phosphorylated to activate other kinases including phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB). The activation of these kinase, results in series of downstream signaling events that ultimately lead to glucose utilization and uptake in cells. Thus, the insulin-mediated glucose uptake occurs through a chain of highly coordinated phosphorylation events as shown in Figure 1.1.

![Insulin-mediated signal transduction cascade](image)

**Figure 1.1.** Role of PTP1B in insulin mediated signal transduction cascade

1.1.3 Role of PTPs in insulin-mediated glucose uptake

The signal transduction cascade initiated by insulin, is negatively regulated by the protein tyrosine phosphatases (PTPs).\textsuperscript{11} PTPs belong to a large family of enzymes that catalyzes the removal of phosphate group from the phosphorylated tyrosine residues. Several PTPs including
receptor protein tyrosine phosphatase-α (rPTP-α), leukocyte antigen-related tyrosine phosphatase (LAR), SH2-domain containing phosphotyrosine phosphatase (SHP2) and protein tyrosine phosphatase 1B (PTP1B) have been shown to downregulate the insulin mediated signal transduction pathway. More specifically PTP1B, localized in the endoplasmic reticulum, has been implicated as a key negative regulator of the insulin mediated signaling cascade. PTP1B dephosphorylates vital signaling components such as IRS and blocks the downstream signaling events that lead to the uptake of glucose (Figure 1.1).

PTPs play an important role in cellular signal transduction by regulating the levels of phosphorylation in the cells. Typically, protein phosphatases and kinases work in tandem for a normal cell function. They play a major role in regulating the cellular signal transduction pathways of various diseases including diabetes, inflammation, rheumatoid arthritis and cancer. PTP-catalyzed removal of phosphyl group from the substrates can serve as an “on”/“off” switch in mediating signal transduction. PTPs possess a conserved active site with a signature sequence containing C(X)_5R(S/T), where X represents any amino acid. The cysteine thiol present at the active site is responsible for the catalytic activity of this class of enzymes. Due to their central role in cellular signal transduction, it is very important to seek an understanding of the molecular processes that govern the activity of PTPs.

1.1.4 Endogenously produced reactive oxygen species can modulate the activity of PTPs

Reactive oxygen species (ROS) generated by the phagocytes are typically considered to be involved in fighting against the invading pathogens. However, mounting evidence suggests that endogenously produced reactive oxygen species (ROS), particularly hydrogen peroxide, can act as cellular signaling agents. Previous studies indicate that binding of external stimuli such as insulin, epidermal growth factor and cytokines to the cell surface can trigger a burst of
hydrogen peroxide production inside cells.\textsuperscript{17} This oxidant burst has been implicated in mediating certain aspects of downstream signaling events that is responsible for cellular growth, proliferation, metabolism, immune response and apoptosis/survival decisions.\textsuperscript{18} The fact that the burst of hydrogen peroxide production leads to the increased levels of tyrosine phosphorylation in cells indicates the inactivation of protein tyrosine phosphatases (PTPs).\textsuperscript{19}

PTPs undergo oxidative inactivation in presence of endogenously produced hydrogen peroxide inside the cells.\textsuperscript{20} During an oxidative burst, the catalytic cysteine thiol undergoes oxidation to the sulfenic acid state to generate a transiently inactivated enzyme (Figure 1.2). Protein-derived sulfenic acids are well characterized class of oxidative intermediates seen at various proteins.\textsuperscript{21}

![Figure 1.2. Insulin triggered production of hydrogen peroxide inactivates PTP1B](image)

The oxidative inactivation of PTP is reversible, and when levels of oxidant decline, the enzyme is reactivated by cellular thiols such as glutathione or other enzymes like glutaredoxin and disulfide reductases to regenerate the active thiolate form of the enzyme, as shown in Scheme 1.1.\textsuperscript{22} The process of oxidation leading to an inactive enzyme and subsequent reduction to regenerate the active enzyme can be called redox regulation. Thus, an endogenous burst of
hydrogen peroxide can help regulate the levels of phosphorylation through transient inactivation of PTPs and thereby mediate corresponding signaling events.

![Scheme 1.1. Redox regulation of protein tyrosine phosphatases (PTPs)](image)

### 1.1.5 Evidences for the role of PTP1B as a negative regulator of insulin signaling cascade

As discussed above, protein tyrosine phosphatase 1B (PTP1B) is a negative regulator of the insulin signal transduction cascade. This is supported by the fact that the knock-out mice lacking functional PTP1B gene exhibited increased insulin sensitivity and are resistant to weight gain. Additionally, overexpression of PTP1B in rat primary adipose tissue has been shown to decrease the insulin sensitivity. Furthermore, experiments by Zinker and coworkers demonstrated that delivering PTP1B antisense oligonucleotide to diabetic obese mice brings back the blood glucose levels from diabetic to normal, as it shows remarkable insulin sensitivity. These facts make PTP1B a validated therapeutic target for the treatment of type II diabetes. Thus, it is very desirable to understand the factors that control the cellular activity of this enzyme. In the following paragraph, we will discuss the catalytic mechanism of PTP1B followed by the insulin mediated redox regulation of the enzyme.

PTP1B is an important member of the large family of tyrosine phosphatases. It shares a signature active site sequence like other members of the family with a conserved catalytic cysteine thiol. The thiol residue has a pKₐ of 5.6 and hence exists mainly in the thiolate form at the physiological pH. The mechanism of dephosphorylation involves an initial attack of the
cysteine thiolate anion on the phosphoryl group of the substrate to form a phospho-cysteine intermediate with the concomitant release of the dephosphorylated tyrosine. The phospho-cysteine intermediate is then hydrolyzed to regenerate the native enzyme back as shown in Scheme 1.2. Aspartate 181 present at the active site acts both as a general acid in assisting the leaving tyrosine residue and as general base aiding the hydrolysis of phospho-cysteine intermediate to regenerate the active enzyme.

![Scheme 1.2. General catalytic mechanism for dephosphorylation of tyrosine residues by PTPs](image)

PTP1B, analogous to other phosphatases containing active site cysteine thiol, undergo redox regulation in presence of hydrogen peroxide. Initial in vitro studies by Denu and coworkers showed that PTP1B can be reversibly inactivated in presence of hydrogen peroxide and the inactivation can be fully reversed by a physiologically relevant glutathione and other thiols. The mechanism for the redox regulation of PTP1B was proposed to be similar to the one shown in Scheme 1.1. This finding was later supported by cellular studies in which Goldstein and coworkers showed that insulin-mediated production of hydrogen peroxide can reversibly inactivate PTP1B. Stimulation of hepatoma and adipose-like cells with insulin resulted in rapid intracellular production of hydrogen peroxide that led to the decreased activity of PTP1B in those cell lines. Consistent with the reversible inactivation of PTP1B, 83% of the
original activity was restored upon treatment of the inactive enzyme with dithiothreitol, DTT. This experiment provided the first direct evidence that the key feature of the insulin mediated signal transduction pathway involves a hydrogen peroxide mediated transient inactivation of PTP1B.

1.1.6 Generation of sulphenyl amide during the oxidative inactivation of PTP1B

The mechanism for the redox regulation of PTP1B in presence of hydrogen peroxide was expected to involve an inactive sulfenic acid intermediate, which was then reactivated in presence of cellular thiols to yield the native enzyme (Scheme 1.1). However, recent X-ray crystallographic studies by two different groups revealed an unexpected chemical transformation underlying the redox regulation of PTP1B. Oxidation of PTP1B crystals in presence of hydrogen peroxide displayed a novel intrastrand protein crosslink, in which the catalytic cysteine thiol was covalently bonded to the neighboring backbone amide nitrogen. This five membered ‘sulphenyl amide’ structure (as shown in Figure 1.3) at the protein backbone had never been observed before.

![Diagram showing the formation of sulphenyl amide during the oxidative inactivation of PTP1B.]

**Figure 1.3.** Formation of sulphenyl amide during the oxidative inactivation of PTP1B

The sulphenyl amide was proposed to be the thiol reversible inactive intermediate of the enzyme. Further incubation of crystals containing protein sulphenyl amide in thiols such as glutathione or DTT resulted in a complete reactivation to the active thiol form of the enzyme (Scheme 1.3).
Identification of this novel thiol reversible sulfenyl amide structure at the protein backbone has given a new twist to the usual redox regulation mechanism of the enzyme.

Scheme 1.3. Novel sulfenyl amide intermediate during the redox regulation of PTP1B

1.1.7 Proposed mechanisms for the formation of sulfenyl amide at the active site of PTP1B

Several possible mechanisms were proposed to explain the formation of this protein derived novel 3-isothiazolidinone (sulfenyl amide) ring as shown in Scheme 1.4. In one of the mechanisms, the intermediate sulfenic acid was proposed to undergo further oxidation by another molecule of hydrogen peroxide to yield sulfenyl peroxide (8). This was followed by an intramolecular attack of the amide nitrogen onto the sulfenyl sulfur to yield the corresponding sulfenyl amide. In another route, sulfenate oxygen was proposed to react with an oxidized thiol, more likely glutathione disulfide in vivo, followed by further oxidation to yield a sulfinothioic acid (9). Compound 8/9 then undergoes an intramolecular attack by the neighboring amide nitrogen atom resulting in the elimination of glutathione sulfinic acid and generating sulfenyl amide (6). In addition, a most direct route for the formation of sulfenyl amide was also proposed that involves an attack of amide nitrogen on to the sulfenic acid, to eliminate a water molecule as shown in Scheme 1.4. At the outset of our studies, the mechanism
for the formation of this new sulfenyl amide moiety was not clear and needed further investigation to better understand the redox regulation of PTP1B.

Scheme 1.4. Different mechanisms for the formation of sulfenyl amide intermediate

1.2 Goals of this Chapter

Herein, we set out to examine the chemical mechanism for the formation of the novel sulfenyl amide intermediate seen at the active site of PTP1B. Among the proposed mechanisms, we support the most direct route involving the attack of amide nitrogen on the sulfenic acid leading to the formation of sulfenyl amide. The chemistry of sulfenic acid, in general, has been studied well and the sulfenyl sulfur of the sulfenic acid is widely accepted to act as an electrophile to eject a water molecule upon nucleophilic attack by thiols and amines. Hence, we did not see the need to invoke other mechanisms to explain the formation of sulfenyl amide. In addition, the proposed mechanisms involve a nucleophilic attack of amide nitrogen on to the sulfenyl sulfur. This is very intriguing because the amide nitrogens are generally considered to be poor
nucleophiles in organic chemistry.\textsuperscript{30} With this background, the goals of this chapter have been outlined as follows:

1) Develop a small organic system that mimics the important functional groups present at the active site of the enzyme

2) Determine whether the sulfenic acid is in fact, a competent intermediate in the cyclization and generate sulfenyl amide when placed in close proximity to the amide nitrogen.

3) Examine whether sulfenyl amide is readily reactive with RSH to regenerate the ring opened mercapto product (active enzyme model).

\textbf{1.3 Design of the PTP1B model system}

It is well known that appropriately designed small organic molecules can serve as a powerful tool to understand the fundamental chemical reactivity of structurally complex biomolecules and natural products.\textsuperscript{31} With this in mind, we set out to design a small organic compound that can mimic the redox sensing functional groups present at the active site of PTP1B. More specifically, we wanted to provide the model compound with the rigidity experienced at the enzyme’s active site and the proximity of the backbone amide nitrogen and the cysteine thiol in a similar way observed at the active site of PTP1B. For this, we decided to employ benzene ring as a scaffold to provide the rigidity and place the amide functionality and a thiol group at the ortho positions of the benzene ring in order to mimic the proximity, as shown in Figure 1.4.
Importantly, we determined that the thiophenol group adjacent to the amide in 2-mercaptobenzanilide has a pK\textsubscript{a} of 5.7 in aqueous solution. This is similar to the reported pK\textsubscript{a} value of 5.6 for the active site cysteine thiol (Cys 215) of PTP1B\textsuperscript{26}. However, chemical basis for the low pK\textsubscript{a} in our model compound is not the same as that causing the low pK\textsubscript{a} of cysteine thiol in PTP1B. With this model compound, we planned to examine the mechanism of formation of sulfenyl amide under physiological conditions. For this, we planned to generate sulfenic acid at the ortho position adjacent to the amide nitrogen and examine whether it leads to cyclization, to yield the corresponding isothiazolidinone heterocycle under physiologically relevant conditions. As a first step to examine this process, we set out to synthesize some precursors for the \textit{in situ} generation of sulfenic acid. The following section will be dedicated to the synthesis of sulfenic acid precursors.

**1.4 Synthesis of sulfenic acid precursors**

Typically, sulfenic acids are very difficult to isolate due to their high instability and there is no literature evidence available for the isolation of simple organic sulfenic acids under mild conditions\textsuperscript{32,\textsuperscript{33,\textsuperscript{34,\textsuperscript{35}}} However, there exist methods for the \textit{in situ} generation of sulfenic acid through the \(\beta\)-elimination of corresponding sulfoxide\textsuperscript{35,\textsuperscript{36}}. Katritzky and coworkers reported the use of a pyridyl ethyl group as a thiol protecting agent. This group has been shown to generate sulfenic acid \textit{in situ} upon oxidation followed by a mild base treatment as shown in Scheme 1.5\textsuperscript{37}.}
Due to the instability of sulfenic acids, we decided to generate them \textit{in situ} via β elimination of sulfoxides. Drawing inspiration from the Katritzky’s chemistry, Dr. Kripa keerthi of our lab synthesized the model compound 19 in three steps. The first step involved refluxing a mixture of vinyl pyridine and 2-mercaptobenzoic acid in benzene to yield a Michael addition product 17. The amide functionality was then incorporated using a dicyclohexyl carbodiimide (DCC) coupling with aniline, to generate 18. The sulfide (18) was oxidized in the next step using dimethyl dioxirane (DMD) to yield the sulfoxide (19). In the final step, compound 19 was treated with excess methyl iodide in presence of 50% methanol/acetone mixture to generate the desired 2-phenyl-1,2-benzisothiazol-3(2H)-one in 58% yield as shown in Scheme 1.6.

Though we obtained the desired isothiazolidinone heterocycle, presumably through the sulfenic acid intermediate, our repeated attempts to isolate the immediate precursor to sulfenic acid (20), was not possible in our hands. We found that, once methylated, compound 20 was very unstable and undergoes decomposition to yield the isothiazolidinone heterocycle. However, this reaction gave us the initial evidence that sulfenic acid can cyclize with adjacent amide nitrogen. We anticipated that the instability of 4-pyridyl ethyl protecting group is due to the high acidity of the methylene protons β to sulfoxide (pKa of 4-methyl pyridine is measured to be 6.0).\textsuperscript{38} Furthermore, an electron-withdrawing sulfoxide and the positive charge on the pyridyl nitrogen together is expected to increase the acidity of protons β to sulfinyl sulfur. Hence, we
chose to incorporate relatively stable and less acidic protecting groups on the sulfur, to gain control over the stability of the sulfenic acid precursor.

![Chemical Structure]

Scheme 1.6. Generation of isothiazolidinone via β elimination of 4-pyridyl ethyl protecting group

Earlier studies in our laboratory and by Anders et al.\textsuperscript{36} have explored the generation of sulfenic acid from ethyl propanal protected sulfinyl sulfur (Keerthi, K. and Gates, K.S, unpublished data). A corresponding sulfoxide product (sulfenic acid precursor, 23) could not be isolated due to its high reactivity. More recently, there have been reports on the use of β-sulfinyl propionic acid ester groups\textsuperscript{39,40} for the \textit{in situ} generation of sulfenic acid via thermolysis (24). Along these lines, we decided to incorporate ethyl propionic ester protecting groups on thiol (24), as we anticipated that the relatively higher pK\textsubscript{a} of the methylene protons β to the sulfinyl sulfur (pK\textsubscript{a} of an ester is ∼ 25 as compared to the pK\textsubscript{a} of an aldehyde, 18) would allow us to isolate the sulfenic acid precursor and perform the reaction under physiologically relevant conditions as well. In the following section, we will discuss the synthesis of sulfenic acid precursors with ethyl acrylate and diethyl maleate as protecting groups on sulfinyl sulfur.
1.4.1 Synthesis of sulfenic acid precursor 27

The synthesis of compound 27 started with the conjugate addition reaction of 2-mercaptobenzoic acid (16) with ethyl acrylate in presence of triethyl amine to generate compound 25. The carboxylic acid was then coupled with aniline using a simple DCC coupling to yield 26. The final step involves oxidation of the sulfide using a mild oxidizing agent DMD, to generate the sulfenic acid precursor 27 in 92% yield, as shown in Scheme 1.8.

As expected, the sulfenic acid precursor was stable enough to be isolated and was characterized by $^1$H and $^{13}$C NMRs and HRMS. Consistent with the expectation that the oxidation of sulfide 26 would generate a chiral sulfoxide, the two diastereotopic methylene protons adjacent to sulfoxide in 27 displayed different splitting patterns and chemical shift values compared to its sulfide precursor (26). Thus, we isolated and characterized the sulfenic acid precursor containing an anilide group at the close proximity to the putative sulfenic acid moiety.
1.4.2 Synthesis of sulfenic acid precursor, 29

The previous model compound 27 had a benzanilide group available for the cyclization reaction with the \textit{in situ} generated sulfenic acid. In addition, we sought sulfenic acid precursor containing a peptide-like amide nitrogen. Accordingly, we decided to incorporate a glycyl ethyl ester amide at the ortho position of the \(\beta\)-sulfinyl propionic ethyl ester group.

The first step of the synthesis involved protecting the thiol group of the 2-mercaptobenzoic acid with the ethyl acrylate in a typical conjugate addition to yield compound 25. The amide group was incorporated by a coupling reaction between the carboxylic acid and the glycyl ethyl ester hydrochloride in presence of DCC, HOBt and \(N\)-methyl morpholine in dry tetrahydrofuran (THF) to generate compound 28. The sulfenic acid precursor 29 was then synthesized by a simple oxidation of the sulfide using DMD as shown in Scheme 1.9.

![Scheme 1.9. Synthesis of sulfenic acid precursor 29](image)

1.4.3 Synthesis of sulfenic acid precursor, 32

In an attempt to slightly increase the lability of propionic ester protecting group, we introduced another ethyl ester moiety \(\alpha\) to the sulfinyl sulfur in order to decrease the pH of the hydrogen \(\beta\) to sulfinyl sulfur. For this, 2-mercaptobenzoic acid was stirred in presence of diethyl maleate and triethyl amine at 25 \(^\circ\)C for 24 h under nitrogen to obtain compound 30. Glycyl
ethyl ester was then incorporated at the carboxylic acid group using a DCC coupling reaction, to generate 31. Oxidation of the sulfide was then carried out using DMD to synthesize another sulfenic acid precursor 32, as shown in Scheme 1.10.

\[
\begin{align*}
\text{OH} & \quad \text{COOEt}\quad \text{TEA} \\
\text{SH} & \quad \text{EtOOC}\quad \text{COOEt} \\
\text{EtOOC} & \quad \text{NHCH}_2\text{COOEt} \\
\text{S} & \quad \text{O} \\
\text{EtOOC} & \quad \text{COOEt} \\
\text{COOEt} & \quad \text{COOEt} \\
\text{S} & \quad \text{O} \\
\text{EtOOC} & \quad \text{COOEt} \\
\text{DMD} & \\
\text{EtOOC} & \quad \text{NHCH}_2\text{COOEt}
\end{align*}
\]

**Scheme 1.10.** Synthesis of sulfenic acid precursor 32

With the sulfenic acid precursors in hand (compounds 27, 29 and 32), we set out to examine whether the incubation of these compounds under physiologically relevant conditions can lead to the generation of the desired 1,2-benzisothiazolin-3-(2H)-one heterocycle.

### 1.5 Generation of 1,2-benzisothiazolin-3-(2H)-one under aqueous buffer conditions

The sulfenic acid precursors were incubated in aqueous buffer solution (250 mM sodium phosphate buffer, pH 7.5 at 37 °C) containing 30% acetonitrile by volume for about 24 - 36 h. Consistent with our expectation, the reaction produced 1,2-benzisothiazolin-3-(2H)-one in good yields. Incubation of β-sulfinyl propionic ethyl ester group in pH 7.5 sodium phosphate buffer presumably resulted in the abstraction of hydrogen β to the sulfoxide to generate a putative sulfenic acid intermediate. The adjacent amide nitrogen then cyclizes on to the sulfinyl sulfur to eject a water molecule and generate the desired benzisothiazolidinone heterocycle as shown in Scheme 1.11. For example, incubation of phenyl substituted amide (27) in aqueous buffer
solution (250 mM sodium phosphate, pH 7.5 containing 30% acetonitrile at 37 °C) gave an 88% yield of 22, while glycinyl ethyl ester substituted amide (32) yielded 92% of 34 under similar conditions.

![Scheme 1.11. Generation of 1,2-benzisothiazolin-3-(2H)-one via the sulfenic acid intermediate](image)

The cyclization reaction was also examined in organic solvents. Reaction of sulfenic acid precursor 32 in dichloromethane (at 45 °C for 48 h) generated benzisothiazolidinone heterocycle (34) in 60 % yield. Taken together, our results suggest that the reaction of the amide nitrogen with the sulfenic acid is rather robust and does not depend strongly upon the nature of the solvent or the substituent on the amide nitrogen. More importantly, our studies emphasize the generality of this unusual chemical transformation seen first at the active site of the enzyme PTP1B. Furthermore, our results indicate the facile nature of this transformation and suggest that it could in principle, occur in any enzyme that possesses a cysteine residue capable of undergoing an oxidation. Consistent with our prediction, there have been recent reports indicating the formation of the five membered isothiazolidinone ring at the active sites of other cysteine containing enzymes.41,42 In the next section, we will discuss some experiments conducted toward confirming the identity of the in situ generated putative sulfenic acid intermediate.

1.6 Trapping the intermediate sulfenic acid under our reaction conditions

Though there have been literature reports confirming the in situ generation of sulfenic acid via the β-elimination of propionic ethyl ester groups, we wanted to verify the identity of the
intermediate in our system, under our reaction conditions. Several attempts to trap the intermediate sulfenic acid using an electrophilic methyl iodide in our model system (32) failed in our hands as it resulted in the generation of the isothiazolidinone heterocycle (34) instead. This is not surprising, given the fact that the reaction of amide nitrogen with sulfenic acid is intramolecular while the trapping reaction with methyl iodide is bimolecular. Hence, in order to block the potential intramolecular cyclization pathway we incorporated a dialkyl amide in place of glycyl ethylester amide and examined the trapping reaction with methyl iodide.

For this purpose, we set out to synthesize a sulfenic acid precursor having diethyl amide functionality at the ortho position to the sulfinyl propionic ethyl ester group of the benzene ring (36). The synthesis was carried out in a manner similar to the previously reported synthesis of the sulfenic acid precursors (see section 1.4). The carboxylic acid (16) was coupled with diethyl amine in presence of DCC as shown in Scheme 1.12 to generate the desired sulfenic acid precursor 36.

![Scheme 1.12. Synthesis of diethyl amide protected sulfenic acid precursor 36](image)

We expected that incubation of the sulfenic acid precursor (36) with methyl iodide, in conditions similar to our model reaction (sodium phosphate buffer, pH 7.5 containing 30 %
acetonitrile) would trap the intermediate sulfenic acid generated from the β elimination. Indeed, the putative sulfenic acid was trapped with methyl iodide as a methyl sulfoxide when compound 36 was treated with excess methyl iodide aqueous buffer conditions as shown in Scheme 1.13. This confirmed the generation of intermediate sulfenic acid under the reaction conditions employed in our model reaction. Furthermore, the sulfenic acid was also trapped efficiently under organic conditions using dichloromethane as a solvent.

![Scheme 1.13. Trapping the intermediate sulfenic acid using methyl iodide](image)

As evident from this reaction, the sulfenyl sulfur acts as a nucleophile to attack methyl iodide, whereas in the cyclization reaction, it behaves as an electrophile. It is well documented in literature that sulfenic acids can act both as an electrophile and as nucleophile. Earlier literature reports indicate that the transiently generated sulfenic acid can be trapped as its sulfoxide by the reaction with alkynes and soft electrophiles. At the same time, reaction of the sulfenic acid with nucleophiles such as thiols and amines generated the corresponding mixed disulfide and sulfenamides respectively, indicating that sulfenic acid exhibits both electrophilic and nucleophilic characters. Thus, we established the intermediacy of the sulfenic acid under our reaction conditions. In the following section, we seek to rule out the intermediacy of a possible dimerization product of sulfenic acid in the cyclization reaction.
1.7 Experiments to rule out the intermediacy of thiosulfinate in the cyclization reaction

1.7.1 Background and set up

Sulfenic acids are very unstable to isolate owing to their propensity to undergo dehydrative dimerization to produce thiosulfinate ester (RS(O)SR). This anhydride form of the sulfenic acid results from the reaction of two molecules of sulfenic acid to form one molecule of thiosulfinate and water (Eqn 1).\textsuperscript{32,44,35} Furthermore, the thiosulfinate can undergo disproportionation to yield a mixture of stable disulfide and thiosulfonate (Eqn. 2)\textsuperscript{45,46}

\[
\begin{align*}
2 \text{RS(O)SR} & \rightarrow \text{RSSR} + \text{RSO}_2\text{SR} \\
2 \text{RSOH} & \rightarrow \text{RS(O)SR} + \text{H}_2\text{O}
\end{align*}
\]

\textbf{Scheme 1.14.} Reactions of sulfenic acid and its derivatives

Along these lines, we wanted to examine if the cyclization that we observe under our reaction conditions could occur via thiosulfinate intermediate as shown in Scheme 1.15. This section will be devoted to the discussion of experiments that argue against this mechanism.

\textbf{Scheme 1.15.} Potential dimerization pathway leading to the generation of compound 34

Our central goal was to examine how the two bi-molecular reactions, a) trapping of sulfenic acid with a potential electrophile methyl iodide and b) dimerization of the sulfenic acid to produce thiosulfinate compete with each other. The dimerization of sulfenic acid is a
bimolecular process involving the reaction between two molecules of sulfenic acid. Similarly, sulfenic acid trapping by methyl iodide is also a bimolecular reaction. Hence, we anticipated that dilution of the reaction mixture along with the addition of large excess of methyl iodide will slow down the self dimerization of sulfenic acid and in turn result in the efficient generation of methyl iodide trapped product (38). Our idea here is to design the conditions to achieve a large excess concentration of methyl iodide, sufficient to block the dimerization reaction. For this purpose, we used the diethyl amide protected sulfenic acid precursor (36), as there is no competing cyclization reaction involved in this model. With the suitable conditions in hand, we planned to examine the cyclization reaction in our real glycinyl ethyl ester amide containing model (32) under the same excess methyl iodide conditions that can block the potential dimerization of sulfenic acid.

1.7.2 Characterization of the authentic products stemming from the dimerization of sulfenic acid

To begin with, we carried out the control reaction to verify the authentic products stemming from the dimerization of sulfenic acid. For this, we incubated the sulfenic acid precursor (36) in pH 7.4 sodium phosphate buffer containing 40% acetonitrile at room temperature. After 48 h, isolation of the products by a flash column chromatography revealed the presence of stable disulfide (47) and thiosulfonate ester (46) as major products along with little amount of isolable thiosulfinate (45), as shown in Scheme 1.16. This further confirmed the formation of sulfenic acid but at the same time, hinted at the slow decomposition of the thiosulfinate (45).
1.7.3 Reaction of 36 under excess methyl iodide conditions that blocks the potential dimerization

After characterizing the dimerization products of sulfenic acid, we set out to examine the concentrations of methyl iodide required to block this dimerization under aqueous buffer conditions. For this purpose, we treated compound 36 under the excess methyl iodide conditions (6.3 mM 36, 6.7 M CH₃I, 165 mM sodium phosphate buffer, pH 7.5 containing 25 % acetonitrile at 37 °C) for 48 h. Constant monitoring of the reaction by TLC did not reveal the formation of the thiosulfinate product (45). More importantly, we obtained the corresponding methyl iodide trapped sulfenic acid product (38) in over 75% yield as shown in Scheme 1.17. This suggested that under this excess methyl iodide conditions, the dimerization of sulfenic acid is slower than the reaction of sulfenic acid with methyl iodide. This was further supported by the fact that we did not observe the presence of signature products resulting from the dimerization of sulfenic acid (46 and 47).

Scheme 1.16. Isolation of products resulting from the dimerization of sulfenic acid

Scheme 1.17. Blocking the self-dimerization of sulfenic acid through reaction with excess methyl iodide
1.7.4 Reaction of 32 under excess methyl iodide conditions that completely block the dimerization of sulfenic acid

With conditions that allow complete methyl iodide trapping of sulfenic acid, we turned to our model reaction to examine if the cyclization can occur under the conditions that block dimerization of sulfenic acid. We anticipated that the intramolecular cyclization in our model (32) should be faster compared to the intermolecular trapping of sulfenic acid by methyl iodide. Accordingly, treatment of the sulfenic acid precursor (32) under excess methyl iodide conditions as mentioned above (6.3 mM 32, 6.7 M CH₃I, 165 mM sodium phosphate buffer, pH 7.5 containing 25 % acetonitrile at 37 °C), resulted in the generation of 1,2-benzisothiazolin-3(2H)-one (34) in 85% yield as shown in Scheme 1.18.

We observed that the cyclization of sulfenic acid to the corresponding isothiazolidinone heterocycle proceeds normally even when there is no potential dimerization of the sulfenic acid is possible. Our results strongly suggest that the unimolecular cyclization is faster than the potential bimolecular dimerization pathway. Taken together, our results clearly indicate that compound 34 probably did not arise via a sequence of reactions involving the formation of thiosulfinate (44) followed by the conversion of 44 to produce 34, as shown in Scheme 1.15. In the following section, we seek to understand whether thiosulfinate generated adjacent to a secondary amide can undergo cyclization to generate benzisothiazolidinone (34).
1.7.5 Reaction of thiosulfinate adjacent to secondary amide (49) under aqueous buffer conditions

Though the experiments in previous section have clearly ruled out the generation of compound 34 via the dimerization of sulfenic acid, we were interested to observe the fate of thiosulfinate generated adjacent to a secondary amide under our reaction conditions. Our goal is to see if the resulting thiosulfinate (49) can cleanly generate the benzisothiazolidinone heterocycle. For this purpose, we synthesized the thiosulfinate (49) by a controlled oxidation of the authentic disulfide of the phenyl benzanilide 48 using DMD.

Scheme 1.19. Reaction of authentic thiosulfinate in aqueous buffer conditions

The thiosulfinate was then incubated under aqueous buffer conditions similar to our model reaction (250 mM sodium phosphate buffer, pH 7.5 containing 30 % acetonitrile at 37 °C) used for the generation of benzisothiazolidinone heterocycle. We observed that compound 49 does not cleanly generate the desired cyclized product (22), as expected. It rather gave rise to various spots on TLC, along with the authentic dimerization products as shown in Scheme 1.19. The fact that these products are not observed in our model cyclization reaction provides further evidence that the cyclization does not proceed through a thiosulfinate intermediate. A possibility that product 22 may arise from the cyclization of the symmetric disulfide (51), cannot be ruled out here. However, this suggested that, if generated in situ, the thiosulfinate (49) will give rise to more than one product. In other words, the mechanism involving dimerization of sulfenic acid
followed by the cyclization will not produce compound \textbf{22/34} in good yields. This fact has led us to investigate the cyclization reaction for the formation of possible intermediates (\textbf{49}, \textbf{50} and \textbf{51}) during the generation of benzisothiazolidinone heterocycle in our model reaction. We will discuss that in the following section.

\textbf{1.7.6 Monitoring the cyclization reaction under HPLC}

To further confirm the absence of stable accumulation of thiosulfinate or disulfide intermediates leading to the generation of benzisothiazolidinone (\textbf{34}) heterocycle, we monitored the formation of \textbf{34} from \textbf{32} under biologically relevant conditions using HPLC. The sulfenic acid precursor (\textbf{32}) was incubated in aqueous buffer solution (250 mM sodium phosphate buffer, pH 7.5 containing 30 % acetonitrile at 37 °C) and the formation of benzisothiazolidinone (\textbf{34}) was constantly monitored over time, in a reverse phase C-18 HPLC column at 254 nm.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{hplc_monitoring.png}
\caption{HPLC monitoring of the reaction of \textbf{32} under aqueous buffer conditions}
\end{figure}
The results provide no evidence for the formation of any detectable intermediates towards generation of compound 34 (Figure 1.5). In principle, the reaction would have generated various products, if it had to undergo cyclization via the thiosulfinate intermediate. The fact that we started seeing the formation of compound 34 within 5 min of initiation of the reaction clearly suggests that the intermediate sulfenic acid can undergo a direct cyclization in presence of neighboring amide nitrogen. Taken together, our results indicate two things 1) the precursors (compounds 27, 29 and 32) decompose in aqueous buffer solution to yield the sulfenic acid intermediate and 2) the sulfenic acid generated in situ can efficiently undergo cyclization to generate the 3-isothiazolidinone heterocycle. Although there are some examples of similar intramolecular cyclization of amide nitrogen on to the sulfenyl sulfur to yield benzisothiazolidinone heterocycle,47 this is the first report that shows sulfenic acid, generated adjacent to amide nitrogen, possesses sufficient electrophilicity to drive the cyclization reaction. Thus, our results argue against the need to invoke any intermediates other than sulfenic acid in the formation of the novel 3-isothiazolidinone heterocycle seen during oxidation of PTP1B. In addition to understanding the mechanism of this novel chemical transformation, we wondered if our simple chemical model can mimic the hydrogen peroxide mediated formation of benzisothiazolidinone (34).

1.8 Can our model mimic the oxidative inactivation chemistry of PTP1B?

In direct analogy to the oxidation of PTP1B’s active site cysteine thiol by hydrogen peroxide, we asked a simple question whether a direct oxidation of the thiol form of our model compound (10) can generate the corresponding benzisothiazolidinone heterocycle? For this purpose, we oxidized compound 52 in aqueous buffer conditions with hydrogen peroxide in presence of excess methyl iodide. We carried out the oxidation reaction in presence of methyl
iodide to effectively block the production of compound 22 arising from the thiosulfinate intermediate, as discussed above. In a typical reaction, incubation of 52 with 1.1 equivalents of hydrogen peroxide in sodium phosphate buffer (pH 7.5) with excess methyl iodide at 25 °C (52, 11.5 mM; H₂O₂, 13.3 mM; sodium phosphate buffer, 166 mM, pH 7.5; methyl iodide, 5.3 M, acetonitrile, 30 % by volume) generated the desired 1,2-benzisothiazolin-3-(2H)-one (22) in 56% yield presumably via a sulfenic acid intermediate, as shown in Scheme 1.20. As expected, we also obtained the S-methylated product (53) resulting from the direct alkylation of the thiol.

![Scheme 1.20. Direct oxidation of 52 in presence of hydrogen peroxide to generate 22](image)

This demonstrates the ability of our model compound to mimic the oxidative inactivation chemistry seen at the enzyme’s active site. Furthermore, oxidation of compound 52 in dichloromethane in presence of m-chloroperbenzoic acid and triethyl amine (TEA) also resulted in the generation of the corresponding isothiazolidinone product (22). Having mimicked the oxidative inactivation chemistry of the enzyme PTP1B, our next goal was to examine reactions of isothiazolidinone heterocycle with thiols.

1.9 Can our chemical model mimic the thiol-mediated reactivation chemistry of PTP1B?

![Scheme 1.21. Reactivation of 34 in presence of thiol](image)
To verify this, we treated compound 34, the oxidatively inactivated mimic of enzyme, in aqueous buffer conditions with 10 equivalents of 2-mercaptoethanol (2-ME). We used 2-mercaptoethanol, as it is a simple thiol and more importantly, the pKₐ of 2-ME (9.2) is close to the pKₐ of a physiologically relevant thiol, glutathione (8.8). We found that 34 was converted rapidly and completely to the corresponding thiol (54). The reaction went to completion within 5 min without the accumulation of any intermediates. We attempted to measure the rate constant for this reaction of 34 to 54 using the reverse phase HPLC. But the reaction was rapid as the starting 34 was completely transformed into product 54 within 1 min of addition of thiol, under aqueous buffer conditions containing 30% acetonitrile. Several attempts to slow down this reaction by decreasing the pH of the buffer or by increasing the concentration of acetonitrile did not help. Overall, this shows that benzisothiazolidinone (34) does indeed react rapidly with thiol to yield the corresponding ethyl 2-(2-mercaptobenzamido) acetate 54, as shown in Scheme 1.22.

![Scheme 1.22](image)

The fact that we did not see accumulation of the mixed disulfide (55) under our conditions of ten equivalents 2-mercaptoethanol indicates that the second step in the reactivation reaction is faster compared to the initial attack of thiol on the benzisothiazolidinone heterocycle. However, we wanted to investigate whether the mixed disulfide intermediate can generate the benzisothiazolidinone via an intramolecular cyclization reaction. We will discuss that in the following section.
1.10 Can mixed disulfide undergo cyclization to generate compound 34

We set out to investigate whether the mixed disulfide (55) can undergo cyclization under aqueous buffer conditions, in the absence of thiol. Typically, the reactivation by cellular thiols must proceed in two steps with an initial attack of thiol on sulfenic acid / on sulfenyl amide leading to the formation of a mixed disulfide. This must be followed by an attack of a second molecule of thiol to regenerate the active thiolate form of the enzyme. The second step of this reactivation depends on various factors like the local environment of the enzyme, availability of thiol and the rate of the reaction. With this in mind, we wanted to examine the possibility of isothiazolidinone (sulfenyl amide) formation via an intramolecular attack of amide nitrogen onto the mixed disulfide, as shown in Scheme 1.23.

![Scheme 1.23. Potential pathway for isothiazolidinone generation via a mixed disulfide](image)

To verify this, we synthesized the mixed disulfide compound 55 via the reaction of 34 with one equivalent of 2-mercapto ethanol in dry THF (Scheme 1.24). The isolated compound 55 was then incubated in the standard aqueous sodium phosphate buffer conditions similar to our model reactions. We observed that the mixed disulfide (55) is very stable (with an apparent t$_{1/2}$ of 35-39 h) under the conditions of formation of benzisothiazolidinone heterocycles (55, 50 µM; sodium phosphate buffer, 100 mM, pH 7.5; acetonitrile, 30% by volume). In addition, compound 55 does not undergo rapid cyclization when dissolved in organic solvent. For example, no other products were identified in NMR spectrum when 55 was allowed to sit in a solution of CDCl$_3$ for over 4 days at room temperature.
Scheme 1.24. Synthesis of mixed disulfide with 2-mercapto ethanol

However, we observed that upon storage of 55 in acetonitrile at 4°C for over a month; it yielded a mixture of cyclized 34, 54 and the disulfide of 2-mercaptoethanol (56) as shown in Scheme 1.25. These results indicate that mixed disulfide is very slow to undergo cyclization.

Scheme 1.25. Products of decomposition of 55 in aqueous buffer solution

This result stands in contrast to other reports noting that symmetric disulfides such as 2,2’-dithiobisbenzamides existing in equilibrium with their corresponding 1,2-benzisothiazolin-3-(2H)-ones.48 This may be due to the high pKa of the leaving 2-mercapto ethanol group (9.2) in our case. Overall, we believe that the cyclization of amide nitrogen onto the mixed disulfide to generate the isothiazolidinone heterocycle is very unlikely under physiologically relevant conditions.

On a quite similar note towards understanding the fate of isothiazolidinone heterocycle in presence of thiols, we wanted to examine the stability of the sulfenyl amide mimic under the conditions of oxidative stress. We will discuss that in the following section.
1.11 Stability of sulfenyl amide under oxidative stress conditions

PTP1B is known to undergo oxidation in presence of hydrogen peroxide to generate the inactive isothiazolidinone heterocycle. Cellular generation of reactive oxygen species can result in the oxidative damage to proteins, lipids and DNA along with the possible generation of sulfenyl amide at the active site of PTP1B. The excessive production of reactive oxygen species in a cell is often referred to a condition called “oxidative stress”. Typically, various cellular mechanisms are available to counter the accumulation of reactive oxygen species and oxidative stress and restore the redox balance in the cell. One of the mechanisms involves the oxidation of cellular reducing agent glutathione (GSH) to its corresponding glutathione disulfide (GSSG). The ratio of GSH/GSSG determines the redox status of the cell and it is reported to be in the range of 100:1 for a normal healthy cell. Under the conditions of oxidative stress, this balance is disturbed as there is an increase in concentration of the oxidized glutathione (GSSG). In the context of our studies, we thought since the isothiazolidinone ring is formed under the conditions of oxidative stress, it would be important for us to verify how changes in the ratio of GSH/GSSG affect the stability of the isothiazolidinone heterocycle. In other words, our major goal was to examine whether the inactive isothiazolidinone form of the enzyme can be reactivated under the conditions of oxidative stress (or) if it acts as a redox sensor to detect the changes in ratio of GSH/GSSH in the cell.

To study this, we incubated compound 34 in aqueous buffer conditions and in the presence of varying RSH/RSSR concentrations to mimic the normal and oxidative stress conditions of the cell. The reaction was constantly followed by NMR.
Scheme 1.26. Structures of possible products stemming from the reaction of thiol with 1,2-benzisothiazolin-3-(2H)-one

To simulate the possible ratios of \( \text{RSH/RSSR} \) in a normal and oxidative stress conditions, we added 34 as the last component to a mixture of sodium phosphate buffer (pH 7) containing 2-mercaptoethanol and disulfide of 2-mercapto ethanol in a ratio of 60:1 for the normal and 5:1 to mimic the conditions of oxidative stress. Changes in the chemical shift of the aromatic protons were constantly monitored over time. We used 2-mercaptoethanol, as it does not contain any protons that can interfere with the chemical shifts in the region of our interest. In addition, \( pK_a \) of 2-mercaptoethanol is very similar to the \( pK_a \) of a physiologically relevant thiol, glutathione. Typically, 2-mercaptoethanol was taken in at least 20 equiv. excess to compound 34 and the concentration of the disulfide was varied accordingly to mimic the normal and oxidative stress conditions of the cell.

We observed that under both normal and oxidative stress conditions, the benzisothiazolidinone was converted within 15 min to the corresponding thiol product 54. This implies that the reactivation of the isothiazolidinone heterocycle in PTP1B can take place even under the conditions of oxidative stress. The factor that determines the stability of 34 is the concentration of available thiol. However, when the experiment was performed under a hypothetically extreme oxidative stress conditions containing an \( \text{RSH/RSSR} \) ratio of 1:5, a mixture of thiol product 54 with a little amount of unreacted starting benzisothiazolidinone (34) was observed in the NMR spectrum. In conclusion, the benzisothiazolidinone (34) was readily
reduced under both normal and oxidative stress conditions to yield ethyl 2-(2-mercaptobenzamido) acetate (54).

![Scheme 1.27. Reaction of thiol with benzisothiazolidinone heterocycle (34)](image)

**Figure 1.6.** NMR spectrum of the reaction mixture when 34 was incubated under various conditions of oxidative stress. a) 60:1 RSH/RSSR b) 5:1 RSH/RSSR c) 1:5 RSH/RSSR. The reaction was monitored 15 min from the beginning of the experiment and up to 3 days. The initially formed mixture appears to be stable over the period of measurement.
1.12 Conclusion

In conclusion, we have developed a small organic molecule that serves as an effective model for the redox-sensing assembly of functional groups found at the active site of the enzyme PTP1B. More importantly, our findings suggest that sulfenic acid possesses enough electrophilicity to drive the cyclization reaction with a neighboring amide nitrogen to generate the isothiazolidinone ring analogous to the one recently characterized at the active site of oxidatively inactivated PTP1B. Protein sulfenic acids are common intermediates generated during the oxidation of cysteine thiol residue in cells. This fact along with the facile nature of sulfenic acid chemistry reported here, suggests that the reversible formation of protein derived isothiazolidinone heterocycle first observed in case of PTP1B represents a potentially general mechanism for the redox switching of protein function. This was well supported in the literature as after our report, researchers have identified the formation of this novel isothiazolidinone heterocycle at the active sites of another protein tyrosine phosphatase and a transcription factor.
1.13. Experimental Procedures

Materials and Methods

Reagents were of highest purity available and were used without further purification unless otherwise noted. Materials were purchased from the following suppliers: HPLC grade solvents, Fisher; silica gel 60 (0.04-0.063 mm pore size) for column chromatography, Merck; all other chemicals were purchased from Aldrich Chemical Company. Thin layer chromatography (TLC) was performed on plates from Aldrich Chemical Company, coated with general purpose silica containing UV$_{254}$ fluorophore and compounds were visualized by illumination of the plates with short-wave (254 nm) UV light. Water was distilled, deionized and glass redistilled. The oxidizing agent, dimethyl dioxirane (DMD), was freshly synthesized according to literature methods$^{52}$ and stored at -18 °C. High Resolution Mass Spectrometry was performed at The Ohio State University Mass Spectrometry facility and low resolution mass spec were performed at the University of Missouri–Columbia. NMR spectra were recorded using Bruker DRX 500, DRX 300, or ARX 250 MHz instruments at the University of Missouri – Columbia. HPLC spectra of compounds were recorded directly without an internal standard. The standard error in the HPLC peak area for 3 injections was estimated to be 2 ± 0.5 %.
2-(3-ethoxy-3-oxopropylthio) benzoic acid, 25. To a stirred solution of thiosalicyclic acid (1 g, 6.4 mmol) in dry THF (10 mL) under nitrogen was added triethylamine (1.3 g, 1.8 mL, 12.8 mmol) and ethyl acrylate (780 mg, 0.85 mL, 7.8 mmol). The resulting dark yellow solution was stirred at 25 °C under nitrogen for 24 h. The resulting pale yellow solution was then acidified to pH 2-3 using 10% sulfuric acid. The white precipitate was removed by filtration and the filtrate extracted with diethyl ether (3 x 30 mL). The combined organic extracts were dried over anhydrous sodium sulfate, filtered, and evaporated to yield a pale yellow oil, which was purified by a flash column chromatography (3:7 methanol/EtOAc) to give 25 as a white powder (1.27 g, 78%). R_f = 0.72 (3:7 methanol/EtOAc). 1H-NMR (CD3OD, 250 MHz) δ 1.18 (3H, t, J = 7.12 Hz), 2.62 (2H, t, J = 7.5 Hz), 3.14 (2H, t, J = 6.25 Hz), 4.08 (2H, q, J = 7.5 Hz), 7.15 (1H, m), 7.41 (2H, m), 7.87 (1H, dd, J = 6.25 Hz, 1.5 Hz); 13C-NMR (CDCl3, 62.9 MHz) δ 173.48, 170.00, 141.54, 133.47, 132.44, 130.33, 127.29, 125.38, 61.89, 34.41, 28.06, 14.48. HRMS (ESI) calcd for C12H14O4S [M + Na]^+ 277.0505, found 277.0495.

Ethyl 3-(2-(phenylcarbamoyl)phenylthio)propanoate, 26. To a stirred solution of 25 (140 mg, 0.55 mmol), DCC (168 mg, 0.824 mmol) and DMAP (13.4 mg, 20 mol%) in dry THF (6 mL) was added aniline (76 mg, 0.82 mmol) and the mixture allowed to stir at 25 °C for 12 h under nitrogen. The solvent was removed by rotary evaporation and the resulting oil taken up in diethyl ether and filtered to remove dicyclohexyl urea (DCU). The filtrate was then washed with 5% sulfuric acid, followed by water (2 x 15 mL). The ether layer was then dried over anhydrous sodium sulfate, filtered,
and evaporated to give a yellow oil, which was then purified by a flash column chromatography (8:1 hexane/EtOAc) to give 26 as a white powder (137 mg, 68.5%). $R_f = 0.12$ (8:1 hexane/EtOAc). $^1$H-NMR (CDCl$_3$, 500 MHz) $\delta$ 1.19 (3H, t, J = 7 Hz), 2.60 (2H, t, J = 7 Hz), 3.17 (2H, t, J = 7 Hz), 4.02 (2H, q, J = 7 Hz), 7.13 (1H, t, J = 7.5 Hz), 7.37 (4H, m), 7.49 (1H, d, J = 7 Hz), 7.67 (2H, d, J = 8 Hz), 7.74 (1H, d, J = 7 Hz), 8.71 (1H, s); $^{13}$C-NMR (CDCl$_3$, 62.9 MHz) $\delta$ 171.62, 165.82, 137.94, 137.92, 132.38, 132.25, 130.85, 129.61, 129.04, 127.52, 124.53, 120.02, 60.87, 34.20, 30.02, 14.06. HRMS (ESI) calcd for C$_{18}$H$_{19}$NO$_3$S [M + Na]$^+$ 352.0978, found 352.0960.

Ethyl 3-(2-(phenylcarbamoyl)phenylsulfinyl)propanoate, 27. To a stirred solution of 26 (50 mg, 0.152 mmol) in dry acetone (2 mL) in an ice bath was added freshly prepared dimethyl dioxirane (DMD) solution. The disappearance of 26 was monitored by TLC (1:1 EtOAc/hexane). The reaction was complete in about 5 min at which time the acetone and excess DMD was evaporated to give a white powder (50 mg, 96%). $R_f = 0.125$ (1:1 EtOAc/hexane). $^1$H-NMR (CDCl$_3$, 300 MHz) $\delta$ 1.23 (3H, t, J = 7.2 Hz), 2.63 (1H, ddd, J = 12.6 Hz, 9.3 Hz, 6 Hz), 2.83 (1H, ddd, J = 15.8 Hz, 9.6 Hz, 6 Hz), 3.26 (1H, ddd, J = 15.3 Hz, 9.3 Hz, 6 Hz), 3.57 (1H, ddd, J = 15.5 Hz, 9.5 Hz, 6 Hz), 4.07 (2H, q, J = 7.2 Hz), 7.20 (1H, m), 7.40 (2H, m), 7.61 (5H, m), 8.06 (1H, dd, J = 7.8 Hz, 1.2 Hz), 8.60 (1H, s, broad); $^{13}$C-NMR (CDCl$_3$, 75.4 MHz) $\delta$ 171.41, 164.17, 145.07, 137.51, 132.65, 132.00, 130.85, 129.19, 127.17, 125.67, 125.03, 120.30, 60.91, 50.49, 26.57, 14.11. HRMS (ESI) calcd for C$_{18}$H$_{19}$NO$_4$S [M + Na]$^+$ 368.0927, found 368.0930.
2-(1,4-diethoxy-1,4-dioxobutan-2-ylthio)benzoic acid, 30. To a stirred solution of thiosalicylic acid (1 g, 6.4 mmol) in dry THF (10 mL) under nitrogen was added triethylamine (1.3 g, 1.8 mL, 12.8 mmol) and diethyl maleate (1.34 g, 1.26 mL, 7.8 mmol). The resulting greenish yellow solution was stirred at 25 °C for 24 h under nitrogen. Water (10 mL) was added and the reaction mixture acidified with 10% sulfuric acid to pH 2. The resulting colorless solution was extracted with diethyl ether (3 x 30 mL) and the combined organic extracts dried over anhydrous sodium sulfate. The solution was then filtered and evaporated to yield a yellow oil that was purified by flash column chromatography (7:3:1 hexane/EtOAc/methanol) to give 30 as a white solid (1.52 g, 72%). Rf = 0.43 (7:3:1 hexane/EtOAc/methanol). 1H-NMR (CDCl3, 300 MHz) δ 1.20 (3H, t, J = 7.2 Hz), 1.26 (3H, t, J = 7.2 Hz), 2.83 (1H, dd, J = 10.8 Hz, 5.4 Hz), 3.10 (1H, dd, J = 17.10 Hz, 9.6 Hz), 4.17 (2H, q, J = 7.2 Hz), 4.17 (2H, q, J = 7.2 Hz), 4.33 (1H, dd, J = 10 Hz, 6 Hz), 7.31 (1H, m), 7.53 (1H, m), 7.71 (1H, d, J = 7.5 Hz), 8.07 (1H, dd, J = 7.8 Hz, 1.5 Hz); 13C-NMR (CDCl3, 62.9 MHz) δ 171.04, 170.58, 170.37, 137.95, 133.03, 132.03, 129.54, 128.72, 126.04, 61.72, 61.09, 44.13, 36.28, 14.00, 13.84. HRMS (ESI) calcd for C15H18O6S [M + Na]+ 349.0716, found 349.0723.

Diethyl 2-(2-((2-ethoxy-2-oxoethyl)carbamoyl)phenylthio)succinate, 31. A mixture of 30 (200 mg, 0.613 mmol), DCC (187.5 mg, 0.919 mmol), HOBt (141 mg, 0.919 mmol) and N-methylmorpholine (93 mg, 94 μL, 0.919 mmol) was stirred in dry THF (10 mL) in an ice bath. In a separate flask, glycinyl ethyl ester hydrochloride (102 mg, 0.735 mmol) in dry DMF (4 mL) was mixed with triethylamine (111 mg, 152 μL, 1.1 mmol) and the resulting white
suspension stirred for 20 min in an ice bath under N₂. This solution of glycinyl ethyl ester was then transferred into the flask containing 30 and the mixture stirred at 25 °C for 15 h. After filtering off the DCU, the THF was removed by rotary evaporation and the DMF was then removed under high vacuum. The resulting oil was taken up in diethyl ether (15 mL) and washed with saturated sodium carbonate solution, followed by 10% citric acid and water (10 mL each). The ether layer was dried over anhydrous sodium sulfate, filtered, and evaporated to yield a yellow oil, which was purified by flash column chromatography (7:3 hexane/EtOAc) to obtain 31 as a white solid (220 mg, 85%). R_f = 0.153 (7:3 hexane/EtOAc). ^1H-NMR (CDCl₃, 250 MHz) δ 1.13 (3H, t, J = 7 Hz), 1.23 (3H, t, J = 7 Hz), 1.31 (3H, t, J = 7 Hz), 2.81 (1H, dd, J = 17.25 Hz, 5.5 Hz), 2.98 (1H, dd, J = 17 Hz, 7.5 Hz), 4.15 (9H, m), 7.29 (1H, s, broad, NH), 7.39 (2H, m), 7.63 (1H, m), 7.71 (1H, m); ^13C-NMR (CDCl₃, 62.9 MHz) δ 170.91, 170.32, 169.65, 167.57, 138.56, 135.12, 130.75, 130.44, 129.32, 128.69, 61.56, 61.49, 60.98, 60.93, 46.38, 41.91, 36.24, 14.12, 14.03, 13.83. HRMS (ESI) calcd for C₁₉H₂₅NO₇S [M + Na]^+ 434.1244, found 434.1254.

**Diethyl 2-(2-((2-ethoxy-2-oxoethyl)carbamoyl)phenylsulfinyl)succinate, 32.** To a stirred solution of 31 (80 mg, 0.195 mmol) in dry acetone (3 mL) in an ice bath was added freshly prepared dimethyl dioxirane (DMD) solution. The disappearance of 31 was monitored by TLC (1:1 EtOAc/hexane).

The reaction was complete in about 5 min at which time the acetone and excess DMD was evaporated to give 32 as a white powder (78 mg, 94%). R_f = 0.152 (1:1 EtOAc/hexane). This material was taken to the next step without further purification. ^1H-NMR (CDCl₃, 250 MHz) δ 1.07 (3H, t, J = 7 Hz), 1.24 (3H, t, J = 7 Hz), 1.31 (3H, t, J = 7 Hz), 3.10 (1H, dd, J = 17.25 Hz, 3.75 Hz), 3.26 (1H, dd, J = 17 Hz, 11.25 Hz), 4.17 (8H, m), 5.35 (1H, dd,
J = 11.25 Hz, 1.75 Hz), 6.55 (1H, t, J = 4.8 Hz, NH), 7.68 (3H, m), 8.02 (1H, d, J = 8.25 Hz); HRMS (ESI) calcd for C_{19}H_{25}NO_{8}S [M + Na]^+ 450.1193, found 450.1188.

**Ethyl 3-(2-((2-ethoxy-2-oxoethyl)carbamoyl)phenylthio)propanoate 28.** A mixture of the starting material 25 (500 mg, 1.96 mmol), DCC (600 mg, 2.94 mmol), HOBt (450 mg, 2.94 mmol) and N-methylmorpholine (300 mg, 2.94 mmol) were stirred in dry THF (15 mL) at 0°C in an ice bath. In a separate flask, glycinyl ethylester hydrochloride (330 mg, 2.35 mmol) was stirred in dry DMF (4 mL) and triethylamine (356 mg, 3.52 mmol) was added to the reaction flask via syringe and the resulting white solution was stirred for 20 min in an ice bath under N₂. The free amine was then syringed out into the flask containing 25 and the mixture was allowed to stir at room temperature for 15 hrs. The DCU that precipitated was filtered off and the light orange filtrate was evaporated to remove THF and DMF was removed under high vacuum. The resulting oil was taken in diethylether (30 mL) and extracted with saturated sodium carbonate solution followed by 10% citric acid and water (25 mL each). The final organic layer was dried under anhydrous sodium sulfate, filtered and evaporated to give a pale yellow oil, which was then purified by a flash column (1.4:0.6 Hex/EtOAc, Rₚ = 0.225) to obtain 28 as a white solid product (554 mg, 83%).

$^{1}$H NMR (CDCl₃, 300 MHz) δ 1.16 (3H, t, J = 7 Hz), 1.23 (3H, t, J = 7 Hz), 2.54 (2H, t, J = 7.35 Hz), 3.10 (2H, t, J = 7.35 Hz), 4.03 (2H, q, J = 7.20 Hz), 4.16 (4H, m), 7.32 (4H, m), 7.62 (1H, dd, J = 6.3 Hz, 1.5 Hz); $^{13}$C NMR (CDCl₃, 62.9 MHz) δ 14.11, 29.70, 33.98, 41.89, 60.75, 61.54, 126.99, 129.37, 130.87, 131.64, 133.37, 136.43, 167.74, 169.74, 171.58. HRMS (EI) calcd for C_{16}H_{21}NO_{5}S [M + Na]^+ 362.1032, found 362.1034.
**Ethyl 3-(2-(2-ethoxy-2-oxoethyl)carbamoyl)phenylsulfinyl)propanoate 29.** To a stirred solution of 13 (60 mg, 0.176 mmol) in dry acetone (3 mL) in an ice bath at 0°C was added freshly prepared dimethyldioxirane (DMD) solution. The disappearance of 28 was constantly monitored by tlc (1.4:0.6 EtOAc/Hex). The reaction was complete in about 5 min after which the excess acetone and DMD was evaporated to give 29 as a white powder (60 mg, 97%) with R<sub>f</sub> = 0.225 (1.4:0.6 EtOAc/Hex). ¹H NMR (CDCl₃, 250 MHz) δ 1.20 (3H, t, J = 7 Hz), 1.32 (3H, t, J = 7 Hz), 2.62 (1H, ddd, J = 12.5 Hz, 7.5 Hz, 3.5 Hz), 2.81 (1H, ddd, J = 15.25 Hz, 9.25 Hz, 7.5 Hz), 3.19 (1H, ddd, J = 15.25 Hz, 9.25 Hz, 7.5 Hz), 3.56 (1H, ddd, J = 12.5 Hz, 7.5 Hz, 3.5 Hz), 4.06 (4H, q, J = 7.25 Hz), 4.21 (4H, m), 6.97 (1H, s), 7.62 (3H, m), 8.18 (1H, d, J = 8 Hz); ¹³C NMR (CDCl₃, 62.9 MHz) δ 14.04, 26.53, 32.6, 41.79, 50.54, 60.77, 61.80, 125.57, 126.79, 130.48, 130.93, 132.25, 145.76, 165.85, 169.52, 171.43. HRMS (EI) calcd for C<sub>16</sub>H<sub>21</sub>NO<sub>6</sub>S [M + Na]<sup>+</sup> 378.0981, found 378.1012.

**Generation of 1,2-benzisothiazol-3(2H)-ones 22 and 34 from 27, 29 and 32 in aqueous buffer and generation of 3b from 1b in organic solvent.**

**Generation of 2-phenyl-1,2-benzisothiazol-3(2H)-one (22) from 27 in aqueous buffer.** To a stirred solution of 27 (20 mg, 0.06 mmol) in a mixture of water (0.5 mL) and acetonitrile (0.75 mL) was added sodium phosphate buffer (1.25 mL, 500 mM, pH 7.5) and the mixture stirred at 37 °C for 48 h (final concentrations: 27 24 mM; buffer 250 mM, pH 7.5; acetonitrile 30% by volume). The reaction mixture was then extracted with ethyl acetate (3 x 5 mL) and the combined extracts dried over anhydrous sodium sulfate. The solution was then filtered and
evaporated to yield a colorless oil that was purified by flash column chromatography (1.5:1 hexane/EtOAc) to give 22 as an off-white solid (11.6 mg, 88%). Rf = 0.54 (1.5:1 hexane/EtOAc). 1H-NMR (CDCl3, 250 MHz) δ 7.30 (1H, m), 7.43 (3H, m), 7.64 (4H, m), 8.10 (1H, m); 13C-NMR (CDCl3, 75.47 MHz) δ 164.11, 139.87, 137.25, 132.33, 129.36, 127.21, 127.04, 125.79, 124.87, 124.58, 120.07. HRMS (ESI) calcd for C13H9NOS [M + Na]+ 250.0297, found 250.0302.

Generation of ethyl 2-(3-oxobenzothiazol-2(3H)-yl)acetate (34) from 32 in aqueous buffer. To a stirred solution of 32 (26 mg, 0.063 mmol) in a mixture of water (0.64 mL) and acetonitrile (0.96 mL) was added sodium phosphate buffer (1.6 mL, 500 mM, pH 7.5) and the mixture was stirred at 37 °C for 36 h (final concentrations: 32 20 mM; buffer 250 mM, pH 7.5; acetonitrile 30% by volume). The product was then extracted into ethyl acetate (3 x 5 mL) and the combined extracts dried over anhydrous sodium sulfate, filtered and evaporated to yield colorless oil that was purified by flash column chromatography (1:1 hexane/EtOAc) to give 34 as an off-white solid (13.6 mg, 92%). Rf = 0.545 (1:1 hexane/EtOAc). 1H-NMR (CDCl3, 300 MHz) δ 1.29 (3H, t, J = 7 Hz), 4.25 (2H, q, J = 7.25 Hz), 4.60 (2H, s), 7.41 (1H, m), 7.60 (2H, m), 8.06 (1H, m); 13C-NMR (CDCl3, 75.47 MHz) δ 167.63, 165.73, 140.84, 132.22, 126.95, 125.55, 123.42, 120.34, 61.87, 44.62, 14.09. HRMS (ESI) calcd for C11H11NO3S [M + Na]+ 260.0352, found 260.0355.
Generation of Ethyl 2-(3-oxobenzoisothiazol-2(3H)-yl)acetate (34) from 29.

To a stirred solution of 29 (42 mg, 10 mM final concentration) in acetonitrile (30% of final volume) was added sodium phosphate buffer at pH 7.5 (250 mM final concentration) and the mixture was stirred at 37°C for 48 hrs. The product was then extracted with ethyl acetate (3 x 5 mL) and the combined extracts were dried over anhydrous sodium sulfate, filtered and evaporated to yield colorless oil that was purified by flash column (1:1 Hex/EtOAc, Rf = 0.545) to give 34 as an off-white solid (26 mg, 89%).

Generation of ethyl 2-(3-oxobenzoisothiazol-2(3H)-yl)acetate (34) from 32 in organic media.

In a dry round bottom flask, 32 (20 mg, 0.047 mmol) was dissolved in dichloromethane (2.0 mL, HPLC grade solvent) and the solution was allowed to stir at 45 °C under nitrogen for 48 h. The solvent was then evaporated and the crude material purified by flash column chromatography (1:1 hexane/EtOAc) to give 34 as an off-white solid (6.8 mg, 60%). Rf = 0.545 (1:1 hexane/EtOAc).

2-(2-Pyridin-4-yl-ethylsulfanyl)benzoic acid, 17.

To a stirred solution of thiosalicylic acid (1 g, 6.4 mmol) in benzene (15 mL) was added 4-vinyl pyridine (0.98 mL, 9.1 mmol) and the mixture allowed to reflux for 12 h. When the reaction was complete as judged by TLC, the benzene was evaporated under reduced pressure to give 17 as a pale pink solid (1.5 g, 89% yield). The compound was
used without further purification. $^1$H-NMR (250 MHz, DMSO-$d_6$) 13.2 (1H, s), 8.47 (2H, dd, J = 3.75 Hz, 1.5 Hz), 7.85 (1H, dd, J = 7.74 Hz, J = 1.45 Hz), 7.52 (2H, m), 7.33 (2H, dd, J = 3.75 Hz, J = 2.5 Hz), 7.21 (1H, dt, J = 7.5 Hz, J = 2.5 Hz), 3.24 (2H, t, J = 7.5 Hz), 2.93 (2H, t, J = 7.5 Hz); $^{13}$C-NMR (62.9 MHz, DMSO-$d_6$) δ 167.41, 149.42, 148.98, 140.17, 132.33, 130.85, 128.45, 125.71, 124.02, 123.99, 32.89, 30.88. HRMS (ESI) calcd for C$_{14}$H$_{13}$NO$_2$S [M + H]$^+$ 260.0740, found 260.0744.

$N$-phenyl-2-(2-pyridin-4-yl-ethylsulfanyl)benzamide, 18. To a stirred solution of 17 (300 mg, 1.1 mmol) in THF (2 mL) under nitrogen, was added DCC (358 mg, 1.73 mmol) and DMAP (28 mg, 0.23 mmol). The reaction mixture was stirred at 25 °C for 15 h. When the reaction was complete as judged by TLC, the DCU precipitate was removed by filtration and the solution evaporated under reduced pressure to give an oily solid, which was purified by flash column chromatography (1:1 hexane/EtOAc) to obtain 18 as a flaky white solid (211 mg, 55%). R$_f$ = 0.15 (1:1 hexane/EtOAc). $^1$H-NMR (250 MHz, CDCl$_3$) 8.48 (1H, s), 8.43 (2H, dd, J = 4 Hz, J = 1.5 Hz), 7.79 (1H, dd, J = 7.75 Hz, J = 2 Hz), 7.60 (2H, d, J = 7.75 Hz), 7.37 (5H, m), 7.14 (1H, tt, J = 7 Hz, J = 1 Hz), 7.04 (2H, dd, J = 4.5, J = 1.5 Hz), 3.16 (2H, t, J = 7.5 Hz), 2.86 (2H, t, J = 7.5 Hz); $^{13}$C-NMR (62.9 MHz, CDCl$_3$) δ 165.66, 149.86, 148.26, 137.74, 137.51, 132.73, 132.23, 130.95, 129.80, 129.09, 127.57, 124.64, 123.76, 119.87, 35.19, 34.47. HRMS (ESI) calcd for C$_{20}$H$_{18}$N$_2$OS [M + Na]$^+$ 357.1032, found 357.1037.
N-phenyl-2-(2-pyridin-4-yl-ethylsulfiny1)benzamide, 19. To a rapidly stirred dilute solution of 18 (50 mg, 0.14 mmol) in HPLC grade acetone (10 ml) was added freshly prepared dimethyl dioxirane (4 mL) in an ice bath. The reaction was fast and careful monitoring by TLC was essential to obtain the product in good yield. Upon completion of the reaction, the solvent was evaporated to give a white solid (46 mg, 88%) that was used without further purification. $^1$H-NMR (250 MHz, CD$_3$OD) 8.81 (2H, m), 8.09 (1H, d, J = 8 Hz), 7.90 (1H, d, J = 7.5 Hz), 7.74 (1H, t, J = 8 Hz), 7.61 (1H, t, J = 6.5 Hz), 7.55 (2H, d, J = 8.5 Hz), 7.28 (4H, m), 7.08 (1H, t, J = 6.5 Hz) 4.77 (1H, s), 3.60 (1H, m), 3.21 (1H, m), 3.05 (2H, m).

**Generation of 2-phenyl-1,2-benzisothiazol-3(2H)-one (22) from 19.** Following the method of Katritsky and coworkers for the in situ generation of sulfenic acids,$^{54}$ to a stirred solution of 19 (40 mg, 0.11 mmol) in 50% methanol/acetone mixture (1 mL) was added excess methyl iodide (156 mg, 70 μL, 1.1 mmol). The reaction was monitored by TLC (1:1 hexane/EtOAc). When all starting material was consumed, the reaction mixture was evaporated and purified by flash column chromatography (9:1 hexane/EtOAc) to give 22 as an off-white solid (15 mg, 58%).

**Diethyl 2-(2-(diethylcarbamoy1)phenylthio)succinate, 35.** To a stirred solution of 30 (300 mg, 0.919 mmol), DCC (225 mg, 1.103 mmol) and DMAP (22.4 mg, 20 mol%) in dry THF (8 mL) at 25 °C under nitrogen was added diethylamine (73.8 mg, 106 μL, 1.01 mmol). The reaction mixture was stirred at 25 °C for 36 h under nitrogen. The DCU precipitate was removed by filtration and the filtrate evaporated to yield a yellow oil that was purified by flash column chromatography (1:1
hexane/EtOAc) to yield 35 as a colorless oil (280 mg, 80%) that became a white solid on standing. \( R_f = 0.25 \) (1:1 hexane/EtOAc). \(^1\)H-NMR (CDCl\(_3\), 300 MHz) \( \delta \) 1.03 (3H, t, \( J = 7.2 \) Hz), 1.23 (9H, m), 2.86 (2H, m), 3.07 (2H, q, \( J = 7.2 \) Hz), 3.57 (2H, s, broad), 4.12 (5H, m), 7.25 (1H, m), 7.35 (2H, m), 7.60 (1H, m); \(^1^3\)C-NMR (CDCl\(_3\), 75.47 MHz) \( \delta \) 171.20, 170.42, 168.81, 141.74, 134.98, 128.96, 128.76, 126.34, 61.32, 60.75, 45.93, 42.70, 38.79, 36.35, 33.81, 13.96, 13.85, 12.49. HRMS (ESI) calcd for C\(_{19}\)H\(_{27}\)NO\(_5\)S [M + Na]\(^+\) 404.1502, found 404.1518.

**Diethyl 2-(2-(diethylcarbamoyl)phenylsulfinyl)succinate, 36.** To a stirred solution of 35 (32 mg, 0.083 mmol) in dry acetone (2 mL) in an ice bath was added freshly prepared dimethyldioxirane solution. The disappearance of 35 was monitored by TLC (1:1 hexane/EtOAc). When the reaction was complete, acetone and excess DMD were evaporated to give a white powder (31 mg, 94%). \( R_f = 0.31 \) (1:1 hexane/EtOAc). The white solid was used without further purification. HRMS (ESI) calcd for C\(_{19}\)H\(_{27}\)NO\(_6\)S [M + Na]\(^+\) 420.1451, found 420.1486.

**Trapping of the sulfenic acid intermediate generated from 36 with methyl iodide in aqueous media.** To a stirred solution of 36 (15 mg, 0.038 mmol) in a mixture of acetonitrile (1 mL) and sodium phosphate buffer (1 mL, 500 mM, pH 7.5) was added methyl iodide (1 mL) and the resulting mixture stirred for 24 h at 25 °C under nitrogen. (initial concentrations: 36 12.7 mM; buffer, 166 mM, pH 7.5; CH\(_3\)I, 5.3 M; acetonitrile, 33% by volume). After the first 24 h, an additional 1 mL of CH\(_3\)I, buffer (1 mL, 500 mM, pH 7.5) and acetonitrile (0.5 mL) was added and the reaction stirred for further 12 h. At that time, an additional 0.5 mL of CH\(_3\)I was added
and the resulting biphasic mixture was stirred vigorously at 25 °C under nitrogen (final concentrations: 36 6.3 mM; buffer, 165 mM, pH 7.5; CH₃I, 6.7 M; acetonitrile, 25% by volume). The colorless reaction mixture turned yellow after 4 days of stirring. Excess methyl iodide was removed first by a stream of nitrogen gas inside a well ventilated hood and then by rotary evaporation. The resulting mixture was then extracted with ethyl acetate (3 x 4 mL) to give a yellow oil that was purified by flash column chromatography (7:3 EtOAc/hexane followed by 7:3 EtOAc/MeOH) to obtain 38 as an yellow gum (6.7 mg, 75%). Rₚ = 0.80 (7:3 EtOAc/MeOH). ¹H-NMR (CDCl₃, 250 MHz) δ 1.11 (3H, t, J = 7 Hz), 1.26 (3H, t, J = 7 Hz), 2.86 (3H, s), 3.22 (2H, q, J = 7.2 Hz), 3.45 (1H, m), 3.65 (1H, m), 7.30 (1H, dd, J = 9.75 Hz, 1 Hz), 7.52 (1H, m), 7.65 (1H, m), 8.12 (1H, dd, J = 7.75 Hz, 1 Hz); ¹³C-NMR (CDCl₃, 75.47 MHz) δ 167.62, 143.98, 134.07, 130.81, 130.42, 125.78, 123.85, 44.27, 43.37, 39.37, 14.12, 12.62. HRMS (ESI) calcd for C₁₂H₁₇NO₂S [M + Na]⁺ 262.0872, found 262.0874.

**Generation of N,N-diethyl-2-(methylsulfinyl) benzamide (38) from 36 in organic media.** To a solution of 36 (32 mg, 0.081 mmol) in dichloromethane (2 mL) was added methyl iodide (2 mL). The reaction was stirred at room temperature under nitrogen. After 24 h, sodium carbonate (2 equiv. in 0.5 mL water) was added and the mixture stirred for another 24 h. The resulting yellow mixture was concentrated by blowing a stream of nitrogen on the solution in a well ventilated hood. The dark yellow paste was taken up in a small volume of dichloromethane and purified by flash column chromatography (7:3 EtOAc/hexane followed by 7:3 EtOAc/MeOH) to obtain 7 (14.3 mg, 82%) as a dark yellow gum.
Decomposition of 36 in the absence of methyl iodide yields characteristic products stemming from the dimerization of sulfenic acid.

To a stirred solution of 36 (80 mg, 0.20 mmol) in a mixture of water (1 mL) and acetonitrile (1.5 mL) was added sodium phosphate buffer (2.5 mL, 500 mM, pH 7.5) and the resulting solution stirred at 25 °C under nitrogen for 48 h (final concentrations: 36, 40 mM; buffer, 250 mM, pH 7.5; acetonitrile, 30% by volume). Water (3 mL) was added to the reaction and the resulting mixture extracted with ethyl acetate (3 x 8 mL). The combined organic extracts were dried over anhydrous sodium sulfate, filtered, and evaporated to yield a colorless oil that was purified by flash column chromatography (1.5:1 EtOAc/hexane) to give the disulfide (47, 28 mg, 35%) and the thiosulfonate (46, 22.5 mg, 26%) as major products. The crude reaction mixture was purified on a silica gel column that had been neutralized with 5% triethylamine. The unstable thiosulfinate (45) was detected using mass spectroscopy. ESI: calcd for C_{22}H_{28}N_{2}O_{3}S_{2} [M + Na]^+ 455, found 455. But the expected disproportionation products arising from 45, the disulfide (47) and the thiosulfonate (46) are obtained as stable products of this reaction.

Characterization of disulfide (47): R_f = 0.35 (1.5:1 EtOAc/hexane). $^1$H-NMR (CDCl$_3$, 250 MHz) $\delta$ 1.06 (6H, t, J = 7 Hz), 1.29 (6H, t, J = 7 Hz), 3.15 (4H, q, J = 7 Hz), 3.59 (4H, q, J = 7.2 Hz), 7.28 (6H, m), 7.69 (2H, dd, J = 7 Hz, 1 Hz); $^{13}$C-NMR (CDCl$_3$, 62.9 MHz) $\delta$ 168.49, 136.79, 133.82, 129.57, 128.20, 127.12, 126.21, 43.00, 39.03, 14.04, 12.72. LRMS (ESI) calcd for C$_{22}$H$_{28}$N$_2$O$_3$S$_2$ [M + H]$^+$ 417, found 417. Characterization of thiosulfonate (46): R_f = 0.13
Conversion of 32 to 34 in the presence of excess methyl iodide. In this reaction, we employed conditions (excess methyl iodide) that completely prevent dimerization of the sulfenic acid intermediate generated by 36 to the thiosulfinate (45) as described above. We find that conversion of 32 to 34 proceeds normally under these conditions. This observation provides good evidence that 34 is not formed via a sequence of reactions involving generation of the sulfenic acid, followed by dimerization to yield the thiosulfinate, followed by conversion of the thiosulfinate to 34. Thus, 32 (21 mg, 0.049 mmol) was dissolved in a mixture of acetonitrile (1.28 mL) and sodium phosphate buffer (1.28 mL, 500 mM, pH 7.5). Methyl iodide (1.28 mL) was added and the reaction mixture was stirred at 25 °C under nitrogen for 24 h (initial concentrations: 32, 12.8 mM; buffer, 166 mM, pH 7.5; CH₃I, 5.4 M; acetonitrile, 33% by volume). After the first 24 h, an additional 1.28 mL of CH₃I, buffer (1.28 mL, 500 mM, pH 7.5) and acetonitrile (0.64 mL) was added and the reaction stirred for 12 h. At that time, an additional 0.64 mL of CH₃I was added and the resulting biphasic mixture was stirred vigorously at 25 °C under nitrogen (final concentrations: 32, 6.3 mM; buffer, 165 mM, pH 7.5; CH₃I, 6.7 M; acetonitrile, 25% by volume). The reaction mixture allowed to stir at 25 °C for 4 days. The excess methyl iodide was then removed by blowing a stream of nitrogen on the solution in a well ventilated hood followed by rotary evaporation. The resulting mixture was extracted with ethyl acetate (4 x 5 mL) and combined organic extracts were dried over sodium sulfate, filtered, and
concentrated to give a yellow oil, which was purified by flash column chromatography (1:1 hexane/EtOAc) to yield 34 as an off-white solid (11.4 mg, 98%).

Formation of cyclized sulfenamide 34 via reaction of thiol 52 with H$_2$O$_2$ in the presence of methyl iodide in aqueous buffer.

Synthesis of 2-mercaptobenzanilide, 52. This compound was prepared according to a literature procedure.$^5$ To a well stirred mixture of mercaptobenzoic acid (200 mg, 1.29 mmol), aniline (0.22 mL, 2.6 mmol) and pyridine (0.13 ml, 1.7 mmol) under nitrogen phosphorous trichloride (78 µL, 0.84 mmol) was added very slowly. This mixture was heated at reflux with stirring for 3 h. The resulting mixture was cooled and hot ethanol (3 mL) was added. The filtrate was cooled and a white precipitate that formed was filtered off. The filtrate was cooled again and cold water was added slowly to precipitate the product 52 (60 mg, 20%) as a pale yellow solid. This was filtered, dried, and further purified by recrystallization from ethanol-water. $^1$H-NMR and $^{13}$C-NMR matched with the literature values.$^6$

Generation of 2-phenyl-1,2-benzisothiazol-3(2$H$)-one (22) via reaction of the thiol 52 with H$_2$O$_2$ in the presence of excess methyl iodide in buffer. This reaction was designed to test whether hydrogen peroxide-mediated oxidation of the thiol 52 leads to the cyclized product 22, presumably via the sulfenic acid intermediate. As above, we performed the reaction in the presence of methyl iodide concentrations that block potential dimerization of the sulfenic acid 21 to the thiosulfinate. Use of these conditions ensures that the observed cyclization product (22) stems directly from the sulfenic acid (21) rather than from dimerization products such as the thiosulfinate, disulfide or thiosulfonate. The thiol 52 (10 mg, 0.044 mmol) in acetonitrile (1.1
mL) was treated with sodium phosphate buffer (1.28 mL, 500 mM, pH 7.4) and methyl iodide (1.28 mL). To this mixture was added hydrogen peroxide (0.205 mL, 250 mM of 30% H2O2 in acetonitrile) and the reaction was vigorously stirred (final concentrations: 52, 11.5 mM; H2O2, 13.3 mM; buffer, 166 mM, pH 7.5; CH3I, 5.3 M; acetonitrile, 33% by volume). The reaction was complete in about 1.5 h as indicated by the disappearance of the starting material on TLC. The excess methyl iodide was removed by passing a stream of nitrogen over the mixture in a well-ventilated hood. The reaction mixture was then extracted with ether twice and the organic layer was washed with water followed by brine. The organic layer was further dried over anhydrous sodium sulfate and the solvent was removed under vacuum to give a pale yellow oil. The crude mixture was then purified by flash column chromatography (12:1 hexane/EtOAc) to yield 22 (5.5 mg, 56% yield) and 53 as flaky colorless needles (4.3 mg, 40% yield). Rf = 0.17 (9:1 hexane/EtOAc). 1H-NMR and 13C-NMR of 53 matches with the literature values.7

10) Synthesis of mixed disulfide 55 from 34 in organic media. A solution of 34 (12 mg, 0.051 mmol) in dry THF (1.5 mL) was stirred with 2-mercaptoethanol (4.35 mg, 4 μL, 0.056 mmol) at 60 °C under nitrogen for 1.5 h with careful monitoring of the reaction by TLC. The solution was then cooled to room temperature and concentrated by rotary evaporation. The crude mixture was purified by gravity column chromatography (1.5:1 EtOAc/hexane) to yield 55 as a white solid (11 mg, 70%) Rf = 0.40 (1.5:1 EtOAc/hexane). 1H-NMR (CDCl3, 500 MHz) δ 1.32 (3H, t, J = 7.2 Hz), 2.08 (1H, t, OH), 2.88 (2H, t, J = 6.0 Hz), 3.82 (2H, q, J = 6.0 Hz), 4.26 (4H, m), 6.6 (1H, broad, NH), 7.28 (1H, m), 7.49 (1H, m), 7.56 (1H, m), 8.00 (1H, dd, J = 8 Hz, 0.9 Hz); 13C-NMR (CDCl3, 125.75 MHz) δ 169.83, 167.69, 137.60, 134.06, 131.38, 128.66, 127.89, 126.65, 61.81, 60.36, 41.87, 40.95, 14.15. HRMS (ESI) calcd for C13H17NO4S2 [M + Na]⁺ 338.0491, found 352.0492.

52
Characterization of the reactivity of the mixed disulfide 55 in aqueous solution: the disulfide does not rapidly cyclize to 34. Compound 55 (5 μL of a 5 mM stock in CH₃CN) was incubated at 25 °C in a mixture of sodium phosphate buffer (100 μL, 500 mM, pH 7.5), water (245 μL) and acetonitrile (150 μL). The mixture (final concentrations: 55, 50 μM; buffer, 100 mM, pH 7.5; acetonitrile, 30% by volume) was vortex mixed and analyzed by reverse phase HPLC at regular time intervals. The disappearance of 55 was monitored by HPLC using a reverse phase C-18 Varian Microsorb-MV column (100 Å sphere size, 5 μm pore size, 25 cm length, 4.6 mm i.d.), eluted with an isocratic mobile phase composed of 70% water containing 0.5% acetic acid v/v and 30% acetonitrile at a flow rate of 1 mL/min (injection volume = 50 μL, detection wavelength = 254 nm). Compound 55 decomposes slowly, with an apparent half-life of 35-39 h to yield a mixture of 34 and 54. In CDCl₃, 55 does not yield any cyclized product as observed by proton NMR over the course of 4 days. A 5 mM solution of 55 in acetonitrile yields the cyclized product 34 (~50%) along with the thiol 54 and the disulfide of 2-mercaptoethanol (56) when refrigerated at 4 °C for one month.

Figure 1.7. A plot of HPLC peak area vs time for the disappearance of 55.
Generation of ethyl 2-(2-mercaptobenzamido) acetate (54) by reaction of 34 with 2-mercaptoethanol in aqueous buffer. To a stirred solution of 34 (11.2 mg, 0.047 mmol) in a mixture of water (0.4 mL) and acetonitrile (0.6 mL) was added sodium phosphate buffer (1 mL, 500 mM, pH 7.5). 2-mercaptoethanol (55 mg, 50 μL, 0.70 mmol) was then added to the reaction mixture and the resulting colorless solution stirred at 25 °C for 10 min (final concentrations: 34 24 mM; buffer 250 mM, pH 7.5; acetonitrile, 30% by volume; thiol, 0.36 M, 15 equiv.). The reaction mixture was acidified to pH 2 using dilute hydrochloric acid, extracted with diethyl ether (3 x 5 mL), and washed with water. The combined organic extracts were dried over anhydrous sodium sulfate, filtered, and evaporated to yield a colorless oil that was purified by flash column chromatography (7:3 EtOAc/hexane) to obtain 54 as a white solid (10.4 mg, 91%). Rf = 0.61 (7:3 EtOAc/hexane). ^1H-NMR (CDCl3, 300 MHz) δ 1.32 (3H, t, J = 7 Hz), 4.24 (4H, m), 4.74 (1H, s), 6.57 (1H, s, broad), 7.17 (1H, m), 7.31 (2H, m), 7.53 (1H, dd, J = 7.6 Hz, 1.0 Hz); ^13C-NMR (CDCl3, 75.4 MHz) δ 169.79, 168.45, 133.37, 132.16, 131.07, 130.94, 128.12, 125.21, 61.76, 41.83, 14.14. HRMS (ESI) calcd for C11H14NO3S [M+H]^+ 240.0694, found 240.0706. The disulfide 55 was not seen in this reaction.

Synthesis of 2-(phenylcarbamoyl)phenyl 2-(phenylcarbamoyl)benzene sulfinothioate 49.

To a stirred solution of the disulfide 48 (20 mg, 0.044 mmol) in a 1:1 mixture of dichloromethane and methanol (8 mL total) in an ice bath at 0°C was added freshly prepared dimethyldioxirane solution. The heterogeneous white solution turned colorless as soon as it got oxidized, which was also monitored constantly by tlc (1:1 Hex/EtOAc). The reaction was complete in about 5 min after which the clear colorless solution was evaporated to remove excess solvents and DMD to give 49 as a white powder (18 mg, 87%). ^1H NMR (CDCl3, 300
MHz) δ 7.14 (8H, m), 7.62 (1H, d, J = 7.2 Hz), 8.47 (1H, s); 13C NMR (CDCl3, 75.4 MHz) δ 119.44, 119.56, 124.30, 128.97, 129.54, 129.58, 130.51, 130.56, 131.23, 131.28, 137.80, 138.55, 138.60, 142.90, 142.96, 165.57. To further confirm its presence, thiosulfinate (49) was taken in methanol and stirred with 2.0 equiv. triphenylphosphine at room temperature under nitrogen for 24 h to get the disulfide (48). This was identical to the standard disulfide 48, prepared using a reported procedure2. 1H NMR (CDCl3, 300 MHz) δ 7.10 (4H, m), 7.35 (6H, m), 7.54 (4H, m), 7.72 (4H, d, J = 8 Hz), 10.44 (2H, s); 13C NMR (CDCl3, 62.9 MHz) δ 120.14, 123.99, 126.29, 126.38, 128.46, 128.74, 131.42, 134.70, 136.43, 138.83, 165.69. The thiosulfinate 49 was oxidized3 further with DMD to get the thiosulfonate (50), which matched the compound obtained from the direct oxidation of the disulfide 48 with excess DMD. 1H NMR (CDCl3, 500 MHz) δ 7.10 (1H, t, J = 7.2 Hz), 7.32 (2H, t, J = 7.5 Hz), 7.55 (2H, m), 7.70 (3H, m), 8.03 (1H, m); 13C NMR (CDCl3, 75.4 MHz) δ 121.40, 124.92, 127.98, 128.40, 129.06, 129.86, 130.44, 130.53, 130.95, 133.25, 135.34, 139.26, 142.71, 169.06.

**Reaction of thiosulfinate 49 under the physiological conditions.** The starting thiosulfinate 49 (15mg, 0.032 mmol, 20 mM final concentration) was dissolved in acetonitrile (30% of final volume) and sodium phosphate buffer (250 mM final concentration at pH 7.5) was added to it. The resulting mixture was stirred at room temperature for 30 min as the progress of the reaction was monitored by TLC using 1.2:0.8 hex/EtOAc. The reaction mixture was then extracted with ethylacetate (3 x 5 mL) and the combined organic extracts were dried under sodium sulfate, filtered and evaporated to yield the disulfide (48) and thiosulfonate (50) in comparable yields along with very low yields of 22. When 1 mM 49 was incubated under the same condition mentioned above, we observed the formation of 22 after 24 h of incubation.
HPLC analysis of the reaction of 34 with 2-mercaptoethanol in aqueous buffer. The reaction was initiated by the addition of ethyl 2-(3-oxobenzoisothiazol-2(3H)-yl) acetate (34) (5 μL of a 1 mM solution in CH$_3$CN) to a solution containing sodium phosphate buffer (50 μL), water (190 μL), acetonitrile (145 μL) and 2-mercaptoethanol (100 μL) at 25 °C (final volume 500 μL; final concentrations: 34, 10 μM; buffer, 50 mM, pH 7.0; 2-mercaptoethanol, 100 μM; acetonitrile, 30% by volume). The disappearance of 34 was monitored by HPLC using a reverse phase C-18 Varian Microsorb-MV column (100 Å sphere size, 5 μm pore size, 25 cm length, 4.6 mm i.d.), eluted with an isocratic mobile phase composed of 70% water containing 0.5% acetic acid v/v and 30% acetonitrile at a flow rate of 1 mL/min (injection volume = 50 μL, detection wavelength = 254 nm).

Figure 1.8: (a) Control: 50 μM 34 in 50mM buffer (b) 10 μM 34 in 50 mM buffer and 100 μM 2-mercaptoethanol, 1 min after the initiation of the reaction.
**Determination of the **pK_a** of the thiol residue in 2-mercaptobenzanilide (52).** In a typical assay, the thiol compound (5 μL of a 5mM stock in acetonitrile, 50 μM final concentration) was added to a mixture containing sodium phosphate buffer (50 μL of a 500 mM stock, 50 mM final concentration) and water (445 μL) in a 1.5 mL eppendorf tube. The mixture was vortexed for 10 s and transferred to a UV cuvette where the absorbance was recorded over the range of 200 – 800 nm at varying pH values of 4.0, 5.0, 6.0, 7.0 and 8.0. The sample was blanked with an appropriate solution containing acetonitrile (5μL) in place of the thiol compound at every pH. The pK_a of the thiol was then calculated by plotting [RSH] and [RS⁻] against pH and was found to be 5.71.

![Figure 1.9.](http://example.com/figure1.9.png)

**Figure 1.9.** A plot of pH versus concentration of thiol and thiolate species in determining pK_a of 54

■ = [RS⁻] and ♦ = [RSH]

**Monitoring the redox sensing behavior of compound 34 by NMR under varying RSH:RSSR.** In a typical assay, compound 34 was dissolved in 120 μL acetone-\textit{d}_6 and to this was added 60 μL of 2-mercaptoethanol (7.5 M), 60 μL of 2-hydroxyethyl disulfide (0.125 M) and 360 μL of sodium phosphate buffer (500 mM, pH 7.0). The mixture was (final concentrations: 34, 0.025 M; thiol, 0.75 M; disulfide, 0.0125 M; buffer, 300 mM; acetone, 20 %
by volume) vortex mixed and taken in an NMR tube. The NMR spectrum was recorded in a 500 MHz Bruker instrument. The initial NMR spectrum was recorded 15 min after the addition of thiol, followed by constant monitoring at varying time intervals (3, 6, 12, 24 and 36 h). The initial concentration of 34 was kept constant and the ratio of thiol/disulfide was changed accordingly to mimic various conditions. Other conditions tried were: 5:1 RSH/RSSR and 1:5 RSH/RSSR.

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The diester protected dialkyl amide system... clean NMR...
Monother protected mercapto-N-ethy lacetyl benzamide.
the overoxdn. rxn. was reacted w/ xs DMD...got a white solid soluble in meOH and insoluble in CDCl3) ? is it thiosulfonate...??

PhHNOC

50

O

S

S

S

O2

NHPh

PhHNOC

50
RXn. of SAI with 15eq. thiol in aq. buffer conditions. Major product at

\[
\begin{align*}
\text{NHCH}_2\text{COOEt}
\end{align*}
\]
U-13 of the mixed disulfide w/ 2-ME in a protein like system.
Chapter 2

Chemical Model Studies Suggests that Sulfenyl amide Protects Against Hydrogen peroxide-Mediated Over-oxidation of enzyme PTP1B

2.1 Introduction

Hydrogen peroxide mediated redox regulation of protein function involves an initial oxidation of cysteine residues to sulfenic acids. The sulfenic acids are typically prone to reaction with cellular thiols or neighboring cysteine residues to generate a mixed disulfide \( \text{(3)} \). The mixed disulfide can regenerate the native protein upon reaction with cellular thiols. Alternatively, the sulfenic acid can undergo further oxidation to generate sulfinic and sulfonic acids, that cannot be converted to the native cysteine by reaction with thiols. Over-oxidation to sulfinic and sulfonic forms are widely considered to be a permanent “turn off”
switch of enzyme activity.\textsuperscript{10} These reactions are illustrated in Scheme 2.1 in the context of a
cysteine dependent enzyme (such as PTP).

![Scheme 2.1. Reversible and irreversible oxidation of cysteine containing enzymes](image)

Recent evidences revealed the formation of a novel sulfenyl amide intermediate during
the hydrogen peroxide mediated redox regulation of protein tyrosine phosphatase 1B (PTP1B).\textsuperscript{11}
The inactive sulfenic acid form of PTP1B undergoes unique chemical transformation involving
an attack by the neighboring amide nitrogen to generate 3-isothiazolidinone ring (sulfenyl amide)
at the active site. The sulfenyl amide, similar to a mixed disulfide, can be reactivated by reaction
with cellular thiols and other thiol reducing enzymes (such as glutathione reductase,
thioredoxins, thioltransferases etc..) to regenerate the active form as shown in Scheme 2.2.\textsuperscript{11}

![Scheme 2.2. Formation of sulfenyl amide during the redox regulation of PTP1B](image)

It has been presumed that the formation of reversibly inactive mixed disulfide (3)\textsuperscript{6,12} and
sulfenyl amide (6)\textsuperscript{11,13} intermediates protect redox-switched proteins against irreversible over-
oxidation. However, this presumption does not rest on firm chemical evidence. Therefore, we undertook model studies to measure the propensity of sulfinic acid (2), mixed disulfide (3) and sulfinyl amide (6) to undergo further oxidation (“over-oxidation”) by hydrogen peroxide. The goals of this work are outlined in the following section.

2.2 Goals of this Chapter

Our central goal is to examine the relative abilities of a sulfinic acid, mixed disulfide and sulfinyl amide to undergo over-oxidation in presence of hydrogen peroxide. Thus, we set out to:

a) Synthesize small molecule models of a protein sulfinic acid, mixed disulfide and sulfinyl amide (Figure 2.1).

![Figure 2.1. Proposed chemical models for sulfinyl amide (34), mixed disulfide (55) and sulfinic acid (61) forms of enzyme](image)

b) Compare the rate constants for over-oxidation of the model compounds in presence of hydrogen peroxide under biologically relevant conditions.

The benzisothiazolidinone heterocycle (34) has been shown to mimic the redox regulation chemistry seen at the active site of PTP1B (Chapter 1). Moreover, the thiol form of this model compound (52) has a pK$_a$ (5.7) comparable to that of an active site cysteine thiol (5.6) of PTP1B. More importantly, the electronic properties of all model compounds (34, 55 and 61) are comparable with an adjacent amide group at the ortho position.
2.3 Synthesis of model compounds

2.3.1 Synthesis of 1,2-benzisothiazolin-3(2H)-one (34)

We recently reported 1,2-benzisothiazolin-3(2H)-one heterocycle (34) as an active site sulfonyl amide mimic of the enzyme PTP1B (Chapter 1). Compound 34 was synthesized as reported previously and used for the oxidation studies in this Chapter.

\[ \text{Scheme 2.3. Synthesis of sulfonyl amide mimic} \]

2.3.2 Synthesis of mixed disulfide analog (55)

Synthesis of mixed disulfide mimic (55) was carried out as reported previously in Chapter 1. 1,2-benzisothiazolin-3(2H)-one was refluxed with one equiv. of 2-mercaptoethanol in dry THF for 1.5 h to yield 70 % of 55, as shown in Scheme 2.4.

\[ \text{Scheme 2.4. Synthesis of a mixed disulfide mimic (55) of enzyme PTP1B} \]

2.3.3 Synthesis of sulfenic acid mimic (61)

We also prepared a model of a protein sulfenic acid containing an ortho substituted amide group (61). However, since free sulfenic acids cannot be isolated, we decided to synthesize a methyl sulfenate, substituting hydrogen atom of the sulfenic acid with a methyl group. Furthermore, to prevent a competing intramolecular cyclization of amide nitrogen onto
the sulfenyl sulfur, we incorporated a diethyl amide to obtain 61, as shown in Figure 2.2.

Importantly, methoxy group is used by physical organic chemists as a substitute for hydroxyl group.\textsuperscript{19,20} Dr. Herschlag has argued that replacement of an H with a methyl group is mechanistically sound and conservative.\textsuperscript{19}

![Figure 2.2. Chemical model for sulfenic acid mimic](image)

The ortho-diethyl amide protected methyl benzenesulfenate (61) was synthesized starting from 2,2′-dithiobis (N,N-diethyl) benzamide (64). Compound 64 on treatment with sulfuryl chloride and pyridine under dry conditions led to the generation of sulfenyl chloride (65). The resulting sulfenyl chloride (65) was stirred in diethyl ether in presence of methanol and triethylamine (TEA) to yield methyl sulfenate (61) as shown in Scheme 2.5. Several initial attempts to purify compound 61 failed due to its extreme instability. Compound 61 was highly unstable both on silica gel column and upon standing in organic solvents, yielding the corresponding disulfide (64). Finally, the crude methyl sulfenate (61) was purified by a flash column chromatography using neutral alumina. It should be noted that compound 61 decomposed even on neutral alumina if eluted over a period of 30 min.

![Scheme 2.5. Synthesis of sulfenate methyl ester (61)](image)
Due to the high instability of compound 61, we wanted to examine if we can measure the rate of hydrogen peroxide mediated decomposition of 61 under biologically relevant conditions. For this purpose, we verified the stability of compound 61 in aqueous buffer solution using HPLC. We found that 61 decomposed rapidly in aqueous buffer solution and in acetonitrile. For example, 100 µM of compound 61 dissolved either in acetonitrile or in 50 mM sodium phosphate buffer (pH 7.0) containing 30 % acetonitrile (v/v), decomposed within a minute to generate a complex HPLC chromatogram (Figure 2.3).

![HPLC chromatogram](image)

**Figure 2.3.** HPLC chromatogram of 100 µM of 61, after 1 min incubation at 25 °C. (A) in acetonitrile (B) in 50 mM sodium phosphate buffer containing 30 % acetonitrile (v/v)

Because compound 61 is highly unstable, we synthesized methyl benzenesulfenate (68) as a sulfinic acid surrogate. Methyl benzenesulfenate (68) has been characterized well in the literature as sulphenyl donors.²¹ Synthesis of 68 was carried out according to the reported procedure starting with phenyl disulfide (66) and sulfuryl chloride to obtain benzene
sulfenylchloride (67). Sulfenyl chloride (67) was very unstable and taken directly to the next step, in which 67 was stirred with methanol in presence of TEA to obtain a crude methyl benzenesulfenate (68). The crude product was purified by vacuum distillation to obtain compound 68 in 40% yield, as shown in Scheme 2.6.

![Scheme 2.6. Synthesis of benzene methylsulfenate ester 68.](image)

With the protein models (34, 55 and 68) in hand, we set out to examine their reactivity towards hydrogen peroxide-mediated oxidation.

### 2.4 Stability of analogs to hydrogen peroxide mediated over-oxidation in aqueous buffer solution

To measure the rate constant for oxidation reaction, we incubated 34, 55 and 68 in presence of ten equiv. hydrogen peroxide in sodium phosphate buffer (pH 7.0) containing 30% acetonitrile (v/v). We monitored the disappearance of starting material over time using C-18 reverse phase HPLC. In the following sections, we will discuss the relative reactivities of the model compounds to further oxidation.

#### 2.4.1 Oxidation of benzisothiazolin-3(2H)-one (34)

In a typical assay, 100 µM 34 was incubated with 1 mM hydrogen peroxide (10 equiv.) in a mixture of sodium phosphate buffer (100 mM, pH 7.0) containing 30% acetonitrile (v/v) at 25 °C. Disappearance of 34 was monitored over time and a semi-log plot of the remaining 34 over time (t) gives the rate constant for the reaction as a slope of the plot (Figure 2.4). We found that
the oxidation of compound 34 under given conditions yielded a pseudo-first order rate constant of $2.45 \times 10^{-4}$ s$^{-1}$ which corresponds to a half-life of 47 min. The reaction was monitored for more than 3 half-lives.

![Figure 2.4](image)

**Figure 2.4.** A plot of ln($a/a_0$) vs time for the hydrogen peroxide mediated oxidation of 34

Oxidation of benzisothiazolin-3(2H)-one (34) in presence of hydrogen peroxide, resulted in a transient formation of the corresponding benzisothiazolin-3(2H)-one 1-oxide (69) (confirmed through the injection of an authentic standard), as shown in Scheme 2.7. Accordingly, compound 34 was stable for over 48 h in a similar aqueous buffer condition containing no hydrogen peroxide. This suggests that compound 34 is susceptible to further oxidation in presence of hydrogen peroxide to generate the corresponding sulfinyl amide (69). A detailed study on the properties of the over-oxidation products of sulfenyl amide will be discussed in Chapter 4. Having measured the rate constant for the oxidation of sulfenyl amide (34), we moved forward to determine the rate constant for the oxidation of mixed disulfide analog (55).

![Scheme 2.7](image)

**Scheme 2.7.** Hydrogen peroxide mediated over-oxidation of sulfenyl amide (34) to generate 69
2.4.2 Oxidation of mixed disulfide compound (55)

In a typical assay, 100 µM 55 was incubated with 1 mM hydrogen peroxide (10 equiv.) in a mixture of sodium phosphate buffer (100 mM, pH 7.0) containing 30% acetonitrile (v/v) at 25 °C. Oxidation of 55 was monitored over time and the resulting semi-log plot of the remaining 55 over time (t) gives the rate constant for the reaction as a slope of the plot. We found that the oxidation of compound 55 under given conditions yielded a pseudo-first order rate constant of $6.8 \times 10^{-5}s^{-1}$ that corresponds to a half life of 168 min. The reaction was monitored for nearly 3 half-lives.

![Figure 2.5](image)

**Figure 2.5.** A plot of ln($a/a_0$) vs time for the hydrogen peroxide mediated oxidation of 55

The results here suggest that the hydrogen peroxide mediated over-oxidation of mixed disulfide analog (55) is 3.5 fold slower than the oxidation of sulfenyl amide (34). It is evident that compound 55 undergoes oxidative decomposition, as we have demonstrated earlier that 55 is stable in aqueous sodium phosphate buffer solution for the entire period of oxidation (Chapter 1). Our next goal was to examine how the rates of over-oxidation of the protective intermediate mimics compare to the over-oxidation of sulfinic acid surrogate (68). In the following section, we will describe our efforts towards determining rate constant for the oxidation of methyl benzenesulenate (68).
2.4.3 Oxidation of methyl benzenesulfonylate (68)

In a typical assay, 100 µM 68 was incubated with 1 mM hydrogen peroxide (10 equiv.) in a mixture of sodium phosphate buffer (100 mM, pH 7.0) containing 30% acetonitrile (v/v) at 25 °C. The disappearance of 68 was monitored over time. We found that compound 68 decomposed within 1 min of incubation under the above conditions. However, it was not clear if 68 was undergoing a H$_2$O$_2$-mediated oxidative decomposition. This led us to examine the stability of methyl benzenesulfonylate (68) in aqueous buffer solution containing no H$_2$O$_2$. We found that 68 (100 µM) decomposed rapidly (< 1 min) in 100 mM sodium phosphate buffer at 25 °C (Figure 2.6). A possible buffer catalyzed decomposition of methyl benzenesulfonylate (68) made it difficult for us to follow the oxidation reaction under biologically relevant conditions. This is further supported by the fact that reactions of methyl benzenesulfonylate (68) are often catalyzed by lewis acids. Since 68 was unstable in aqueous buffer solution, we tested its stability in water.

![Figure 2.6. HPLC chromatogram of 68 after 1 min incubation in (A) acetonitrile (B) 50 mM sodium phosphate buffer, pH 7.0 containing 30% acetonitrile (v/v)]](image)
We found that 68 decomposed slowly in water as compared to its rapid reaction in sodium phosphate buffer solution. For example, only 15% of 68 (100 µM) was lost after 4 h of incubation in a mixture of water containing 30 % acetonitrile (v/v). Hence, we planned to measure the rate of hydrogen peroxide mediated oxidation of 68 in an aqueous-acetonitrile solution. This method will allow us to compare the relative stabilities of all analogs to further oxidation, under similar conditions.

2.5 Stability of analogs to hydrogen peroxide mediated over-oxidation in aqueous acetonitrile mixture

We set out to measure the reactivity of model compounds (34, 55 and 68) to “over-oxidation” in aqueous-acetonitrile mixture. The disappearance of the starting material was monitored over time using C-18 reverse phase HPLC. We compared the resulting rate constants for the over-oxidation of analogs in the following sections.

2.5.1 Oxidation of sulfenyl amide (34)

In a typical assay, 100 µM 34 was incubated with 2 mM hydrogen peroxide (20 equiv.) in a mixture of water containing 30% acetonitrile (v/v) at 25 °C. The oxidation of 34 was monitored over time and the resulting semi-log plot of the remaining 34 over time (t) gives the rate constant for the reaction as a slope of the plot. The oxidation of 34 under given conditions followed a second order kinetics, as a resulting plot of (1/a) versus time yielded a second order rate constant of 0.38 ± 0.1 M⁻¹ s⁻¹. This corresponds to a half life of 5 h when the concentration of sulfenyl amide is 100 µM. The reaction was monitored for nearly 3 half-lives.
2.5.2 Oxidation of mixed disulfide analog (55)

In a typical assay, 100 µM 55 was incubated with 2 mM hydrogen peroxide (20 equiv.) in a mixture of water containing 30% acetonitrile (v/v) at 25 °C. Oxidation of 55 was monitored over time and the resulting semi-log plot of the remaining 55 over time (t) gives the rate constant for the oxidation as a slope of the plot. Oxidation of 55 under given conditions yielded a pseudo-first order rate constant of 5.8 ± 0.1 x 10^{-6} s^{-1} that corresponds to a half life of 33 h. The reaction was monitored for close to 3 half-lives. The equivalent second-order rate constant for oxidation is 2.9 ± 0.7 x 10^{-3} M^{-1} s^{-1}.

Figure 2.7. A second-order plot of (1/a) vs time for the hydrogen peroxide mediated oxidation of 34

Figure 2.8. A plot of ln(a/a_0) vs time for the hydrogen peroxide mediated oxidation of 55
2.5.3 Oxidation of methyl benzenesulenate (68)

In a typical assay, 100 µM 68 was incubated with 2 mM hydrogen peroxide (20 equiv.) in a mixture of water containing 30% acetonitrile (v/v) at 25 °C. Oxidation of 68 was monitored over time and the resulting semi-log plot of the remaining 68 over time (t) gives the rate constant for the oxidation as a slope of the plot. We found that the oxidation of compound 68 under given conditions yielded a pseudo-first order rate constant of 1.8 ± 0.08 x 10^{-4} s^{-1} that corresponds to a half life of 63 min (Figure 2.9). The reaction was monitored for more than 3 half-lives. The equivalent second-order rate constant for oxidation is 9.0 ± 0.4 x 10^{-2} M^{-1} s^{-1}.

![Figure 2.9. A representative plot of ln(a/a_0) vs time for the oxidation of 68](image)

The results obtained here suggest that sulfenyl amide mimic (34) and the mixed disulfide analog (55) is 5 and 33 fold more resistant to hydrogen peroxide-mediated over-oxidation respectively, when compared to the sulfenic acid surrogate (68). Overall, we found that mixed disulfide 55 resists over-oxidation better than the sulfenyl amide (~ 7 fold) or sulfenic acid. In the following section, we will examine if the sulfenyl amide formed at the active site of PTP1B protects the enzyme against over-oxidation.

2.6 PTP1B resists irreversible over-oxidation

As part of this work, we qualitatively examined whether PTP1B resists irreversible over-oxidation by hydrogen peroxide. For this purpose, we compared the oxidation of PTP1B to
another phosphatase enzyme Cdc25 that is not known to form an intermediate sulfenyl amide. Cdc25 phosphatases belong to a family of protein tyrosine phosphatases (PTPs), which share a conserved active site amino acid sequence similar to PTP1B.\textsuperscript{24} Cdc25 is known to undergo reversible oxidation in presence of hydrogen peroxide.\textsuperscript{25} The active site cysteine thiolate (Cys 473) of Cdc25 undergoes oxidation to sulfenic acid in presence of hydrogen peroxide. The intermediate sulfenic acid is then reduced by a backdoor cysteine (Cys 426), proximal to the active site Cys 473 to form a mixed disulfide, as shown in Scheme 2.8 (path A). It has been established that a mutant of backdoor Cys 426 (to Ser-426) leads to a rapid over-oxidation of the active site cysteine sulfenic acid (Cys\textsuperscript{473}-SOH) to an irreversibly inactivated sulfinic acid (Cys\textsuperscript{473}-SO\textsubscript{2}H) as shown in Scheme 2.8 (path B).\textsuperscript{26}

\begin{align*}
\text{A} & : & \text{Cys}^{426}\text{-SH} & \xrightarrow{\text{H}_2\text{O}_2} & \text{Cys}^{473}\text{-SH} & \xrightarrow{\text{H}_2\text{O}} & \text{Cys}^{473}\text{-SOH} \\
& & \text{Cys}^{473}\text{-SH} & & & & \text{Cys}^{473}\text{-S} \\
\text{B} & : & \text{Ser}^{426}\text{-OH} & \xrightarrow{\text{H}_2\text{O}_2} & \text{Ser}^{426}\text{-OH} & \xrightarrow{k = 110 \text{M}^{-1} \text{s}^{-1}} & \text{Ser}^{426}\text{-OH} \\
& & \text{Cys}^{473}\text{-SH} & & & & \text{Cys}^{473}\text{-SOH} \\
& & & & & & \text{Cys}^{473}\text{-SOH} \\
\end{align*}

\textbf{Scheme 2.8.} Hydrogen peroxide mediated oxidation of Cdc25 phosphatase enzyme. A) Oxidation of enzyme leading to the mixed disulfide formation. B) Oxidation of mutant (Cys426S) enzyme leading to an irreversible sulfinic acid.

Assuming that the rate constant for over-oxidation of sulfenic to sulfinic acid for Cdc25-(C426S) is comparable to PTP1B, we anticipated that formation of sulfenyl amide in PTP1B (which does not form an intramolecular mixed disulfide intermediate due to the lack of proximal cysteine) may resist an irreversible over-oxidation. With this in mind, we examined the oxidation of PTP1B under conditions similar to the oxidation of a Cdc25-(C426S), and compared the amounts of recoverable activity.
PTP1B was oxidized in presence of 1 mM hydrogen peroxide at 25 °C for a given time (0, 20, 40, 60 and 120 s) after which an aliquot of enzyme was removed and reactivated in a mixture of catalase and DTT. Reactivation with DTT is expected to regenerate the reversibly oxidized forms (sulfenic acid and sulfenyl amide) to the active thiol form of the enzyme. Remaining activity of the enzyme was then measured by reaction with a standard phosphatase substrate, \( p \)-nitrophenyl phosphate (pNPP). The enzyme-catalyzed release of \( p \)-nitrophenolate anion was monitored at 410 nm over time in a UV-vis spectrometer. The percent recoverable activity was calculated from a control reaction containing no hydrogen peroxide and compared to the values reported for the oxidation of Cdc25-(C426S) by Rudolph and coworkers. A resulting plot comparing the % recoverable activity for PTP1B and Cdc25-(C426S) over time is shown in Figure 2.10.

![Graph](Figure 2.10. Comparison of % recoverable activities at various times after the oxidation of Cdc25-(C426S) and PTP1B in presence of 1 mM hydrogen peroxide)

Our results clearly suggest that PTP1B resists irreversible over-oxidation as compared to Cdc25-(C426S). For example, incubation of PTP1B with 1 mM hydrogen peroxide for 2 min completely inactivates the enzyme (90% of the enzyme activity was lost). However, a subsequent DTT treatment led to 82% recovery of the enzyme activity, whereas only 20% of the
activity was recoverable in case of Cdc25-(C426S). This indicates two things a) Cdc25 is more prone to irreversible inactivation than PTP1B and b) the irreversible over-oxidation was prevented in PTP1B. Based upon the results of our chemical model studies, we presume that the protection of PTP1B from over-oxidation stems, at least in part, from the relative resistance of sulfenyl amide to hydrogen peroxide-mediated further oxidation.

2.7 Conclusion

In conclusion, our results support the notion that the oxidatively inactivated sulfenyl amide and mixed disulfide intermediates protect the enzyme against irreversible over-oxidation. Our chemical model studies suggest that the sulfenic acid surrogate (68) undergoes a rapid hydrogen peroxide mediated over-oxidation compared to sulfenyl amide (34) and mixed disulfide mimics (55) of the enzyme. We found the mixed disulfide mimic (55) to be highly resistant to hydrogen peroxide mediated over-oxidation, followed by the novel sulfenyl amide. Finally, we found that enzyme PTP1B resists hydrogen peroxide mediated irreversible over-oxidation. Comparison of oxidative inactivation of PTP1B and another phosphatase enzyme Cdc25, that does not form sulfenyl amide, revealed that the presence of sulfenyl amide in PTP1B may serve to protect the enzyme against irreversible loss of activity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Aqueous buffer conditions</th>
<th>Aqueous-acetonitrile conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>2.4 x 10^{-4} s^{-1}</td>
<td>0.24 M^{-1} s^{-1}</td>
</tr>
<tr>
<td></td>
<td>+ 0.38 ± 0.01 M^{-1} s^{-1}</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>6.8 x 10^{-5} s^{-1}</td>
<td>0.068 M^{-1} s^{-1}</td>
</tr>
<tr>
<td></td>
<td>+ 5.8 ± 0.1 x 10^{-6} s^{-1}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ 2.9 ± 0.7 x 10^{-3} M^{-1} s^{-1}</td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>--</td>
<td>1.8 ± 0.08 x 10^{-4} s^{-1}</td>
</tr>
<tr>
<td></td>
<td>+ 9.0 ± 0.4 x 10^{-2} M^{-1} s^{-1}</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1. Rate constants for the hydrogen peroxide mediated oxidation of active site mimics
2.8 Experimental Procedures

Materials and Methods

Reagents were of highest purity available and were used without further purification unless otherwise noted. Materials were purchased from the following suppliers: HPLC grade solvents, Fisher; silica gel 60 (0.04-0.063 mm pore size) for column chromatography, Merck; all other chemicals were purchased from Aldrich Chemical Company. Thin layer chromatography (TLC) was performed on plates from Aldrich Chemical Company, coated with general purpose silica containing UV254 fluorophore and compounds were visualized by illumination of the plates with short-wave (254 nm) UV light. Neutral alumina (active 90 grade) was purchased from EM reagents. Water was distilled, deionized and glass redistilled. The oxidizing agent, dimethyl dioxirane (DMD), was freshly synthesized according to reported methods\textsuperscript{28} and stored at -18 °C. High Resolution Mass Spectrometry was performed at The University of Illinois, Urbana-Champaigne Mass Spectrometry facility and low resolution mass spec were performed at the University of Missouri–Columbia. NMR spectra were recorded using Bruker DRX 500, DRX 300, or ARX 250 MHz instruments at the University of Missouri–Columbia. Catalase was obtained from Roche Biosciences (Palo Alto, CA). Recombinant PTP1B (a.a. 1-322) was prepared in our laboratory as reported by us earlier and the concentration was determined by a DTNB burst assay as described previously\textsuperscript{29}. Typically, free thiols were removed just before the experiments, from a stock of purified PTP1B using Zeba mini centrifugal buffer exchange column (Pierce, catalog no. 89882) according to the manufacturer’s protocol. The exchange buffer contained 100 mM sodium acetate, 50 mM Bis-Tris and 50 mM Tris, 10 mM DTPA and 0.05% NP-40 (detergent) at pH 7.0. The assay buffer used in our experiments contained 100 mM sodium acetate, 50 mM Bis-Tris and 50 mM Tris at pH 7.0. The thiol free enzyme stock
was diluted to four fold using an exchange buffer, before being used in the assays. The enzyme catalyzed release of p-nitrophenolate from the substrate pNPP was monitored at 410 nm in a Hewlett Packard, model 8453 spectrophotometer at 25 °C. HPLC spectra of compounds were recorded directly without an internal standard. The standard error in the HPLC peak area for 3 injections was estimated to be 2 ± 0.5 %.

2,2'-dithiobis(N,N-diethylbenzamide) (64). The synthesis was carried out according to the reported procedure. A solution of dithiobis benzoic acid (4g, 13 mmol) in thionyl chloride (15 mL, 0.2 moles) was refluxed at 75 °C. The resulting dark brown liquid was refluxed for further 5 h under nitrogen. Excess SOCl₂ was removed under reduced vacuum first and further traces were removed under high vacuum for 2 h to obtain the acid chloride as dark brown gum. The gum was not purified further and taken directly to the next step.

The brown gum obtained above was taken in hot benzene (8 mL). In a separate flask was taken diethyl amine (4.72 g, 0.08 mol) in dry THF and the resulting solution was stirred in an ice bath under nitrogen. To this was added hot benzene solution containing the acid chloride, slowly via syringe. The resulting brownish yellow gum was stirred in an ice bath for an hour and further 16 h at 25 °C. Excess solvent was removed using rotovap and the resulting solution was extracted with ethyl acetate (3 x 20 mL). Combined organic layer was washed with satd. sodium carbonate (30 mL) followed by 20% citric acid (30 mL) and water (30 mL). The resulting organic layer was dried under anhydrous sodium sulfate, filtered and evaporated to yield a dark brown oil which was purified by a flash column chromatography (1:1 Hexane/EtOAc) to obtain 64 as a yellow gum (3.5 g, 48%) that became a yellow solid upon standing. \( R_f = 0.24 \) (1:1
Hex/EtOAc). $^1$H-NMR (CDCl$_3$, 250 MHz) $\delta$ 1.05 (6H, t, $J = 7$ Hz), 1.28 (6H, t, $J = 7$ Hz), 3.14 (4H, q, $J = 7$ Hz), 3.58 (4H, q, $J = 7$ Hz), 7.28 (6H, m), 7.69 (2H, m); $^{13}$C-NMR (CDCl$_3$, 62.9 MHz) $\delta$ 168.47, 136.78, 133.80, 129.55, 128.18, 127.11, 126.19, 42.99, 38.98, 14.00, 12.68. LRMS (ESI) calcd for C$_{22}$H$_{28}$N$_2$O$_2$S$_2$ [M + H]$^+$ 417.16, found 417.05, found [M + Na]$^+$ 439.02.

2-(N,N-diethylcarbamoyl)phenyl methanesulfenate (61). To a stirred solution of 64 (500 mg, 1.2 mmol) in dry dichloromethane (2 mL) in a flame dried flask under nitrogen, was added pyridine (0.06 mL) via a syringe. The resulting yellow solution was stirred at 25 °C for further 10 min. To this was added sulfuryl chloride (178 mg, 1.32 mmol) dropwise via a syringe over 10 min and the resulting mixture was allowed to stir for further 2.5 h at 25 °C. Methylene chloride was removed under aspirator vacuum for 30 min and excess sulfuryl chloride under high vacuum for 3 h to obtain the sulphenyl chloride (65) as yellow gum. Compound 65 was not purified further and taken directly to the next step. Care should be taken not to expose the contents of the flask to air at any time during the experiment as the resulting sulfenyl chloride (65) can dimerize very quickly to yield the corresponding disulfide (64).

In a flame dried flask was taken a mixture of anhydrous diethyl ether (2 mL), freshly distilled triethyl amine (0.29 g, 2.88 mmol) and dry methanol (0.12 mL, 2.88 mmol). In a separate flask, product 65 from above reaction was taken in anhydrous ether (2.5 mL) and stirred in an ice bath for 20 min. To this was flask was added the solution containing TEA in methanol slowly over 5 min via a syringe. The yellow solution turned pale initially and became colorless. The resulting colorless solution was stirred for further 30 min in ice bath and filtered using a cotton plug. The precipitate was washed with ether (4 x 3 mL) and filtrate concentrated in a
rotovap to obtain yellow oil, which was purified by a flash column chromatography (4:1 hexane/EtOAc) in neutral alumina. Rf = 0.40 (4:1 hexane/EtOAc). $^1$H-NMR (CDCl$_3$, 300 MHz) δ 1.20 (6H, t, J = 7.05 Hz), 3.42 (4H, q, J = 7.0 Hz), 3.73 (3H, s), 7.17 (1H, td, J = 7.25, J = 1 Hz), 7.31 (1H, dd, J = 7.75, J = 1 Hz), 7.44 (1H, dd, J = 7.5 Hz, 1.5 Hz), 7.54 (1H, dd, J = 9 Hz, J = 1 Hz), $^{13}$C-NMR (CDCl$_3$, 75.4 MHz) δ 168.90, 141.5, 130.44, 129.79, 126.78, 124.93, 123.02, 65.10, 13.54. LRMS (APCI) calcd for C$_{12}$H$_{14}$O$_4$S [M + H]$^+$ 240.10, found 239.82.

**Methyl benzenesulfenate (68).** To a stirred solution of phenyl disulfide (8g, 36 mmol) in a flame dried 2 necked flask in dry dichloromethane (25 mL) was added pyridine (1 mL). The resulting pale yellow solution was stirred under nitrogen for 10 min at 25 °C. To this was added sulfuryl chloride (5.44 g, 3.3 mL, 40.3 mmol) dropwise via addition funnel slowly over 30 min. The resulting dark orange solution was stirred for further 1.5 h at 25 °C. Excess dichloromethane was removed in an aspirator vacuum (not the rotovap) for 30 min and sulfuryl chloride under high vacuum for 2-3 h. The resulting dark orange liquid was subjected to vacuum distillation (60 °C) to obtain benzene sulfonyl chloride (67) as a blood red liquid (4.2 g, 79.5%). Compound 67 was stored under nitrogen in a freezer for further use. (CAUTION: The contents of the reaction should not be exposed to air at any stage. Compound 67 is highly unstable and should not be stored in the freezer for more than 12-18 h).

In a flame dried 2 necked flask fitted with pressure equalizing addition funnel and nitrogen balloon was taken a mixture of anhydrous ether (20 mL), dry TEA (4.5 mL, 32 mmol) and dry methanol (1.4 mL, 32 mmol). The resulting solution was stirred in an ice bath for 15 min. To this was added the benzene sulfonylchloride (67) dropwise via addition funnel over 30
min. The resulting pale white solution was stirred for further 30 min in an ice bath, filtered and washed with anhydrous ether (3 x 5 mL). Combined filtrate was evaporated to obtain a pale yellow solution which was vacuum distilled (86-88 °C, 0.1 mm Hg) to obtain 68 as pale yellow oil (1.3 g, 32.5%). The $^1$H and $^{13}$C NMRs matched the reported ones.\textsuperscript{22}

**NOTE:** Dilute solution of 68 (5 mM) in acetonitrile decomposes slowly, if stored at room temperature for 12–24 h. 15 % of 68 was lost, when stored in water over a period of 4 h. Compound 68 undergoes immediate decomposition within a minute upon incubation with 100 mM sodium phosphate buffer (pH 7.0). However, in presence of 10 mM sodium phosphate buffer (pH 7.0), 68 was stable for 10 min at 25 °C. Typically, a 100 mM stock solution was prepared in acetonitrile and stored in the freezer.

**Hydrogen peroxide mediated over-oxidation of analogs in aqueous buffer solution.**

**Oxidation of methyl benzenesulfenate (68).** Compound 68 (10 μL of a 5 mM stock in CH$_3$CN) was incubated at 25 °C in a mixture of sodium phosphate buffer (100 μL, 500 mM, pH 7.0), hydrogen peroxide (100 μL of 5 mM stock in water), water (150 μL) and acetonitrile (140 μL). The mixture (final concentrations: 68, 100 μM; hydrogen peroxide, 1 mM; buffer, 100 mM, pH 7.0; acetonitrile, 30% by volume) was vortex mixed and analyzed by reverse phase HPLC at regular time intervals. The disappearance of 68 was monitored by HPLC using a reverse phase C-18 Varian Microsorb-MV column (100 Å sphere size, 5 μm pore size, 25 cm length, 4.6 mm i.d.), eluted with a mobile phase composed of water and acetonitrile as given below with a flow rate of 0.8 mL/min (injection volume = 40 μL, detection wavelength = 254 nm).
Oxidation of methyl 1,2-benzisothiazolin-3(2H)-one (34). Compound 34 (10 μL of a 5 mM stock in CH₃CN) was incubated at 25 °C in a mixture of sodium phosphate buffer (100 μL, 500 mM, pH 7.0), hydrogen peroxide (100 μL of 5 mM stock in water), water (150 μL) and acetonitrile (140 μL). The mixture (final concentrations: 34, 100 μM; hydrogen peroxide, 1 mM; buffer, 100 mM, pH 7.0; acetonitrile, 30% by volume) was vortex mixed and analyzed by reverse phase HPLC at regular time intervals. The disappearance of 34 was monitored by HPLC using a reverse phase C-18 Varian Microsorb-MV column (100 Å sphere size, 5 μm pore size, 25 cm length, 4.6 mm i.d.), eluted with a mobile phase composed of water and acetonitrile as mentioned in the previous experiment with a flow rate of 0.8 mL/min (injection volume = 40 μL, detection wavelength = 254 nm)

Oxidation of mixed-disulfide analog (55). Compound 55 (10 μL of a 5 mM stock in CH₃CN) was incubated at 25 °C in a mixture of sodium phosphate buffer (100 μL, 500 mM, pH 7.0), hydrogen peroxide (100 μL of 5 mM stock in water), water (150 μL) and acetonitrile (140 μL). The mixture (final concentrations: 55, 100 μM; hydrogen peroxide, 1 mM; buffer, 100 mM, pH 7.0; acetonitrile, 30% by volume) was vortex mixed and analyzed by reverse phase HPLC at regular time intervals. The disappearance of 55 was monitored by HPLC using a reverse phase C-18 Varian Microsorb-MV column (100 Å sphere size, 5 μm pore size, 25 cm length, 4.6 mm i.d.), eluted with a mobile phase composed of water and acetonitrile as mentioned in the previous

<table>
<thead>
<tr>
<th>Time(min)</th>
<th>Water</th>
<th>Acetonitrile</th>
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<tbody>
<tr>
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<td>30</td>
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Hydrogen peroxide mediated over-oxidation of analogs in an aqueous solution.

**Oxidation of methyl benzenesulfenate (68).** Compound 68 (10 μL of a 5 mM stock in CH$_3$CN) was incubated at 25 °C in a mixture of hydrogen peroxide (100 μL of 5 mM stock), water (250 μL) and acetonitrile (140 μL). The mixture (final concentrations: 68, 100 μM; hydrogen peroxide, 1 mM; acetonitrile, 30% by volume) was vortex mixed and analyzed by reverse phase HPLC at regular time intervals. The disappearance of 68 was monitored by HPLC using a reverse phase C-18 Varian Microsorb-MV column (100 Å sphere size, 5 μm pore size, 25 cm length, 4.6 mm i.d.), eluted with a mobile phase composed of water and acetonitrile using similar method as mentioned in the earlier experiments.

**Oxidation of 1,2-benzisothiazolin-3(2H)-one (68).** Compound 68 (10 μL of a 5 mM stock in CH$_3$CN) was incubated at 25 °C in a mixture of hydrogen peroxide (100 μL of 5 mM stock), water (250 μL) and acetonitrile (140 μL). The mixture (final concentrations: 68, 100 μM; hydrogen peroxide, 1 mM; acetonitrile, 30% by volume) was vortex mixed and analyzed by reverse phase HPLC at regular time intervals. The disappearance of 68 was monitored by HPLC using a reverse phase C-18 Varian Microsorb-MV column (100 Å sphere size, 5 μm pore size, 25 cm length, 4.6 mm i.d.), eluted with a mobile phase composed of water and acetonitrile using a similar method as mentioned in the earlier experiments.

**Oxidation of mixed disulfide analog (68).** Compound 68 (10 μL of a 5 mM stock in CH$_3$CN) was incubated at 25 °C in a mixture of hydrogen peroxide (100 μL of 5 mM stock), water (250 μL) and acetonitrile (140 μL). The mixture (final concentrations: 68, 100 μM; hydrogen
peroxide, 1 mM; acetonitrile, 30% by volume) was vortex mixed and analyzed by reverse phase HPLC at regular time intervals. The disappearance of 68 was monitored by HPLC using a reverse phase C-18 Varian Microsorb-MV column (100 Å sphere size, 5 µm pore size, 25 cm length, 4.6 mm i.d.), eluted with a mobile phase composed of water and acetonitrile using a similar method as mentioned in the earlier experiments.

Characterization of % recoverable activity in hydrogen peroxide mediated oxidative inactivation of PTP1B. An aliquot of thiol-free enzyme [(25 µL of a 4 µM solution in 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris, DTPA (10mM) and NP-40 (0.05%, v/v)] was combined with the assay buffer (20 µL) and hydrogen peroxide (5 µL of a 10 mM stock solution) at 25 °C. At varying times of 0 s, 20 s, 40 s, 60 s and 120 s, an aliquot (11 µL) from the above mixture (final concentrations: PTP1B, 2 µM; hydrogen peroxide, 1 mM; buffer, 90 mM sodium acetate, 45 mM Bis-Tris and 45 mM Tris and 9 mM DTPA) was quenched in an assay buffer (11 µL) containing catalase and DTT. The resulting mixture (final concentrations: catalase, 100 units; DTT 50 mM; buffer, 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris, 10 mM DTPA) was incubated at 25 °C for 25 min. Remaining activity was measured by adding 20 µL of the above enzyme to a cuvette containing pNPP (980 µL) in assay buffer at pH 7.0 (final volume, 1 mL; final concentration: pNPP, 10 mM; buffer, 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris, DTPA (10mM)). Immediately following the addition of enzyme, the assay was mixed by repeated inversion and the enzyme catalyzed release of p-nitrophenol monitored at 25 °C by measuring the increase in absorbance at 410 nm. Data points were taken every 2 s for a period of 2 min. The % recoverable activity was calculated with respect to a
control containing no hydrogen peroxide. The control experiment was performed in a similar way as mentioned here.

References


22 Armitage, D.A. Synthesis 1984, 12, 1042-1044.


the top major spot (has some baseline spot).


![Chemical Structure Diagram]

- **Chemical Formula**: \( \text{NEt}_2 \text{SOCH}_3 \)
- **Relative Abundance**
- **Mass To Charge Ratio (m/z)**

- **Values**:
  - 166.65
  - 179.89
  - 208.84
  - 209.80
  - 239.82
  - 417.08
  - 418.14
  - 419.12
  - 420.12
  - 449.09
batch 2.78 g.

![Chemical Structure](image)
A pale yellow liquid that distilled at 68°C under high vacuum.
Chapter 3

Insights into the Properties of Sulfenyl amide

3.1 Introduction

Redox regulation of protein tyrosine phosphatase 1B (PTP1B) involves oxidation of catalytic cysteine thiolate to sulfenic acid.\(^1,2\) The intermediate sulfenic acid undergoes a novel chemical rearrangement with neighboring amide nitrogen to generate 3-isothiazolidinone ring (sulfenyl amide, 6) at the active site of PTP1B.\(^3\) This novel intrastrand protein crosslink between amide nitrogen and a neighboring thiol residue had never been observed before 2003. The sulfenyl amide form of the enzyme is inactive and can be reactivated by reaction with thiols (Scheme 3.1).\(^4,5,3\)

![Scheme 3.1. Formation of sulfenyl amide during oxidative inactivation of PTP1B](image-url)
Although the presence of sulfenyl amide at the active site of PTP1B crystals has been confirmed, the details of its formation and its reactivity under physiological conditions are not clearly understood. With this in mind, we set out to characterize important chemical properties of sulfenyl amide formed at the active site of PTP1B.

3.2 Goals of this Work

Herein, we set out to understand chemical properties of the sulfenyl amide formed during the oxidative inactivation of PTP1B under physiologically relevant conditions. The major goals of this work are:

1) To identify the presence of sulfenyl amide under biologically relevant aqueous buffer solution.

2) To measure rate constant for the formation of sulfenyl amide from sulfenic acid at the enzyme backbone.

3.3 Generation of sulfenyl amide by hydrogen peroxide mediated oxidation of PTP1B in aqueous buffer

The conditions for the generation of sulfenyl amide in solution were lacking in literature. In the following sections, we will present a method to selectively oxidize enzyme PTP1B to its sulfenyl amide form under biologically relevant conditions.

3.3.1 Thiol reversible oxidative inactivation of PTP1B in presence of hydrogen peroxide

Our goal was to oxidize PTP1B using the minimum hydrogen peroxide concentration necessary to completely convert the enzyme to its reversibly oxidized form (sulfenic acid and/or sulfenyl amide). For this purpose, we wanted to a) use low concentrations of hydrogen peroxide
with long incubation time, as this would limit over-oxidation of enzyme to its irreversibly inactive forms (sulfinic and sulfonic acid) and b) to achieve complete inactivation of the enzyme to the reversibly oxidized forms (sulfenic acid or sulfenyl amide), that can be reactivated upon treatment with an organic thiol, dithiothreitol (DTT).

We arrived at a method involving incubation of a ~2 µM solution of enzyme in buffer (50 mM Tris, 50 mM Bis-Tris and 100 mM sodium acetate) containing a known concentration of hydrogen peroxide for a specific time at 25 °C. The percentage of enzyme in the reversibly inactive form within this oxidized pool was determined as follows. Typically after initial oxidation, an aliquot of enzyme was quenched in a solution of catalase, which catalyzes the conversion of hydrogen peroxide to water and molecular oxygen, to prevent further oxidation. The remaining activity was measured by mixing the inactivated enzyme with a substrate \( p \)-nitrophenyl phosphate (pNPP). The resulting enzyme-catalyzed release of \( p \)-nitrophenol was measured by monitoring the increase in absorbance at 410 nm over time. This step measures the amount of residual activity remaining after the oxidation. Similarly, an aliquot of oxidized enzyme was incubated in a mixture of catalase containing 50 mM DTT to convert the reversibly oxidized forms (sulfenic acid and sulfenyl amide) to the active enzyme. This step is called “reactivation”, and it measures the amount of residual and reversibly oxidized forms of the enzyme (Scheme 3.2). The difference in remaining activities measured after reactivation and inactivation provides the amount of enzyme present in reversibly inactivated forms. Thus, we can readily measure the amount of PTP1B converted to sulfenyl amide (thiol recoverable) and sulfinic/sulfonic (thiol unrecoverable) forms by reaction of native enzyme with hydrogen peroxide (Scheme 3.2).
Using this partitioning protocol (Scheme 3.2), we found that incubation of 2 µM PTP1B in an assay buffer containing 50 µM hydrogen peroxide for 35 – 40 min converted approximately 75 ± 5% of enzyme to a thiol-recoverable inactive form. Under these conditions, 15 ± 5% of the enzyme still remained unmodified and 15 ± 5% was converted to thiol-unrecoverable forms (sulfinic and sulfonic acids) of the enzyme.
Figure 3.1. A plot of measured remaining activity over time after the oxidation of PTP1B with 50 µM H₂O₂ for 40 min. The corresponding slopes are: control (blue); reactivation (pink) and inactivation (yellow).

Use of higher hydrogen peroxide concentration or longer incubation times resulted in an increase in the yield of thiol unrecov erable forms of the enzyme (59 and/or 60). This is not surprising given that the over-oxidation of sulfenic to sulfinic acid is second-order in the concentration of hydrogen peroxide and will compete with the cyclization reaction, as shown in Scheme 3.3.

Scheme 3.3. Hydrogen peroxide mediated gentle oxidation of PTP1B towards sulfenyl amide generation

After identifying conditions that provide a good yield of inactive, thiol-recoverable PTP1B, we focused on characterizing the chemical nature of the reversibly inactive species. The thiol reversible inactive form of the enzyme could in principle, be either the sulfenic acid (-SOH) or cyclized sulfenyl amide (6). An intramolecular mixed disulfide intermediate (-S-S-) is not possible, as PTP1B does not possess a proximal cysteine residue.⁶,⁷ Based on the literature precedents, we expected to observe the sulfenyl amide⁷ because, the sulfenic acid was not
observed when Bradford and coworkers oxidized PTP1B crystals in presence of hydrogen peroxide. However, it was important to confirm the absence of sulfenic acid in aqueous solution. In the following section, we will discuss experiments aimed towards characterizing the identity of inactive, thiol-recoverable form of the enzyme generated during the oxidation of PTP1B.

### 3.3.2 Trapping the oxidatively inactivated intermediate using NBD-Cl

Detection of sulfenic acids can be difficult due to their extreme instability. Sulfenic acids often react readily with nucleophiles/electrophiles making their detection or isolation very difficult. However, methods exist to selectively trap cysteine sulfenic acids formed at protein active sites. Ellis and Poole demonstrated the use of an electrophilic 7-chloro-4-nitrobenzo-2-oxa-1,2-diazole (NBD-Cl, 76) to detect the sulfenic acid formed at the active site of alkyl hydroperoxide reductase (AhpC) and native NADH peroxidase. It has been shown that NBD-Cl reacts with both the reduced cysteine thiolate and cysteine sulfenic acid in these proteins to generate two spectrally distinct species by UV-vis. The thiol adduct Cys-S-NBD (78) has a $\lambda_{\text{max}}$ at 420 nm while the sulfenic acid adduct Cys-S(O)-NBD (77) has an absorption maxima at 347 nm, as shown in Scheme 3.4. Since then, NBD-Cl has been used in various proteins to detect the presence of cysteine sulfenic acids (Cys-SOH).
Herein, we used NBD-Cl to search for the presence of a sulfenic acid during the oxidative inactivation of PTP1B under our experimental conditions. We anticipated that the absorbance spectrum of NBD-Cl treated oxidized PTP1B will help us determine the chemical nature of the thiol recoverable, inactive species. PTP1B and the hydrogen peroxide-inactivated PTP1B were treated with NBD-Cl for 1 h and excess NBD-Cl was then removed by extensive dialysis. The UV spectrum of the resulting proteins were determined and shown in Figure 3.2. The characteristic Cys-S-NBD peak at 420 nm was observed for the reaction between native PTP1B and NBD-Cl. Incorporation of NBD-Cl into the native enzyme accompanied with the loss of enzyme activity, suggesting that the active site Cys-215 of PTP1B has been labeled by NBD-Cl. Furthermore, inactivation of enzyme by NBD-Cl is slowed by the addition of a competitive PTP1B inhibitor phosphatase, indicating that the NBD labeling is active site directed. When the oxidized PTP1B was treated with NBD-Cl, the resulting spectra revealed a considerable loss in absorbance of Cys-S-NBD peak at 420 nm, indicating that the active site cysteine residue has
been oxidatively modified. However, a corresponding increase in absorbance at 347 nm that would arise from Cys-S(O)-NBD peak was not observed. This provides evidence for the absence of a stable sulfenic acid and suggests that the inactive, thiol recoverable form of the enzyme is not in sulfenic acid (-SOH) form. The observed Cys-S-NBD labeling in the oxidized enzyme corresponds to the residual enzyme activity.

Figure 3.2. UV spectrum of the NBD modified PTP1B. Reaction of native PTP1B with NBD-Cl (blue) and reaction of oxidized PTP1B with NBD-Cl (pink). Inset: UV-vis spectrum of 50 µM NBD-Cl showing no absorption at 420 nm

The extinction coefficients of Cys-S-NBD and Cys-S(O)-NBD adducts are reported to be identical.11 Using the extinction coefficient of 13400 M⁻¹ cm⁻¹ for Cys-S-NBD adduct, we estimated that approximately one equivalent of NBD has been incorporated into the active site of PTP1B. PTP1B has five other cysteine thiol residues apart from the active site Cys-215, the possibility that NBD-Cl has labeled cysteine residues other than Cys-215 is rendered unlikely based on several lines of evidence. 1) The reaction of protein with NBD-Cl completely inactivates the enzyme and the inactivated Cys-S-NBD conjugate could be reactivated by a treatment with 50 mM DTT 2) the inactivation reaction is slowed by a competitive phosphatase inhibitor and 3) the oxidation of PTP1B under similar conditions results in the loss of one cysteine thiol residue, as determined by a DTNB titration (Labutti, J.N. and Gates, K.S., unpublished data). Thus, the results presented here suggest that NBD-Cl is active site directed.
In conclusion, we devised conditions that selectively oxidize PTP1B to the sulfenyl amide form (6). Having provided an indirect evidence for the formation of sulfenyl amide during the oxidative inactivation of PTP1B, we focused on determining the rate constant for the cyclization of amide nitrogen onto the sulfenic acid.

3.4 Determining the rate constant for the cyclization reaction (k₃)

We employed competition kinetics to estimate the rate constant for sulfenyl amide formation at the active site of PTP1B. Hydrogen peroxide-mediated inactivation of PTP1B oxidizes the catalytic cysteine thiol to sulfenic acid. The sulfenic acid may undergo either intramolecular cyclization to generate the sulfenyl amide (k₂) or can undergo “over-oxidation” to the sulfinic acid (k₃), in presence of excess hydrogen peroxide, as shown in Scheme 3.5. It is important to note that the inactive sulfenyl amide can be converted to active thiol form by reaction with DTT, whereas sulfinic acid form cannot be recovered by DTT treatment.

Scheme 3.5. Partitioning of the oxidized PTP1B to sulfenyl amide and sulfinic acid

To measure the rate constant for cyclization (k₃), the enzyme was inactivated with various concentrations of hydrogen peroxide. After which, the relative amounts of DTT-recoverable (sulfenyl amide) versus DTT-unrecoverable enzyme (sulfinic acid and higher oxidation states) were determined. The amount of unrecoverable activity was expected to increase with the increasing concentration of hydrogen peroxide. The value of k₂ (110 M⁻¹ s⁻¹)
can be estimated from the over-oxidation of a related enzyme Cdc25, (C426S mutant that is not known to form a mixed disulfide intermediate) as reported by Rudolph and coworkers. Finally, assuming that there is no stable sulfinic acid present under our experimental conditions (that is, the observed DTT-recoverable activity arises solely from the sulfinyl amide), a plot of $[\text{H}_2\text{O}_2]$ versus ratio of unrecoverable/recoverable activity is used to obtain $k_3$ as shown below (Eqn. 4).

\[
\begin{align*}
\% \text{unrecoverable} &= k_2 [\text{H}_2\text{O}_2] \\
\% \text{recoverable} &= k_3 \\
\% \text{unrec} / \% \text{rec} &= k_2 [\text{H}_2\text{O}_2] / k_3 \\
\text{slope} &= k_2 / k_3
\end{align*}
\]

In a typical assay, PTP1B was oxidized with varying concentrations of hydrogen peroxide (0.2 – 1.5 mM) for 8 min at 25°C. The amount of inactivated enzyme present in the thiol-recoverable sulfinyl amide form was determined by the difference in recoverable activities between DTT-treated and DTT-untreated samples as follows. In a typical assay, an aliquot of oxidized enzyme was simultaneously incubated in a solution of catalase, with and without DTT (50 mM) for 25 min followed by measuring the remaining activity using pNPP (see section 3.3.1). The activity that cannot be recovered by DTT treatment was taken as unrecoverable activity. More importantly, we determined that the measured unrecoverable activity does not arise from the irreversible over-oxidation of sulfinyl amide under our experimental conditions. We observed only 1% loss in thiol-recoverable activity when sulfinyl amide, synthesized under mild oxidizing conditions, was incubated with hydrogen peroxide (1.5 mM) for 8 min. Finally, the estimated rate constant for cyclization reaction obtained from the plot of $[\text{H}_2\text{O}_2]$ versus ratio of unrecoverable/recoverable activity was measured to be $0.4 \pm 0.2 \text{ s}^{-1}$ (Figure 3.3). The
observed $k_3$ is an average from three sets of experiments and it corresponds to a half-life of 2 s. A similar value for $k_3$ was obtained, even when the oxidation was carried out at higher hydrogen peroxide concentrations (1, 2.5, 5 and 10 mM).

![Figure 3.3](image)

**Figure 3.3.** A representative plot of $[H_2O_2]$ vs unrecoverable/recoverable activity towards measuring $k_3$

The results obtained here suggest that cyclization reaction is extremely rapid compared to the over-oxidation of sulfenic acid under physiologically relevant conditions. For example, under the physiological concentrations of hydrogen peroxide generated in a normal healthy cell ($< 1 \mu M$)\textsuperscript{14}, the cyclization is expected to be more than 3000 fold faster than the competing over-oxidation reaction. Having measured the rate constant for cyclization reaction, we next set out to determine whether physiological thiol glutathione can intercept the intermediate sulfenic acid.

### 3.5 Can glutathione trap the intermediate sulfenic acid?

In this section, we wanted to examine if physiologically relevant thiol glutathione (GSH), can trap the intermediate sulfenic acid, and thus play a role in preventing irreversible over-oxidation to sulfinic acid (Scheme 3.6). For this purpose, we performed the oxidation in presence of several concentrations of glutathione. We anticipated that an efficient trapping of
intermediate sulfenic acid by glutathione would yield an increase in the amount of recoverable activity (with an accompanying decrease in the amount of unrecoverable activity).

**Scheme 3.6.** Trapping intermediate sulfenic acid by a physiological thiol glutathione

To measure the rate constant for the reaction of GSH with sulfenic acid, enzyme PTP1B was oxidized with 1.5 mM hydrogen peroxide for 8 min at 25 °C, in presence of several concentrations of GSH (2 – 10 mM). After 8 min, an aliquot of enzyme was reactivated in a mixture of catalase (100 units) and DTT (50 mM) for 20 min, to regenerate the glutathionylated forms of enzyme to the active thiol form. The remaining activity was measured using pNPP (10 mM) as described earlier. The percentage recoverable and unrecoverable activities were compared to a control oxidation containing no GSH. The rate constant for sulfenic acid trapping by GSH ($k_6$), if it occurred, could be obtained from the slope of a plot (Eqn. 8), [GSH] versus ratio of recoverable/unrecoverable activities as shown below.
We observed that the presence of GSH during the oxidation of PTP1B, does not have any influence on the % recoverable activity (Figure 3.4). This indicates that the reaction of GSH with the intermediate sulfenic acid is slower compared to the over-oxidation, under our experimental conditions. The possibility of a hydrogen peroxide mediated oxidation of GSH is rendered unlikely based on the following observation. The hydrogen peroxide-mediated oxidation of PTP1B (10–20 M⁻¹ s⁻¹) is much faster than the oxidation of glutathione¹⁵ (0.87 M⁻¹ s⁻¹). Moreover, if GSH depleted the concentration of hydrogen peroxide, it would result in an increase in the amount of recoverable activity. As the percent recoverable activity did not change significantly compared to a control with no GSH, the possibility of a hydrogen peroxide mediated oxidation of GSH can be ruled out under our experimental conditions. However, counterintuitively, a decrease in recoverable activity was observed at higher concentrations of GSH (15 mM). We speculate that this could be due to the formation of a more stable protein glutathione adduct (PTP-S-SG). It is possible that the reaction of GSH with sulfinic acid/sulfenyl amide, which is governed by the concentration of GSH, occurred at a higher [GSH] and the resulting adduct (PTP-S-SG) is not completely reactivated by DTT under our experimental conditions. Overall, our results suggest that the intermediate sulfinic acid is not intercepted by physiological concentrations of GSH (1-10 mM).¹⁶ This is not surprising, given that Barford and coworkers observed the formation of sulfenyl amide in presence of GSH.³
Finally, it can be said that the measurement of the rate constant for the reaction of GSH with sulfinic acid ($k_6$) is complicated.

![Figure 3.4](image)

**Figure 3.4.** A plot of [GSH] vs recoverable activity during the oxidation of PTP1B

### 3.6 Conclusion

In conclusion, we developed a method to cleanly generate the sulfinyl amide form of PTP1B. In addition, we obtained an estimate for the rate constant of the cyclization of amide nitrogen onto sulfinic acid, to generate sulfinyl amide at the enzyme backbone. We also found that the physiological concentrations of GSH do not intercept sulfinic acid during the oxidative inactivation of PTP1B. Our results show that under physiologically relevant conditions, the sulfinyl amide formation is rapid compared to the reaction of intermediate sulfinic acid with either hydrogen peroxide or glutathione.
3.7 Experimental procedures

Materials and Methods

All reagents were purchased from Sigma-Aldrich chemical company (St. Louis, MO). Catalase was obtained from Roche Biosciences (Palo Alto, CA). Recombinant PTP1B (a.a. 1-322) was prepared in our laboratory as reported by us earlier\textsuperscript{17} and the concentration was determined by a DTNB burst assay as described previously.\textsuperscript{17} Typically, free thiols were removed just before the experiments, from a stock of purified PTP1B using Zeba mini centrifugal buffer exchange column (Pierce, catalog no. 89882) according to the manufacturer’s protocol. The exchange buffer contained 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris, 10 mM DTPA and 0.05% NP-40 (detergent) at pH 7.0. The assay buffer used in our experiments contained 100 mM sodium acetate, 50 mM Bis-Tris and 50 mM Tris at pH 7.0. The thiol free enzyme stock was typically diluted to four fold using an exchange buffer before being used in the assays, unless otherwise indicated. The enzyme catalyzed release of p-nitrophenolate from the substrate pNPP was monitored at 410 nm in a Hewlett Packard, model 8453 spectrophotometer at 25 °C.

Generation of sulfenyl amide via a hydrogen peroxide mediated gentle oxidation of PTP1B.

An aliquot of thiol-free enzyme [(25 µL of a 4 µM solution in 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris, 10 mM DTPA and NP-40 (0.05%, v/v)] was combined with the assay buffer (20 µL) and hydrogen peroxide (5 µL of a 500 µM solution) at 25 °C for 35 min (final concentrations: PTP1B, 2 µM; hydrogen peroxide, 50 µM; buffer, 90 mM sodium acetate, 45 mM Bis-Tris, 45 mM Tris, 9 mM DTPA).
**Reactivation:** An aliquot of oxidized enzyme (12 µL) was added to an assay buffer (12 µL) containing a mixture of catalase and DTT and incubated for 20 min (final concentrations: catalase, 100 units; DTT, 50 mM) at 25 °C. From the above mixture, 20 µL was added to a cuvette containing pNPP (980 µL) in assay buffer at pH 7.0 (final volume, 1 mL; final concentrations: buffer, 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris, 10 mM DTPA; pNPP, 10 mM). Immediately following the addition of enzyme, the assay was mixed by repeated inversion and the enzyme catalyzed release of p-nitrophenol monitored at 25 °C by measuring the increase in absorbance at 410 nm. Data points were taken every 2 s for a period of 2 min. Enzyme activity was measured from the initial slope of the plot.

**Inactivation:** An aliquot of oxidized enzyme (12 µL) was added to an assay buffer (12 µL) containing catalase (100 units) and the resulting solution incubated for 20 min at 25 °C. From above mixture, 20 µL was added to a cuvette containing pNPP (980 µL) in assay buffer at pH 7.0 (final volume, 1 mL; final concentrations: buffer, 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris, 10 mM DTPA; pNPP, 10 mM). Immediately following the addition of enzyme, the assay was mixed by repeated inversion and the enzyme catalyzed release of p-nitrophenol monitored at 25 °C by measuring the increase in absorbance at 410 nm. Data points were taken every 2 s for a period of 2 min.

Untreated enzyme subjected to reactivation conditions was used as a control and the % recoverable activities were calculated with respect to the control reaction. The % of inactive, thiol recoverable form (sulfenyl amide) of the enzyme is calculated as follows:

\[
\% \text{ converted to sulfenyl amide} = \left(\frac{\text{Slope reactivation} - \text{slope inactivation}}{\text{slope control}}\right) \times 100
\]
**NBD-Cl labeling of oxidatively inactivated PTP1B.** An aliquot of thiol-free enzyme [80 µL of a 16 µM solution (used directly with no further dilution)] was added to assay buffer (25 µL) and hydrogen peroxide (3 µL of a 10.8 mM stock) at 25 °C and the resulting mixture incubated for 40 min (final concentrations: PTP1B, 12 µM; hydrogen peroxide, 300 µM, buffer, 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris, 7.5 mM DTPA). Catalase (10 µL, 100 units) was then added to eliminate residual hydrogen peroxide. After 10 min of catalase treatment, NBD-Cl (1.92 µL in DMSO) was added to a final concentration of 0.8 mM. The labeling reaction was allowed to proceed at 25 °C for 1 h. A control reaction treated with water (3 µL) instead of hydrogen peroxide was carried out simultaneously. After NBD-Cl labeling, the samples were dialyzed extensively against four exchanges of 20 mM sodium phosphate (pH 7.2) containing 150 mM NaCl. The absorbance spectra of modified protein were then recorded (in a 200 µL cuvette) from 750 to 250 nm in a UV-vis spectrometer.

**Determining the rate constant for the cyclization reaction (k₃).** An aliquot of thiol-free enzyme [(12.5 µL of a 4 µM solution in 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris, 10 mM DTPA and NP-40 (0.05%, v/v))] was combined with the assay buffer (10 µL) and varying concentrations (0.2, 0.5, 1 and 1.5 mM) of hydrogen peroxide (2.5 µL of 10x stock) at 25 °C for 8 min (final volume, 25 µL).

**Reactivation:** An aliquot from above mixture (11 µL) was added to an assay buffer (11 µL) containing catalase and DTT and the resulting mixture incubated for 25 min (final concentrations: catalase, 100 units; DTT, 50 mM) at 25 °C. After 25 min, 20 µL from above mixture was added to a cuvette containing pNPP (980 µL) in assay buffer at pH 7.0 (final volume, 1 mL; final concentrations: buffer, 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM
Tris, 10 mM DTPA; pNPP, 10 mM). Immediately following the addition of enzyme, the assay was mixed by repeated inversion and the enzyme catalyzed release of p-nitrophenol monitored at 25 °C by measuring the increase in absorbance at 410 nm. Data points were taken every 2 s for a period of 2 min.

**Inactivation:** An aliquot of oxidized enzyme (11 µL) was added to an assay buffer (11 µL) containing catalase (100 units) and the resulting solution incubated for 25 min at 25 °C. After 25 min, 20 µL from above mixture was added to a cuvette containing pNPP (980 µL) in assay buffer at pH 7.0 (final volume, 1 mL; final concentrations: buffer, 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris, 10 mM DTPA; pNPP, 10 mM). Immediately following the addition of enzyme, the assay was mixed by repeated inversion and the enzyme catalyzed release of p-nitrophenol monitored at 25 °C by measuring the increase in absorbance at 410 nm. Data points were taken every 2 s for a period of 2 min.

The % recoverable activities were calculated with respect to a control containing no hydrogen peroxide treated in an identical way. The % sulphenyl amide was calculated as described previously and taken as thiol recoverable activity. The remaining activity that cannot be recovered by DTT treatment was taken as thiol unrecoverable activity. A resulting plot of unrecoverable/recoverable vs [H$_2$O$_2$] yields $k_2/k_3$ as a slope. The rate constant for cyclization ($k_3$) was obtained after substituting the value of 110 M$^{-1}$ s$^{-1}$ for $k_2$.

% recoverable activity = $\left[\frac{\text{slope reactivation} - \text{slope inactivation}}{\text{slope control}}\right] \times 100$

% unrecoverable activity = 100 – (slope reactivation / slope control) x 100
Figure 3.5. Oxidation of PTP1B with increase in concentration of hydrogen peroxide. PTP1B was incubated with varying [H$_2$O$_2$] for 8 min at 25 °C followed by reactivation in a mixture containing catalase, with (A) and without (B) 50 mM DTT for 20 min. The remaining activity was measured using 10 mM pNPP in assay buffer.

**Hydrogen peroxide mediated oxidation of sulfenyl amide.** An aliquot of thiol-free enzyme [(12.5 µL of a 4 µM solution in 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris, 10 mM DTPA and NP-40 (0.05%, v/v))] was combined with the assay buffer (10 µL) and hydrogen peroxide (2.5 µL of a 500 µM solution) at 25 °C for 35 min (final concentrations: PTP1B, 2 µM; hydrogen peroxide, 50 µM; buffer, 90 mM sodium acetate, 45 mM Bis-Tris, 45 mM Tris, 9 mM DTPA) to obtain sulfenyl amide. To 10 µL of above mixture containing sulfenyl amide was added hydrogen peroxide to a final concentration of 750 µM and the resulting mixture incubated for 8 min at 25 °C. After 8 min, 11 µL of the enzyme solution was reactivated in a buffer (11 µL) containing a mixture of catalase and DTT for 25 min at 25 °C (final concentrations: catalase, 100 units; DTT, 50 mM; buffer, 90 mM sodium acetate, 45 mM Bis-Tris, 45 mM Tris, 9 mM DTPA at pH 7.0). After 25 min, 20 µL from above mixture was added to a cuvette containing pNPP (980 µL) in assay buffer at pH 7.0 (final volume, 1 mL; final concentrations: buffer, 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris, 10 mM DTPA; pNPP, 10 mM). Immediately following the addition of enzyme, the assay was mixed by repeated inversion and the enzyme
catalyzed release of p-nitrophenol monitored at 25 °C by measuring the increase in absorbance at 410 nm. Data points were taken every 2 s for a period of 2 min.

The amount of enzyme present in sulfenyl amide form after 35 min of initial oxidation was determined as mentioned before. The percent recoverable after hydrogen peroxide-mediated oxidation of sulfenyl amide was calculated from the mock reaction containing no hydrogen peroxide. Overall, we obtained 1% of average loss in sulfenyl amide, under the conditions used for measuring the rate constant of the cyclization reaction ($k_3$).

**Figure 3.6.** Oxidation of sulfenyl amide under the extreme oxidation conditions used to measure $k_3$.

**Oxidation of PTP1B in presence of glutathione: measuring the rate constant for the reaction of glutathione with protein sulfenic acid.** An aliquot of thiol-free enzyme [(12.5 µL of a 4 µM solution in 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris, DTPA (10mM) and NP-40 (0.05%, v/v)] was combined with the assay buffer (7.5 µL), hydrogen peroxide (2.5 µL of a 3 mM solution) and varying concentrations (2, 5, 8 and 10 mM) of GSH (2.5 µL of 10x stock) at 25 °C for 8 min (final volume, 25 µL; final concentrations: PTP1B, 2 µM; hydrogen peroxide, 1.5 mM; buffer, 90 mM sodium acetate, 45 mM Bis-Tris, 45 mM Tris, 9 mM DTPA). An aliquot (11 µL) of above mixture was reactivated in an assay buffer (11 µL) containing catalase
(100 units) and DTT (50 mM) for 20 min at 25 °C. 20 μL of this enzyme solution was added to a cuvette containing pNPP (980 μL, 10 mM). Immediately after addition, the assay was mixed by repeated inversions and the enzyme catalyzed release of p-nitrophenol monitored by measuring the increase in absorbance at 410 nm. Data points were taken every 2 s for a period of 2 min.

![Figure 3.7](image.png)

**Figure 3.7.** Oxidation of PTP1B in presence of varying concentrations of GSH. PTP1B incubated with 750 UM H₂O₂ and varying [GSH] for 8 min at 25 °C. An aliquot of oxidized enzyme reactivated in a mixture of catalase and DTT for 20 min before assaying for the remaining activity using 10 mM pNPP.

The % recoverable activities were calculated with respect to a control oxidation performed in a similar way containing no GSH. The calculation was carried out as follows:

\[
\text{% recoverable at specific [GSH]} = \left( \frac{\text{slope after GSH treatment}}{\text{slope of control}} \right) \times 100
\]

\[
\text{% unrecoverable activity} = 100 - \text{% recoverable}.
\]
References


Chapter 4

Chemical Model Studies Predict that “Over-oxidation” of the Active site Cysteine of PTP1B to the Sulfinyl Oxidation State Yields a Thiol Reversible but Hydrolytically Labile form of the enzyme

4.1 Introduction

4.1.1 Background

Hydrogen peroxide acts as a secondary messenger\textsuperscript{1} modulating various signal transduction cascades through the oxidation of cysteine thiol residues.\textsuperscript{2,3} The physiological significance of hydrogen peroxide as a signaling agent gained more attention after characterization of redox regulation of cysteine containing PTPs, as discussed in Chapter 1.\textsuperscript{4} Oxidation of cysteine thiols leads to the formation of a sulfenic acid\textsuperscript{5} that can either be reduced by a proximal cysteine residue or by cellular thiols to form a mixed disulfide intermediate.\textsuperscript{6,7,8} Further reduction of this disulfide by another molecule of cellular thiols or disulfide reductases will regenerate the active cysteine thiol.\textsuperscript{3} The functional properties of a protein can be switched “on” or “off” by this reversible cysteine modification. This process is referred to as redox regulation of protein function (Scheme 4.1).
Typically, as part of a redox regulation mechanism, the intermediate sulfenic acid can be further oxidized to sulfinic and sulfonic acids. Oxidation of the catalytic cysteine residue to higher oxidation states has been observed in various proteins including glyceraldehyde-3-phosphate dehydrogenase,\textsuperscript{9} carbonic anhydrase III,\textsuperscript{10} peroxiredoxins (Prx)\textsuperscript{11} and protein tyrosine phosphatase 1B (PTP1B).\textsuperscript{12,13} Further oxidation of sulfenic acid to sulfinic or sulfonic acid is expected to generate a stable, over-oxidized cysteine residue at the protein backbone.\textsuperscript{14} These higher oxidation states of sulfur often are called “over-oxidized” because they represent an irreversibly inactive form of the enzyme, as they cannot be reactivated by spontaneous or enzyme catalyzed reaction with cellular thiols.\textsuperscript{15} Thus, the over-oxidation of sulfenyl sulfur is considered to be an unwanted detour in a redox cycle (Scheme 4.1).

![Scheme 4.1. Hydrogen peroxide mediated redox switch of cysteine containing enzymes](image)

4.1.2 Over-oxidation to sulfinic acid is reversible in peroxiredoxins (Prxs)

Prx is an antioxidant enzyme that catalyzes the reduction of hydrogen peroxide through reversible oxidation of its catalytic cysteine thiol residues.\textsuperscript{16} During oxidative stress, the sulfenic acid intermediate of Prx is over-oxidized to sulfinic acid (82). This over-oxidation to the sulfinic oxidation state was believed to be a permanent “turn off” of the catalytic activity of Prx.\textsuperscript{17} However, recent studies have identified an eukaryotic enzyme sulfiredoxin (Srx1), that selectively reduces cysteine sulfinic acid in certain isoforms of peroxiredoxins, to regenerate the active thiol form of the enzyme.\textsuperscript{18} This reversible oxidation of sulfenic to sulfinic acid adds
another regulatory step in the usual redox regulation mechanism of Prxs.\textsuperscript{19,20} Nonetheless, the sulfinic acid formed in the active site of other enzymes still remain irreversibly inactivated, as Srx dependent reduction is specific only to Prxs.\textsuperscript{21} However, this reversible over-oxidation underlines the critical role played by sulfinic acid in the redox regulation of Prx (Scheme 4.2).

This is the first known example of a reduction of the over-oxidized sulfinic form of cysteine residue in a protein.

\textbf{Scheme 4.2.} Reversible over-oxidation of sulfinic acid in presence of sulfiredoxin (Srx1)

\textbf{4.1.3 Hydrogen peroxide-mediated oxidation of PTP generates a novel sulfenic oxidation state}

Protein tyrosine phosphatases (PTPs), an important class of cellular signaling enzymes, undergo redox regulation by oxidation of the catalytic cysteine thiol residue.\textsuperscript{3,22} Recent reports suggest a new intermediate in the usual redox regulation mechanism of protein tyrosine phosphatase 1B (PTP1B)\textsuperscript{13} and membrane bound receptor protein tyrosine phosphatase (R-PTP).\textsuperscript{23} In these enzymes, an oxidation of cysteine residue leads to the formation of an intrastrand protein crosslink to generate a sulfenyl amide at the active site. The formation of sulfenyl amide has been discovered by X-ray crystallography during the oxidative inactivation of PTP1B and R-PTP in the presence of hydrogen peroxide. It is important to notice that the sulfenyl amide represent a novel sulfenic oxidation state of the cysteine thiol. Importantly, this
oxidatively inactivated novel sulenic oxidation state can be reactivated by reaction with thiols. However, the functional relevance of this novel sulfenyl amide is not clearly understood.

4.2 Goals of this chapter

In this chapter, we consider the functional role of the novel sulfenyl amide heterocycle, seen at the active site of some PTPs. Sulfenyl amide is generated via an intramolecular cyclization of amide nitrogen onto the sulenic acid. Typically, over-oxidation of sulenic acid to sulfinic acid leaves the enzyme irreversibly inactive. However, it is unclear whether further oxidation of sulfenyl amide, a novel sulenic oxidation state, to its sulfinic state is reversible or irreversible by reaction with thiols (Scheme 4.3). To examine this, the sulfenyl amide, like sulenic acid, is subjected to over-oxidation. For this purpose, we used our recently reported 1,2-benzisothiazolin-3(2H)-one, as an active site mimic of this novel sulfenyl amide. We oxidized the sulfenyl amide to its corresponding sulfinyl and sulfonyl oxidation states. It becomes important to characterize the fundamental, biologically relevant reactions of this new post translationally modified protein structures. Herein, we will discuss the synthesis and chemical properties of the oxidized mimics and their reactions with thiol and water.

Scheme 4.3. Projected over-oxidation of the novel sulfenyl amide during redox regulation
4.3 Properties of 1,2-benzisothiazolin-3(2H)-one 1-oxide

4.3.1 Synthesis of 1,2-benzisothiazolin-3(2H)-one 1-oxide (69)

We synthesized compound 34 through the methods discussed in Chapter 1 and also by the reaction of 1,2-benzisothiazolin-3(2H)-one with iodoethyl acetate in presence of triethyl amine (TEA). The resulting compound was then oxidized using dimethyl dioxirane (DMD) to obtain the desired sulfinyl amide (69) in over 90% yield as shown in Scheme 4.4.

Scheme 4.4. Synthesis of 1,2-benzisothiazolin-3(2H)-one 1-oxide

4.3.2 Reaction of compound 69 with thiol

With the desired over-oxidized enzyme mimic (69) in hand, we explored its reaction with thiol. For this, we incubated compound 69 (10 mM) under physiologically relevant (sodium phosphate buffer, 300 mM, pH 7.0) conditions with 15 equiv. of 2-mercaptoethanol (150 mM) at 25 °C. We found that compound 69 resulted in the production of the corresponding ring-opened thiol product (54) in 85% yield as shown in Scheme 4.5. In addition, similar yields of compound 54 were obtained when the reaction was conducted in methylene chloride in presence of TEA.

Scheme 4.5. Thiol mediated conversion of compound 69 to the active form of the enzyme

The identity of the thiol product (54) was further confirmed by in situ alkylation of 54 with methyl iodide under aqueous buffer conditions to obtain the S-methylated product (87).
Additionally, the isolated product (54) was treated with excess methyl iodide in methylene chloride, in the presence of TEA to yield the corresponding S-methylated product (87) similar to the one obtained under the aqueous buffer conditions. Overall, these results suggest that the 1,2-benzisothiazolin-3(2H)-one 1-oxide heterocycle (69) can undergo a facile ring opening reaction in presence of 2-mercaptoethanol to generate the corresponding thiol product (54). This is very intriguing as it indicates that an over-oxidation of the novel sulfenyl amide to its sulfinic oxidation state is in fact reversibly active in presence of thiols.

![Scheme 4.6. Derivatization of product 54 using methyl iodide](image)

We next focused on characterizing the intermediates in the thiol-mediated “reactivation” of the 1,2-benzisothiazolin-3(2H)-one 1-oxide compound (69). For this purpose, we incubated 69 in 50 mM sodium phosphate buffer, pH 7.0 containing 30% acetonitrile (v/v) at 25 °C with 10 equiv. 2-mercaptoethanol. The reaction course was monitored by reverse phase HPLC. We found that compound 69 was converted to the thiol product 54 in less than a minute. A transient intermediate that was completely converted to the product 54 within 5 min was observed. We anticipated that this might be the mixed-disulfide conjugate of the benzisothiazolidinone with 2-mercaptoethanol (55). Indeed consistent with our expectations, the intermediate was identified to be compound 55 (having a mass of 315 a.m.u) by the LC/MS experiment.

4.3.3 Possible mechanism for the thiolysis reaction of compound 69

Base upon these results, we envisioned a mechanism for the thiolysis of compound 69 involving four equiv. of 2-mercaptoethanol. The initial attack of thiol on the sulfinyl sulfur is
expected\textsuperscript{24} to yield an unstable thiosulfinate intermediate (88), which upon attack by a second molecule of thiol will generate the sulfenic acid (33). The sulfenic acid is then anticipated to react with excess thiol to yield mixed disulfide intermediate (55). Finally, compound 55 is reduced by another molecule of thiol to yield the mercapto product 54 (Scheme 4.7). A similar mechanism involving an initial attack of thiol on the sulfinyl sulfur was proposed for some aliphatic sulfinamides.\textsuperscript{25,26}

Scheme 4.7. Proposed mechanism for the thiol mediated reactivation of 69

In order to further confirm the intermediacy of the mixed disulfide compound (55) during the thiol mediated reactivation of 69, we performed the experiment with less than ten equivalents of thiol under the aqueous buffer conditions. Our central goal is to examine whether we can cleanly detect the mixed disulfide (55). For this purpose, we incubated 69 in 50 mM sodium phosphate buffer containing 2 equiv. of 2-mercaptoethanol and monitored the reaction by HPLC. We observed the formation of the mixed disulfide intermediate (55) as a major product along with the unreacted starting material (69) and the mercapto product (54). In fact, mixed disulfide (55) was stable for a period of over 6 h. However, in presence of three equiv. of thiol, we observed the mercapto compound (54) as a major product along with the formation of the mixed disulfide intermediate (55). These results indicate that the reactivation of 69 proceeds via a
mixed disulfide intermediate (55). A similar observation has previously been made by Clarke and coworkers for the thiol mediated conversion of aliphatic thiosulfinates to its corresponding thiol product (Scheme 4.8).  

\[
\begin{align*}
\text{Ph-S}^\text{N} + \text{PhSH} & \rightarrow \text{Ph-S}^\wedge\text{Ph} + \text{PhNH}_2 \\
\text{Ph-S}^\wedge\text{Ph} + \text{RSH} & \rightarrow [\text{PhSOH}] + \text{PhSSR} \\
[\text{PhSOH}] + \text{RSH} & \rightarrow \text{PhSSR} + \text{H}_2\text{O}
\end{align*}
\]

**Scheme 4.8. Reaction of thiol with sulfinamide**

Similar to thiolysis, hydrolysis of sulfinamides is well documented in the literature. In the following section, we will discuss the possible products resulting from the hydrolysis of the compound 69 under aqueous buffer conditions.

### 4.3.4 Hydrolysis of 1,2-benzisothiazolin-3(2H)-one 1-oxide

Based upon literature precedents, we anticipated a reaction of 69 with water would generate the corresponding sulfonic acid (97). If so, the hydrolysis product would represent an irreversibly inactivated form of the enzyme. Consistent with our expectations, incubation of 69 in 50 mM sodium phosphate buffer (pH 7.0) at 25 °C for 12 h in the absence of thiol, resulted in the production of sulfonic acid 97 in 88% yield. The sulfonic acid was characterized as its methyl ester derivative prepared via reaction with methyl iodide as shown in Scheme 4.9. This suggests that the over-oxidized form of sulfenyl amide (85) is hydrolytically labile and that hydrolysis leads to a stable and irreversibly inactivated sulfonic acid (59). In contrast, we found the parent benzisothiazolin-3(2H)-one heterocycle (34) to be very stable under similar aqueous buffer conditions (50 mM sodium phosphate, pH 7.0) for over 48 h.
However, as mentioned in the previous section, reaction of compound 69 with thiols will regenerate the active thiol mimic (54) of the enzyme. Nonetheless, this inspired us to measure the rates of these two reactions under similar conditions. This is important, because the relative rates of hydrolysis and thiolysis reactions will determine whether the over-oxidized intermediate (69) can be regenerated to the active form of the enzyme. The measurement of the rate constants for hydrolysis and thiolysis reactions will be discussed in the following section.

### 4.4 Measuring rate constants for the reaction of 69 with water and thiol under aqueous buffer conditions

#### 4.4.1 Rate constant for the reaction of 69 with water

In order to gain information regarding the rates of the competing hydrolysis and thiolysis reactions, we incubated compound 69 in 50 mM sodium phosphate buffer mixture both in the presence and absence of 2-mercapto ethanol. We monitored the disappearance of the starting 69 using reverse phase HPLC. A resulting semi-log plot of the remaining starting material (69) versus time (t) yielded the rate constant of the reaction (k) as a slope of the plot. Under the conditions of 50 mM sodium phosphate buffer (pH 7.0) containing 50% acetonitrile by volume (final concentrations: 69, 50 µM; sodium phosphate buffer, 50 mM, pH 7.0; acetonitrile, 50% by volume) the pseudo first order rate constant for the hydrolysis of compound 69 was measured to be $0.027 \pm 0.001 \text{ min}^{-1}$, which corresponds to a half life of \(~26 \text{ min}\) (Figure 4.1).
Figure 4.1. A representative plot of ln(a/a₀) vs time for the hydrolysis of 69

4.4.2 Rate constant for the reaction of 69 with thiol

After measuring the rate constant for the hydrolysis, we performed the thiolysis of 69 in presence of 20 equiv. of 2-mercaptoethanol, under conditions similar to the hydrolysis reaction (final concentrations: 69, 50 µM; 2-ME, 1 mM; sodium phosphate buffer, 50 mM, pH 7.0; acetonitrile, 50% by volume). We observed that the reaction of 69 with thiol proceeded rapidly to yield an approximate second order rate constant of 5.5 ± 0.2 M⁻¹ s⁻¹. This corresponds to a half life of 2 min when the concentration of thiol is 1 mM. These results show that the reaction of compound 69 with thiol occurs much faster compared to the hydrolysis reaction. For example, in presence of 5 mM 2-mercaptoethanol, the thiolysis reaction is expected to be 60 fold faster than the corresponding hydrolysis. Overall it is evident from our findings that, if steric access for thiol and water is equal, the thiolysis of 69 vastly predominates over hydrolysis under physiologically reducing conditions.
After determining the properties of the sulfinic oxidation state (85) of the novel sulfenyl amide, we examined the properties of the sulfonic oxidation state of the isothiazolidinone heterocycle (86). In the following section, we will look into the synthesis and stability of the benzisothiazolidinone 1,1-dioxide (99), an over-oxidized mimic of the novel sulfenyl amide form of the enzyme.

4.5 Properties of 1,2-benzisothiazolin-3(2H)-one 1,1-dioxide

4.5.1 Synthesis of 1,2-benzisothiazolin-3(2H)-one 1,1-dioxide (99)

Compound 99 was obtained in over 90% yield by oxidation of the 1,2-benzisothiazolin-3(2H)-one heterocycle in presence of excess DMD, as shown in Scheme 4.10.

With the over-oxidized sulfonic form of the sulfenyl amide in hand, we set out to investigate its stability under aqueous buffer conditions both in the presence and absence of thiol.
4.5.2 Stability of 99 in presence of water and thiol

Compound 99 was incubated in 50 mM sodium phosphate buffer containing 40% acetonitrile by volume and the disappearance of 99 was monitored over time by reverse phase HPLC. We found that 99 was very stable in aqueous buffer for a period of over 48 h. This is not surprising, given that the sulfonamides are known to be highly resistant to hydrolysis. Similarly, compound X was found to be stable when incubated in sodium phosphate buffer in presence of 10 equiv. 2-mercaptoethanol, for 48 h. These results reveal that the over-oxidized sulfonic form of the sulfenyl amide (99) is extremely unreactive towards water or thiol (Scheme 4.11). This predicts that sulfonyl oxidation state, if formed, would be irreversibly inactive form of the enzyme. The stability of compound 99 in water and thiol was further confirmed by NMR, thin layer chromatography (TLC) and LC/MS experiments.

4.6 Discussion

In this chapter, we explored the chemical properties of “over-oxidized” forms of sulfenyl amide through simple chemical models. Typically, over oxidation of sulfenic acid intermediate to its corresponding sulfinic or sulfonic acids leads to the generation of an irreversibly inactive (dead) enzyme. In case of PTP1B, the intermediate sulfenic acid undergoes a novel chemical transformation to generate an isothiazolidinone heterocycle (sulfenyl amide) without changing the oxidation state of the sulfur. However the functional properties of the intermediates are not clearly understood, when sulfenyl amide is subjected to over-oxidation. Hence we wanted to
investigate the consequences of over-oxidation of this novel sulfenyl amide. For this purpose, we used a small molecule model of the PTP1B active site thiol/cysteine (Chapter 1), to understand the consequences of further oxidation to the sulfenyl amide moiety seen at the active sites of PTP1B and R-PTP.

We oxidized 1,2-benzisothiazolin-3(2H)-one (34) using DMD, to obtain the corresponding over oxidized sulfanyl (69) and sulfonyl (99) oxidation states. Treatment of the sulfinic oxidation state (69) in presence of excess 2-mercaptoethanol under aqueous buffer conditions resulted in the complete conversion of 69 to the mercapto product (54). This offers prediction that sulfenyl amide can be returned to active enzyme via reaction with thiols. However, compound 69 also undergoes hydrolysis under aqueous buffer conditions to generate the corresponding sulfinic acid (97), which represents an irreversibly inactive form of the enzyme. Under physiological concentrations of thiol, we estimated that the thiolytic reaction will vastly predominate over hydrolysis and expected to regenerate the active form of the enzyme, provided steric access of thiol and water are equal.

Taken together, our results suggest that a further oxidation of the novel sulfenyl amide to its sulfinic form is still reversible in presence of thiols. This reversible “over-oxidation” of sulfenyl amide may indicate a novel protection mechanism employed by Nature against the irreversible over-oxidation of PTP1B, as shown in Scheme 4.12.

Scheme 4.12. Chemical model studies suggesting a “reversible over-oxidation” of the novel sulfenyl amide heterocycle
Recent X-ray crystal structure analysis of hydrogen peroxide mediated inactivation of PTPs revealed the formation of sulfenyl amide after 40 min of incubation. Further oxidation of crystals of PTP1B containing sulfenyl amide resulted in the generation of an irreversibly over-oxidized sulfinic and sulfonic acid forms of the enzyme. However, the chemical mechanisms for the possible generation of the sulfinic and sulfonic acids from the intermediate sulfenyl amide are not clearly understood. Our findings herein, suggest a likely mechanism involving a sequence of oxidation followed by hydrolysis of the intermediate sulfenyl amide to generate the irreversibly inactive sulfinic and sulfonic acids, as shown in Scheme 4.13.

![Scheme 4.13](image1)

**Scheme 4.13.** A sequence of oxidation followed by hydrolysis towards generation of compound 97 & 100

The proposed mechanism involving hydrolysis of the sulfenyl amide (34) to generate the sulenic acid, followed by further oxidation (Scheme 4.14), is rendered unlikely based on our model studies.

![Scheme 4.14](image2)

**Scheme 4.14.** Mechanism proposed by Barford et al. for the generation of sulfinic acid from sulfenyl amide

We also explored the properties of the over-oxidized sulfonic form of the sulfenyl amide. Incubation of 1,2-benzisothiazolin-3(2H)-one 1,1-dioxide (99) under aqueous buffer conditions in presence and absence of thiol revealed that compound 99 is highly resistant to hydrolysis or thiolysis. This finding indicates that the over-oxidation of the intermediate sulfenyl amide to its
sulfonic state results in an irreversibly inactive (dead) form of the enzyme. However, according to our model studies, formation of this sulfonic state (99) of sulfenyl amide seems less likely under physiological conditions, as several attempts to oxidize compound 34 under aqueous buffer conditions in presence of hydrogen peroxide failed to produce 99. This is because of the fact that the hydrolysis of the intermediate sulfinyl amide (69) is faster compared to its further oxidation to generate 99 as shown in Scheme 4.15. Nonetheless, formation of this irreversibly inactive sulfonic form of the sulfenyl amide at the active site of the enzyme cannot be ruled out altogether.

Scheme 4.15. Oxidation of 34 under aqueous buffer conditions

Overall, the results from our chemical model studies predict some likely roles for the over-oxidized sulfinyl and sulfonyl amide intermediates (85 and 86) in the redox regulation of PTP1B (Scheme 4.16). It would be interesting to probe the presence and properties of these novel intermediates at the enzyme backbone. In addition, our findings may provide scope for potential research in multidisciplinary fields involving toxicology, medicinal chemistry, drug discovery and structural biology.
4.7 Experimental methods

Material and Methods

Reagents were of highest purity available and were used without further purification unless otherwise noted. Materials were purchased from the following suppliers: HPLC grade solvents, Fisher; silica gel 60 (0.04-0.063 mm pore size) for column chromatography, Merck; all other chemicals were purchased from Aldrich Chemical Company. Thin layer chromatography (TLC) was performed on plates from Aldrich chemical company, coated with general purpose silica containing UV$_{254}$ fluorophore and compounds were visualized by illumination of the plates with short-wave (254 nm) UV light. Water was distilled, deionized and glass redistilled. The oxidizing agent, dimethyl dioxirane (DMD), was freshly synthesized according to reported methods$^{1}$ and stored in the freezer. High resolution mass spectrometry was performed at the facility in the University of Illinois–Urbana Champaigne. LC/MS analysis was performed by Dr. Nathan Leigh at the University of Missouri-Columbia mass spectrometry facility, on a Thermo-Finnigan LC system connected to an integrated Thermo-Finnigan TSQ7000 triple-quadrupole
instrument with ESI/API2 source and performance Pack (ThermoFinnigan, San Jose, CA). NMR spectra were recorded using Bruker DRX 300, ARX 250 or DRX 500 MHz instruments at the University of Missouri-Columbia. HPLC spectra of compounds were recorded directly without an internal standard. The standard error in the HPLC peak area for 3 injections was estimated to be 2 ± 0.5%.

Synthesis of 1,2-benzisothiazol-3(2H)-one 1-oxide, 69. To a stirred solution of 34 (25 mg, 0.105 mmol) in dry acetone (2 mL) in an ice bath was added freshly prepared dimethyl dioxirane (DMD) solution. The disappearance of 34 was constantly monitored by TLC (3:2 hexane/EtOAc). The reaction was complete in about 5 min at which time the acetone and excess DMD was evaporated to yield colorless oil (25 mg, 94%). \( R_f = 0.38 \) (3:2 hexane/EtOAc). \(^1\)H-NMR (CDCl\(_3\), 300 MHz) δ 1.30 (3H, t, J = 7 Hz), 4.25 (2H, q, J = 7.2 Hz), 4.37 (1H, d, J = 18 Hz), 4.80 (1H, d, J = 18 Hz), 7.81 (2H, m), 7.95 (1H, m); 8.04 (1H, m); \(^{13}\)C-NMR (CDCl\(_3\), 75.47 MHz) δ 167.64, 165.18, 145.90, 134.45, 133.25, 127.55, 126.36, 125.25, 62.03, 41.24, 14.01. HRMS (ESI) calcd for C\(_{11}\)H\(_{12}\)NO\(_4\)S \([\text{M+H}]^+\) 254.0487, found 254.0493.

Generation of 2-(2-mercaptobenzamideo)acetate (54) by reaction of 69 with 2-mercapto ethanol under aqueous buffer conditions. To a stirred solution of 69 (15 mg, 0.059 mmol) in acetonitrile (178 mL) was added a mixture of sodium phosphate buffer (3.56 mL, 500 mM, pH 7.0), water (538 µL) and 2-mercapto ethanol (62.2 µL, 14.3 M). The resulting colorless solution was stirred at 25 °C (final concentrations: 69, 10 mM; buffer, 300 mM, pH 7.0; thiol, 150 mM; acetonitrile, 30% by volume). The starting material disappeared in less than 5 min as indicated by the TLC and the reaction mixture was let stir for further 2 h. It was then acidified to pH 2 using dilute hydrochloric acid, extracted with diethyl ether (3 x 5 mL), and washed with water. The combined organic extracts were dried over anhydrous sodium sulfate, filtered and
evaporated to yield a colorless oil that was purified by flash column chromatography (7:3 EtOAc/hexane) to obtain 54 (11 mg, 80%). Rf = 0.61 (7:3 EtOAc/hexane). \(^1\)H-NMR (CDCl\(_3\), 300 MHz) \(\delta\) 1.32 (3H, t, J = 7 Hz), 4.24 (4H, m), 4.74 (1H, s), 6.57 (1H, s, broad), 7.17 (1H, m), 7.31 (2H, m), 7.53 (1H, dd, J = 7.6 Hz, 1.0 Hz); \(^1^3\)C-NMR (CDCl\(_3\), 75.4 MHz) \(\delta\) 169.79, 168.45, 133.37, 132.16, 131.07, 130.94, 128.12, 125.21, 61.76, 41.83, 14.14. HRMS (ESI) calcd for C\(_{11}\)H\(_{14}\)NO\(_3\)S [M+H]+ 240.0694, found 240.0706.

**Generation of Ethyl 2-(2-(methoxysulfinyl)benzamido)acetate (98) upon reaction of 69 in aqueous buffer followed by the treatment with excess methyl iodide.** To a stirred solution of 69 (20 mg, 0.08 mmol) in a mixture of water (632.4 µL) and acetonitrile (948.6 µL) was added sodium phosphate buffer (1581 µL) and the resulting solution stirred at 25 °C for 12 h (final concentrations: 69, 25 mM; buffer, 250 mM, pH 7.0; acetonitrile, 30% by volume). To this was added methyl iodide (1 mL) and the resulting solution was stirred at 25 °C for further 24 h. The reaction mixture was then extracted with ethyl acetate (3 x 6 mL) and the combined organic extracts were dried over anhydrous sodium sulfate, filtered and evaporated to yield an yellow oil, which was purified by a flash column chromatography (3:2 hexane/EtOAc) to yield 98 as a white solid (20 mg, 88.8%). Rf = 0.25 (3:2 hexane/EtOAc). \(^1\)H-NMR (CDCl\(_3\), 250 MHz) \(\delta\) 1.32 (3H, t, J = 7 Hz), 3.36 (3H, s), 4.25 (4H, m), 6.63 (1H, s), 7.65 (3H, m), 8.11 (1H, m); \(^1^3\)C-NMR (CDCl\(_3\), 62.9 MHz) \(\delta\) 169.37, 168.05, 138.29, 136.39, 133.72, 130.53, 129.66, 128.61, 61.75, 45.16, 41.95, 14.09. HRMS (ESI) calcd for C\(_{12}\)H\(_{16}\)NO\(_5\)S [M+H]+ 286.0749, found 286.0755.

**Determination of the rate constant for the decomposition of 69 in aqueous buffer.** Compound 69 (2.5 µL of a 10 mM stock in CH\(_3\)CN) was incubated at 25 °C in a mixture of sodium phosphate buffer (50 µL, 500 mM, pH 7.0), water (200 µL) and acetonitrile (247.5 µL). The mixture (final concentrations: 69, 50 µM; buffer, 50 mM, pH 7.0; acetonitrile, 50% by
volume) was vortex mixed and analyzed by reverse phase HPLC at regular time intervals. The disappearance of 69 was monitored by HPLC using a reverse phase C-18 Varian Microsorb-MV column (100 Å sphere size, 5 µm pore size, 25 cm length, 4.6 mm i.d.), with a gradient elution system containing water with 0.5% acetic acid v/v and acetonitrile as mobile phase at a flow rate of 0.8 mL/min (injection volume = 40 µL, detection wavelength = 254 nm). The compound was eluted with an initial linear gradient of 70:30 aqueous/acetonitrile for 4 min followed by an increase to 50:50 aqueous/acetonitrile over 8 min. The method was continued as a linear gradient over 15 min at 50/50 aqueous/acetonitrile and then ramped down to 70:30 aqueous/acetonitrile by 18 min and continued till 22 min as the column was equilibrated before the next run. The kinetic plot revealed a pseudo-first order rate constant of 0.0272 min⁻¹, suggesting an apparent half life of 25.5 min for the decomposition of 69 under aqueous buffer conditions. The reaction was repeated twice and the rate constant reported is the average of two experiments.

**Characterization of the reactivity of 69 in presence of excess 2-mercaptoethanol in aqueous buffer condition:** Compound 69 (2.5 µL of a 10 mM stock in CH₃CN) was added at the end to a mixture containing sodium phosphate buffer (50 µL, 500 mM, pH 7.0), water (175 µL), 2-mercapto ethanol (25 µL of a 10 mM stock in water) and acetonitrile (247.5 µL), incubated at 25 °C. The mixture (final concentrations: 69, 50 µM; buffer, 50 mM, pH 7.0; thiol, 500 µM; acetonitrile, 50% by volume) was vortex mixed and analyzed by reverse phase HPLC at regular time intervals. The disappearance of 69 was monitored by HPLC using a reverse phase C-18 Varian Microsorb-MV column (100 Å sphere size, 5 µm pore size, 25 cm length, 4.6 mm i.d.), with a gradient elution system containing water with 0.5% acetic acid v/v and acetonitrile as mobile phase at a flow rate of 0.8 mL/min (injection volume = 40 µL, detection wavelength =
254 nm) using the same method as described in the previous experiment. Compound 69 degraded rapidly to yield 54. The kinetic plot revealed a second-order rate constant of 5.5 M\(^{-1}\) s\(^{-1}\), suggesting an apparent half life of 2 min (when thiol = 1 mM) for the thiolysis of 69 under aqueous buffer conditions. An intermediate peak at 10.1 min that appeared after 1 min incubation gave a mass of 316 a.m.u corresponding to [M+H]\(^+\) of a mixed disulfide with 2-mercaptoethanol (55).

**Synthesis of 1,2-benzisothiazol-3(2H)-one 1,1-dioxide, 99.** To a stirred solution of 34 (20 mg, 0.085 mmol) in dry acetone (2 mL) in an ice bath was added freshly prepared dimethyl dioxirane (DMD) solution. The disappearance of 34 was constantly monitored by TLC (3:2 hexane/EtOAc). After 12 h of stirring at 25 °C, excess DMD was evaporated to yield colorless oil (22 mg, 98%). \(R_f = 0.43\) (3:2 hexane/EtOAc). \(^1\)H-NMR (CDCl\(_3\), 300 MHz) \(\delta\) 1.30 (3H, t, \(J = \) 7 Hz), 4.27 (2H, q, \(J = 7.2\) Hz), 4.45 (2H, s), 7.90 (3H, m), 8.10 (1H, dd, \(J = 7.65\) Hz, 0.9 Hz); \(^1\)C-NMR (CDCl\(_3\), 75.47 MHz) \(\delta\) 165.85, 158.77, 137.78, 135.05, 134.47, 127.10, 125.47, 121.21, 62.27, 39.11, 14.04. HRMS (ESI) calcd for C\(_{11}\)H\(_{12}\)NO\(_5\)S [M+H]\(^+\) 270.0436, found 270.0446.

**Characterization of the reaction of 99 under aqueous buffer conditions in the presence and absence of 2-mercaptoethanol.**

**Reaction of 99 in aqueous buffer in the absence of 2-mercapto ethanol:** Compound 99 (10µL of a 5 mM stock in CH\(_3\)CN) was added at the end to a mixture containing sodium phosphate buffer (100 µL, 500 mM, pH 7.0), water (500 µL), and acetonitrile (390 µL). The solution (final concentrations: 99, 50 µM; buffer, 50 mM, pH 7.0; acetonitrile, 40% by volume) was vortex mixed and analyzed by reverse phase HPLC at regular time intervals at 25 °C. The
disappearance of 99 was monitored by the HPLC using a similar method mentioned above. Compound 99 appears to be very stable under the aqueous buffer conditions as only 3% of the initial starting material was found to degrade over an 8 h incubation period. The identity of 99 was further confirmed by an LC/MS experiment under these conditions.

![Figure 4.3](image.png)

**Figure 4.3.** A plot of HPLC peak area vs time for the hydrolytic decomposition of 99.

**Reaction of 99 in aqueous buffer in the presence of 2-mercaptoethanol:** Compound 99 (10 µL of a 5 mM stock in CH₃CN) was added at the end to a mixture containing sodium phosphate buffer (100 µL, 500 mM, pH 7.0), 2-mercapto ethanol (50 µL of a 10 mM stock in H₂O), water (450 µL), and acetonitrile (390 µL). The solution (final concentrations: 99, 50 µM; buffer, 50 mM, pH 7.0; thiol, 500 µM; acetonitrile, 40% by volume) was vortex mixed and analyzed by reverse phase HPLC at regular time intervals at 25 °C. The disappearance of 99 was monitored by the HPLC using a similar method mentioned in the previous experiment. Only 2% of compound 99 degraded over a 6 h incubation period. The identity of 99 was further confirmed by an LC/MS experiment under these conditions. This suggests that the reaction of 99 with thiol is very slow compared to the reaction of 69 and thiol under similar conditions.
Verifying the stability of 99 in presence and absence of thiol by thin layer chromatography and NMR spectroscopy. 9 mg of compound 99 was dissolved in a mixture of sodium phosphate buffer (474 µL, 500 mM, pH 7.0), deuterium oxide (191 µL) and acetonitrile (287 µL). The mixture (final concentrations: 99, 35 mM; buffer, 250 mM, pH 7.0; acetonitrile, 30% by volume) was vortex mixed and taken in a NMR tube. The NMR spectrum was recorded in a Bruker 500 MHz instrument at 15 min, 24 h and 36 h after the initiation of the experiment. The reaction mixture was also monitored by TLC for the disappearance of the starting 99 using a 3:2 (hexane/EtOAc) solvent mixture.

Similarly, 14 mg of 99 was dissolved in sodium phosphate buffer (1248 µL, 500 mM, pH 7.0), deuterium oxide (172 µL) and acetonitrile (624 µL). The reaction was initiated by the addition of 2-mercapto ethanol (36.4 µL) and the resulting mixture (final concentrations: X, 25 mM; buffer, 300 mM; thiol, 250 mM; acetonitrile, 30% by volume) was vortex mixed and taken in a NMR tube. The NMR spectrum was recorded in a Bruker 500 MHz instrument at 15 min, 24 h and 36 h after the addition of 2-mercapto ethanol. The reaction was simultaneously monitored by TLC for the disappearance of the starting 99 using a 3:2 (hexane/EtOAc) solvent mixture.
References


II spot in the oxon of amide-diester protected w/ DMD. [14mg]
1 spot in the Rf of cleaner protected amide # E057. I think the top spot and second major product on tlc.

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Chapter 5

Evidence for the Presence of a Thiol Reversible but Hydrolytically Labile Intermediate during the Oxidative Inactivation of PTP1B

5.1 Introduction

Protein tyrosine phosphatase 1B (PTP1B) undergoes oxidative inactivation in presence of hydrogen peroxide to generate a novel sulfenyl amide intermediate via a cysteine sulfinic acid.\(^1,2,3\) The presence of sulfenyl amide intermediate has been characterized well by X-ray crystal structure that shows formation of a five membered isothiazolidinone heterocycle (sulfinyl amide) upon incubation of crystals of PTP1B in the presence of hydrogen peroxide.\(^3\) Further incubation of PTP1B crystals containing sulfinyl amide under oxidative conditions resulted in the formation of over-oxidized sulfinic and sulfonic acid forms of the enzyme. A similar observation has recently been made at the active site of a membrane bound receptor protein tyrosine phosphatase (R-PTP).\(^4\) The chemical mechanisms leading to the formation of the over-
oxidized forms of the enzyme from the novel sulfenyl amide is not clearly understood (Figure 5.1).

![Figure 5.1](image)

**Figure 5.1.** Electron density map of RPTPα during oxidation. a) sulfenyl amide after 90 min. of incubation b) oxidized to SO$_2$ upon 16 h incubation in H$_2$O$_2$. (borrowed from Barford et al. *Biochemistry* 2007, 46, 709)

However, recent findings from our chemical model studies (Chapter 4) have revealed the potential formation of sulfinyl amide (isothiazolidinone 1-oxide) that can undergo facile hydrolysis to generate the corresponding sulfenic acid, as shown in Scheme 5.1. Herein, we wanted to extend the knowledge gained from our model studies and investigate the possible formation of this novel sulfinyl amide heterocycle in the backbone of the enzyme PTP1B.

![Scheme 5.1](image)

**Scheme 5.1.** Generation of irreversibly over-oxidized forms of the enzyme from sulfenyl amide

### 5.2 Goals of this Chapter

Our central goal of this chapter is to identify the presence of sulfinyl amide at the enzyme active site and to understand some of the properties of this novel heterocycle, as outlined below:
1) To investigate the presence of a thiol reversible but hydrolytically unstable species during the oxidative inactivation of PTP1B

2) To measure the rate constant for the hydrolysis of sulfinyl amide formed at the enzyme backbone

3) To measure the rate constant for the hydrogen peroxide mediated oxidation of sulfenyl amide

5.3 Determining the generation of sulfinyl amide at the active site of enzyme PTP1B

We set out to examine if the sulfenyl amide formed at the active site of the enzyme can get oxidized to produce the sulfinyl amide. For this, we utilized the inherent chemical properties of the sulfinic form of isothiazolidinone ring (69), to distinguish sulfinyl amide (85) from the sulfenyl amide (6) form of the enzyme. Our chemical model studies suggest that the benzisothiazolidinone 1-oxide (69), an inactive sulfinyl amide mimic of the enzyme, can be reactivated by reaction with thiols. At the same time, 69 can undergo facile hydrolysis to generate the sulfinic acid mimic of the enzyme, whose activity is unrecoverable by reaction with thiols. However, thiolysis of sulfinyl amide vastly predominates over the hydrolysis reaction under any given conditions. On the other hand, the sulfenyl amide mimic of the enzyme, 1,2-benzisothiazolin-3(2H)-one (34) is highly resistant to hydrolysis. We utilized this difference in reactivity of sulfinyl amide towards water and thiol, to identify its potential formation in the active site of PTP1B, as shown in Scheme 5.2. The experimental strategy towards measuring the sulfinyl amide at the active site of PTP1B will be discussed in the following paragraphs.
Scheme 5.2. The difference in reactivity of sulfinyl amide towards water and thiol

5.3.1 Experimental strategy to measure sulfinyl amide at the active site of PTP1B

It is known that PTP1B undergoes oxidation in the presence of hydrogen peroxide to generate sulfenyl amide.\textsuperscript{1,2,3} We anticipated the oxidation of PTP1B to generate a mixture of sulfenyl amide, sulfinyl amide and sulfinic/sulfonic acids. Herein, we report an indirect method to detect the formation of a novel sulfinyl amide during the oxidative inactivation of PTP1B. Typically, after an initial oxidation, reactivation of the oxidized mixture in the presence of catalase and DTT will lead to a complete recovery of the reversibly oxidized forms (both sulfenyl and sulfinyl amide) of the enzyme (path A, Figure 5.2).\textsuperscript{8} With the hydrolytic instability of sulfinyl amide in mind, we introduced a step in which the oxidized forms were allowed to hydrolyze in the absence of DTT. At this stage, the sulfinyl amide (85) is expected to undergo hydrolysis to yield the unrecoverable sulfinic acid (59), while sulfenyl amide (6) remains unreactive (path B, Figure 5.2). This was followed by a treatment with DTT to reactivate the sulfenyl amide (6), and the remaining activities for both pathways were measured using a substrate p-nitrophenyl phosphate (pNPP). The presence of sulfinyl amide (85) is indicated by a decrease in remaining activity in the hydrolysis pathway compared to reactivation. This loss of activity is expected to have come from the hydrolysis of thiol recoverable sulfinyl amide. Finally, a comparison of the difference in % recoverable activities with a control reaction with no hydrogen peroxide will provide a measure of sulfinyl amide present. In the following paragraph
we will explain the terms reactivation and hydrolysis, which will be used frequently in this chapter.

**Reactivation:** In this step, the oxidized forms of enzyme are treated in an assay buffer (50 mM Bis-Tris, 50 mM Tris and 100 mM sodiumacetate, pH 7.0) containing a mixture of catalase and 50 mM DTT for 35 min. Catalase is expected to quench the excess hydrogen peroxide and prevent further oxidation during the incubation period while DTT is added to reactivation the reversibly oxidized forms of the enzyme (sulfenyl and sulfinyl amide forms). The over-oxidized sulfinic and sulfonic acids remain unreactive after this step.9

**Hydrolysis:** In this step, the oxidized forms of enzyme are treated in an assay buffer containing catalase alone for 20 min. Sulfinyl amide (85) is anticipated to undergo selective hydrolysis to generate the unrecoverable sulfinic acid (59). This step was followed by a treatment with 50 mM DTT for 15 min to convert the remaining sulfenyl amide (6) back into the active thiol form of the enzyme.

With this plan, we set out to verify the formation of sulfinyl amide during the oxidative inactivation of PTP1B.
Figure 5.2. A schematic diagram showing the experimental strategy towards identifying sulfinyl amide (85) at the active site of PTP1B
5.4 Evidence for a thiol reversible but hydrolytically labile intermediate during the oxidative inactivation of PTP1B

Our goal is to examine whether the oxidation of PTP1B generates an intermediate whose activity is recoverable by reaction with thiol and unrecoverable upon hydrolysis in aqueous buffer. For this purpose, we incubated PTP1B with 250 μM hydrogen peroxide for 15 min in an assay buffer mixture. An aliquot from this mixture was reactivated for 35 min followed by measuring the remaining activity using a standard phosphatase substrate p-nitrophosphoryl phosphate (pNPP). Similarly, another aliquot of the oxidized enzyme was allowed to hydrolyze for 20 min (catalase was added to prevent further oxidation) followed by the incubation in DTT for 15 min, before measuring the remaining activity using pNPP, as shown in figure 5.3. The difference in % recoverable activity between reactivation and hydrolysis yielded the amount of hydrolytically labile form (sulfinyl amide) of the enzyme.

![Figure 5.3](image)

Figure 5.3. Strategy to measure the thiol reversible and hydrolytically labile intermediate upon oxidation of PTP1B

A plot of remaining activity, measured as an increase in absorbance of the enzyme catalyzed release of p-nitrophenol (at 410 nm), over time (Figure 5.4) clearly showed a significant decrease in the activity of PTP1B between hydrolysis and reactivation.
A resulting plot of % recoverable activity for reactivation and hydrolysis reactions showed a 20% decrease in recoverable activity due to hydrolysis (Figure 5.5). The loss in enzyme activity can be envisioned to have come from a hydrolytically labile intermediate. We know from our model studies that the other oxidized forms of the enzyme (sulfenyl amide and sulfinic acids) are stable to hydrolysis for the given incubation time (20 min). Thus our results clearly point toward the presence of a thiol reversible and hydrolytically labile intermediate, which we project to be the novel over-oxidized sulfinyl amide form of the enzyme.

This new method has provided us with an advantage of being able to measure the amounts of various oxidized forms of the enzyme present at any specific hydrogen peroxide

Figure 5.4. A plot showing the remaining activity of oxidized PTP1B after reactivation and hydrolysis

Figure 5.5. Decrease in recoverable activity upon hydrolysis of the oxidized PTP1B
concentration. For example, the % recoverable activity after hydrolysis gives a measure of the amount of sulfenyl amide present. Similarly, the difference in the recoverable activities between hydrolysis and reactivation reactions will provide the % of sulfinyl amide form of the enzyme. With this method in hand, we wanted to examine whether the observed decrease in recoverable activity arises from the background hydrolysis of sulfenyl amide, under our experimental conditions.

5.4.1 Sulfenyl amide formed at the active site of PTP1B is hydrolytically stable at pH 7

Our model studies revealed that 1,2-benzisothiazolin-3(2H)-one (34) is stable for over 48 h under aqueous buffer conditions. However, we wanted to examine if the sulfenyl amide formed at the active site of PTP1B is stable to hydrolysis under our experimental conditions. For this purpose, we first determined the % sulfenyl amide formed when PTP1B was oxidized gently with 50 µM hydrogen peroxide for 40 min (see section 3.3.1). This was followed by an incubation of PTP1B containing sulfenyl amide in aqueous buffer solution for a further 80 min (catalase was added to stop further oxidation during incubation) before measuring the remaining sulfenyl amide present. A comparison of the percent sulfenyl amide present after 80 min of incubation in assay buffer to a control reaction, revealed no significant decrease (Figure 5.6) in the amount of sulfenyl amide. This suggests that the sulfenyl amide formed at the enzyme active site is stable in aqueous buffer for at least 80 min. This is not surprising given that crystals of PTP1B containing sulfenyl amide are found to be stable for more than 5 h in aqueous buffer.3
Measuring the hydrolytic stability of sulfenyl amide (SA)

Figure 5.6. Hydrolytic stability of sulfenyl amide after 80 min of incubation in assay buffer

Taken together, our results suggest that the oxidation of PTP1B leads to the formation of a thiol reversible but hydrolytically labile intermediate. The decrease in recoverable activity upon hydrolysis is not due to the background decomposition of the sulfenyl amide under our experimental conditions. Thus we provided evidence that suggests the formation of a potential sulfinyl amide species at the active site of PTP1B. This preliminary observation has inspired us to further understand some crucial properties of this novel heterocycle. Hence, we focused on determining the rate constant for the hydrolysis of sulfinyl amide.

5.5 Rate constant for the hydrolysis of sulfinyl amide ($k_9$)

Herein, we wanted to examine the rate constant for the hydrolysis of sulfinyl amide ($k_9$). For this purpose, we synthesized sulfenyl amide (see Chapter 3) and oxidized it using known concentration of hydrogen peroxide for a specific time to generate a mixture of sulfenyl and sulfinyl amide. Catalase was added to prevent further oxidation and the resulting mixture was
incubated at 25 °C to allow for the hydrolysis reaction. At varying time intervals, an aliquot of enzyme was reactivated in DTT and the remaining activity was measured using pNPP (Figure 5.7). We anticipated that the generated sulfinyl amide will undergo hydrolysis to yield thiol unrecoverable sulfinic acid, while the sulfenyl amide will remain stable throughout the incubation time. A semi log plot of the decrease in remaining activity over time provides the rate constant for the hydrolysis of sulfinyl amide (k₉) as a slope.

![Diagram of reaction](image)

**Figure 5.7.** Experimental strategy to measure the rate constant for hydrolysis of sulfinyl amide (k₉)

In a typical assay, sulfenyl amide was treated with 2 mM hydrogen peroxide for 8 min at 25 °C. After 8 min, catalase (200 units) was added to quench the excess hydrogen peroxide and prevent further oxidation. An aliquot of above mixture was taken at varying time intervals (0 – 130 min) and reactivated in DTT (100 mM) for 12 min before measuring the remaining activity using pNPP as described earlier. The reaction was monitored until no change in the remaining activity was observed (which indicates a complete hydrolysis of sulfinyl amide or the presence of
a stable sulfenyl amide). The measured remaining activity at any given time was corrected for the amount of sulfenyl amide, to obtain the % of hydrolytically labile species (sulfinyl amide). Finally, a semi-log plot of decrease in % sulfinyl amide over time provides $k_9$ as a slope of the plot (Figure 5.8).

![Figure 5.8. Plot of ln(a/a_0) vs time towards measuring the rate constant for hydrolysis of sulfinyl amide (k_9)](image)

We measured a rate constant of $4.07 \pm 1 \times 10^{-4}$ s$^{-1}$ for the hydrolysis of sulfinyl amide. This corresponds to a half-life of 28 min. The reported rate constant ($k_9$) is an average of three sets of experiments and the reactions were monitored for more than 3 half-lives. With the value of $k_9$ in hand, we next wanted to determine the rate constant for the hydrogen peroxide mediated over-oxidation of sulfenyl amide.

### 5.6 Determining the rate constant for the over-oxidation of sulfenyl amide ($k_4$)

![Scheme 5.4. Hydrogen peroxide mediated oxidation of sulfenyl amide to sulfinyl amide](image)

In this section, we wanted to examine the rate constant for the hydrogen peroxide mediated oxidation of sulfenyl amide at the active site of PTP1B (Scheme 5.4). For this purpose, we incubated sulfenyl amide (generated under mild conditions: see section 3.3.1) with excess
hydrogen peroxide at 25 °C. At varying time points (1 – 160 min), an aliquot of enzyme was
taken and quenched in catalase (200 units). The resulting mixture was incubated at 25 °C for 2 h
(> 3 half-lives) to allow for the complete hydrolysis of any sulfinyl amide (85) form of the
enzyme. After 2 h, the mixture was reactivated in DTT (100 mM) for 12 min, before measuring
the remaining activity using pNPP (10 mM). The measured activity represents the amount of
thiol recoverable, sulfinyl amide form of the enzyme present at that time. An experimental
strategy to perform the oxidation reaction is outlined below (Figure 5.9).

Figure 5.9. An experimental strategy for oxidation of sulfinyl amide towards measuring k₄

We anticipated that the amount of sulfinyl amide will decrease over time, due to
hydrogen peroxide mediated oxidation followed by hydrolysis (Scheme 5.1). Finally, a semi-log
plot of the remaining sulfinyl amide over time will provide the pseudo I order rate constant (kₗobs)
for oxidation, as a slope of the plot. A second order rate constant for the hydrogen peroxide
mediated oxidation of sulfinyl amide (k₄) was then estimated from kₗobs. The oxidative
decomposition of sulfinyl amide was monitored for more than 2 half lives or over 75 %
decomposition. It is interesting to note that a complete oxidation of sulfenyl amide could not be achieved. This may be due to a possible depletion in concentration of hydrogen peroxide over the time of incubation or due to an extremely slow hydrolysis of sulfinyl amide. However, our results clearly suggest that the sulfenyl amide formed at the active site of PTP1B resists further oxidation.

![Graph](image)

**Figure 5.10.** A representative plot of ln(a/a₀) vs time for the oxidation of sulfenyl amide

In presence of 10 mM hydrogen peroxide, the second order rate constant for oxidation of sulfenyl amide (k₄) was measured to be 0.016 ± 0.001 M⁻¹ s⁻¹, which corresponds to a half-life of 73 min (at 10 mM hydrogen peroxide). It is important to note that the half-life for irreversible over-oxidation of sulfinic acid is 0.6 s, under similar conditions (calculated from a k₂ of 110 M⁻¹ s⁻¹ for Cdc25 phosphatase). Thus our findings here provide evidence that the formation of sulfenyl amide protects the enzyme against irreversible over-oxidation. This was also supported by our model studies, which suggested that a sulfenyl amide mimic (34) is 13 fold more resistant to over-oxidation as opposed to a sulfinic acid surrogate (68), under similar conditions (Chapter 4).
5.7 Conclusion

In conclusion, we extended the knowledge gained from our model studies and investigated the presence of a novel sulfinyl amide intermediate at the active site of PTP1B. We developed a new method that includes an additional hydrolysis step, to measure the partitioning of the oxidized enzyme between sulfenyl and sulfinyl amide forms. Consistent with our hypothesis, we have provided evidences that the oxidative inactivation of PTP1B leads to the generation of a thiol reversible and hydrolytically labile intermediate. We propose this intermediate to be the over-oxidized sulfinyl amide form of the enzyme PTP1B. Though the presence of protein sulfinamides are characterized in various proteins and peptides, this is the first known evidence of a protein derived cyclic sulfinyl amide. Finally, our new strategy has offered us a way to determine the amounts of various oxidized intermediates formed during the hydrogen peroxide mediated inactivation of PTP1B. We estimated the rate constant for the hydrolysis of sulfinyl amide. In addition, we also obtained an estimate for the rate constant of the hydrogen peroxide mediated over-oxidation of sulfenyl amide at the enzyme backbone.

5.8 Experimental Procedures

Materials and Methods

All reagents were purchased from Sigma-Aldrich chemical company (St. Louis, MO). Catalase was obtained from Roche Biosciences (Palo Alto, CA). Recombinant PTP1B (a.a. 1-322) was prepared in our laboratory as reported by us earlier and the concentration was determined by a DTNB burst assay as described previously. Typically, free thiols were removed just before the experiments, from a stock of purified PTP1B using Zeba mini centrifugal buffer exchange column (Pierce, catalog no. 89882) according to the manufacturer’s protocol. The exchange buffer contained 100 mM sodium acetate, 50 mM Bis-Tris and 50 mM
Tris, 10 mM DTPA and 0.05% NP-40 (detergent) at pH 7.4. The assay buffer used in our experiments contained 100 mM sodium acetate, 50 mM Bis-Tris and 50 mM Tris at pH 7.0. The thiol free enzyme stock was diluted to four fold using an exchange buffer, before being used in the assays. The enzyme catalyzed release of p-nitrophenol from the substrate pNPP was monitored at 410 nm in a Hewlett Packard, model 8453 spectrophotometer at 25 °C.

**Characterization of the thiol reversible but hydrolytically labile intermediate during the oxidation of PTP1B.** An aliquot of thiol-free enzyme [(25 µL of a 4 µM solution in 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris, 10mM DTPA and NP-40 (0.05%, v/v)] was combined with the assay buffer (20 µL) and hydrogen peroxide (5 µL of a 2.5 mM stock solution) at 25 °C for 15 min (final concentrations: PTP1B, 2 µM; hydrogen peroxide, 250 µM; buffer, 90 mM sodium acetate, 45 mM Bis-Tris, 45 mM Tris, 9 mM DTPA). At this point, aliquots of enzyme were subjected to reactivation and hydrolysis as described below.

**Reactivation:** An aliquot (12 µL) was added to an assay buffer (12 µL) containing a mixture of catalase and DTT for 25 min (final concentrations: catalase, 160 units; DTT, 50 mM). After 25 min, 3 µL of assay buffer was added and the resulting mixture was incubated for further 15 min at 25 °C. Remaining activity was measured by adding 20 µL of the above enzyme to a cuvette containing pNPP in assay buffer (980 µL) at pH 7.0 (final volume, 1 mL; final concentrations: pNPP, 10 mM; buffer, 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris and 4.5 mM DTPA). Immediately following the addition of enzyme, the assay was mixed by repeated inversion and the enzyme catalyzed release of p-nitrophenol monitored at 25 °C by measuring the increase in absorbance at 410 nm. Data points were taken every 2 s for a period of 2 min.
**Hydrolysis**: An aliquot (12 µL) was added to an assay buffer (12 µL) containing catalase (160 units) and incubated for 25 min at 25 °C. After which 3 µL assay buffer containing DTT (final concentration, 50 mM) was added and the resulting mixture incubated for further 15 min at 25 °C. Remaining activity was measured by adding 20 µL of the above enzyme to a cuvette containing pNPP in an assay buffer (980 µL) at pH 7.0 (final volume, 1 mL; final concentrations: pNPP, 10 mM; buffer, 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris and 4.5 mM DTPA) as described above.

A control reaction was performed in an identical manner with no hydrogen peroxide and subjected to reactivation. The remaining activity was then measured as described above. The percent hydrolytically labile form of the enzyme was calculated as shown below.

\[
\text{% hydrolytically labile} = \left[ \frac{\text{slope reactivation} - \text{slope hydrolysis}}{\text{slope control}} \right] \times 100
\]

**Determining the rate constant for the hydrolysis of sulfinyl amide (k₀)**. An aliquot of thiol-free enzyme [(50 µL of a 4 µM solution in 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris, 10mM DTPA and NP-40 (0.05%, v/v)] was combined with the assay buffer (40 µL) and hydrogen peroxide (10 µL of a 1 mM solution) at 25 °C for 35 min (final concentrations: PTP1B, 2 µM; hydrogen peroxide, 100 µM; buffer, 90 mM sodium acetate, 45 mM Bis-Tris, 45 mM Tris, 9 mM DTPA). To this was added additional hydrogen peroxide (4 µL, 52 mM) to a final concentration of 2 mM and the resulting mixture incubated for further 8 min at 25 °C. After 8 min, catalase in assay buffer (200 units) was added to quench excess hydrogen peroxide. Immediately after addition, aliquots (11 µL) were taken at varying time intervals (0 – 130 min) and reactivated in an assay buffer containing DTT (11 µL, 200 mM) for 12 min (final concentration of DTT, 100 mM). 20 µL of this reactivated mixture was added to a cuvette
containing pNPP (980 µL) and immediately after addition, the assay was mixed by repeated
inversions (final volume, 1 mL; final concentrations: pNPP, 10 mM; buffer, 100 mM sodium
acetate, 50 mM Bis-Tris, 50 mM Tris and 4.5 mM DTPA) and the enzyme catalyzed release of
p-nitrophenol monitored by measuring the increase in absorbance at 410 nm. Data points were
taken every 2 s for a period of 2 min.

The reaction was monitored until there was no change in remaining activity, to ensure the
complete hydrolysis of sulfinyl amide form of the enzyme. The remaining activity measured at
time 0 is taken as control. This final slope is considered as the % of enzyme remaining in
sulphenyl amide form. The amount of enzyme in hydrolytically labile form (sulfinyl amide) is
then calculated as follows:

\[
\text{% of sulfinyl amide} = \left[ \frac{\text{slope at given time} - \text{final slope}}{\text{slope control}} \right] \times 100
\]

A semi-log plot of decrease in % sulfinyl amide over time provided the pseudo I order rate
constant for the hydrolysis of sulfinyl amide (k₉) as a slope (see Figure 5.8 in section 5.5).
Alternatively, a decrease in % sulfinyl amide form of enzyme over time is shown in Figure 5.11.

![Figure 5.11](image.png)

*Figure 5.11. A representative plot of decrease in [sulfinyl amide] over time*
Determining the rate constant for the over-oxidation of sulfenyl amide ($k_4$). An aliquot of thiol-free enzyme [(50 µL of a 4 µM solution in 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris, 10mM DTPA and NP-40 (0.05%, v/v)] was combined with the assay buffer (40 µL) and hydrogen peroxide (10 µL of a 1 mM solution) at 25 °C for 35 min (final concentrations: PTP1B, 2 µM; hydrogen peroxide, 100 µM; buffer, 90 mM sodium acetate, 45 mM Bis-Tris, 45 mM Tris, 9 mM DTPA). To this was added hydrogen peroxide (5.3 µL, 200 mM) to a final concentration of 10 mM. The oxidation reaction was allowed to proceed at 25 °C. Aliquots (10 µL) were removed at varying time intervals (0, 5, 20, 40, 60, 90, 120 and 160 min) and quenched in an assay buffer containing catalase (8 µL, 200 units) at 25 °C. After 120 min, DTT (4 µL, 550 mM) was added and the mixture (final concentration: DTT, 100 mM) reactivated for 12 min at 25 °C. After 12 min, 20 µL of reactivated enzyme was added to a cuvette containing pNPP (980 µL) and immediately after addition, the assay was mixed by repeated inversions (final volume, 1 mL; final concentrations: pNPP, 10 mM; buffer, 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris and 4.5 mM DTPA) and the enzyme catalyzed release of p-nitrophenol monitored by measuring the increase in absorbance at 410 nm. Data points were taken every 2 s for a period of 2 min.

The measured remaining activity is taken as the % of enzyme in sulfenyl amide form. A semi-log plot of decrease in % sulfenyl amide over [H$_2$O$_2$] yielded the pseudo 1 order rate constant ($k_{obs}$) as a slope (Figure 5.10). A plot of decrease in % sulfenyl amide over time displays a classic exponential curve for the oxidative decomposition of sulfenyl amide, as shown in Figure 5.12.
Figure 5.12. A representative plot of decrease in [sulfenyl amide] vs time

References


Chapter 6
Possible Chemical Mechanisms Underlying the Biological Activity of $S$-deoxy leinamycin

6.1 Introduction
6.1.1 Leinamycin – a novel structure with a potent biological activity

Leinamycin (101) is a potent DNA-damaging agent isolated from the broth of Streptomyces in 1989 by a group of researchers in the pharmaceutical company, Kyowa Hokko Kogyo (KHK) in Japan. The structure of this molecule was established by spectroscopic analysis and later confirmed by both X-ray crystallography and a total synthesis approach. The molecule possesses an 18-membered macromolecular ring attached to a five membered 1,2-dithiolan-3-one-1-oxide moiety via a spiro linkage.

Figure 6.1. Leinamycin
Leinamycin displays potent antibacterial and antitumor activity (IC$_{50}$ of 27 nM against HeLa S3 tumor cells, 57% increased life span of mice with murine leukemia P388 at 0.38 mg/kg). Biological experiments have shown that DNA is the main target of this molecule as it is found to inhibit the DNA synthesis in *Bacillus subtilis* at significantly lower concentrations of 1 µg/mL. Very recently, Dr. Sun and coworkers have reported the cytotoxic effect of leinamycin on human pancreatic cancer cell lines MiaPaCa. They show that 101 is highly toxic with an IC$_{50}$ value of 50 nM against MiaPaCa cells. The toxicity of leinamycin arises from its ability to cause extensive DNA strand breaks inside the cells leading to apoptotic cell death. These results indicate that DNA is the important cellular target of leinamycin.

### 6.1.2 Chemical mechanisms of DNA damage by leinamycin

Initial investigation by Hara and coworkers revealed that leinamycin is capable of cleaving circular plasmid DNA at low concentrations of 5-10 µM. The DNA damage was reported to be thiol dependent as the efficiency of the cleavage increased with the increase in concentration of thiol. Further experiments suggested an initial nucleophilic attack of thiol on the 1,2-dithiolan 3-one 1-oxide heterocycle of leinamycin leading to the generation of active DNA damaging intermediates.

Early experiments by our group on the reaction of simple analogs of the crucial 1,2-dithiolan 3-one 1-oxide heterocycle (Figure 6.2) with thiol revealed the generation of cell killing reactive oxygen species (ROS) under physiological conditions. This was indicated by the fact that the DNA cleavage was inhibited by 1) radical scavengers such as methanol, ethanol and mannitol 2) hydrogen peroxide destroying enzyme catalase 3) diethylenetriamine pentaacetic acid (DETAPAC), a chelator of adventitious trace metals
and 4) by the removal of molecular oxygen. Also consistent with the generation of the superoxide radical, superoxide dismutase (SOD), an enzyme that catalyzes the dismutation of superoxide radicals, increased the DNA cleavage. These results suggested a possible involvement of ROS leading to the oxidative DNA damage.

![Figure 6.2. Analogs of leinamycin’s active dithiolanone heterocycle](image)

In a quest to find out the possible reactive intermediates that could generate the potential DNA damaging species, our group explored the reaction of simple dithiolanone model compounds 3H-1,2-benzodithiol-3-one 1-oxide (102) and 5,5-dimethyl-1,2-dithiolan-3-one 1-oxide (103) with thiol. Products formed were rationalized by the generation of an electrophilic oxothiolanone moiety (104a) and a highly reducing hydrodisulfide/persulfide intermediates (105) as shown here in Scheme 6.1.

![Scheme 6.1. Reaction of 102 in presence of thiol](image)

The hydrodisulfide intermediate is believed to be responsible for the generation of DNA damaging reactive oxygen species (ROS). Reaction of hydrodisulfides with excess thiol in presence of oxygen generates superoxide radical anion with the subsequent
formation of polysulfides. The polysulfides thus produced are converted back to hydrodisulfides by their reaction with thiols as shown in Scheme 6.2.

\[
\text{RSH} + \text{RSSR} \rightarrow [\text{RSSH}] + \text{RSSR} \\
\text{HO}^* \leftrightarrow \text{O}_2^* \\
\text{O}_2^- + \text{H}^+ \leftrightarrow \text{HO}_2^* \\
\text{HO}_2^* + \text{O}_2^- + \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \\
\text{H}_2\text{O}_2 + \text{M}^{n+} \rightarrow \text{HO}^* + \text{HO}^- + \text{M}^{(n+1)+} \\
\text{M}^{(n+1)+} + \text{O}_2^- \rightarrow \text{M}^{n+} + \text{O}_2
\]

**Scheme 6.2:** Hydrodisulfide mediated production of ROS

The superoxide anion thus produced can lead to the generation of DNA damaging reactive oxygen species by a typical Fenton chemistry in presence of metal ions, as shown in Scheme 6.3. Thus the hydrodisulfide produced by the reaction of thiol on leinamycin’s 1,2-dithiolan 3-one 1-oxide heterocycle can lead to the overall oxidative DNA damage.

\[
\begin{align*}
\text{O}_2^- + \text{H}^+ & \leftrightarrow \text{HO}_2^* & (1) \\
\text{HO}_2^* + \text{O}_2^- + \text{H}^+ & \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 & (2) \\
\text{H}_2\text{O}_2 + \text{M}^{n+} & \rightarrow \text{HO}^* + \text{HO}^- + \text{M}^{(n+1)+} & (3) \\
\text{M}^{(n+1)+} + \text{O}_2^- & \rightarrow \text{M}^{n+} + \text{O}_2 & (4)
\end{align*}
\]

**Scheme 6.3:** Reactions involved in the production of reactive oxygen species (ROS)

At the same time Gates group elucidated ability of dithiolanone heterocycles (102, 103 and 104) to produce ROS, Asai and coworkers reported a thiol mediated DNA alkylation by leinamycin. They isolated and characterized a novel N7-guanine alkylated adduct of leinamycin that led to the proposal of a new DNA damaging mechanism. The guanine adduct 111 was observed when leinamycin reacted with thiol in the presence of duplex DNA. Characterization of the product by 2D NMR revealed a novel rearrangement with the nucleophile attached at the C6 carbon. This finding has
supported the DNA alkylation by leinamycin that involves the initial attack of thiol on dithiolanone heterocycle, leading to the formation of a sulfenate anion (107), which undergoes a ring closure on to the carbonyl carbon to generate a putative oxothiolanone intermediate (108) along with the release of hydrodisulfide (105), as suggested earlier by our group. The compound 108 was then attacked by the C6-C7 double bond of the macrocycle, to form an electrophilic species, presumably an episulfonium ion (109) that can alkylate the N7 guanine of DNA as shown in Scheme 6.4

Scheme 6.4. Mechanism of thiol mediated DNA alkylation by leinamycin

Leinamycin causes DNA strand breaks by both thiol mediated alkylation at the N-7 guanine residues and by the production of reactive oxygen species through the hydrodisulfide intermediate. Both the known mechanisms of DNA damage involve an initial attack of the thiol on the crucial 1,2-dithiolan 3-one 1-oxide heterocycle of
leinamycin. Thus, the chemical mechanisms leading to the biological activity of leinamycin are well characterized. However, little is known about the mode of action of the $S$-deoxy leinamycin, a possible bio-synthetic precursor to leinamycin, that lacks the sulfoxide oxygen at the crucial 1,2-dithiolan 3-one 1-oxide heterocycle (Figure 6.3).

Initial studies carried out by Nakano and coworkers revealed that $S$-deoxy leinamycin possess less biological activity ($IC_{50}$ of 4 µM against HeLa S3 cancer cells) compared to the leinamycin ($IC_{50}$ of 27 nM). Indeed, reaction of $S$-deoxy leinamycin with plasmid DNA did not produce significant strand breaks under similar conditions used for leinamycin, further indicating that $S$-deoxy leinamycin is less potent than the leinamycin itself. Although, the cytotoxicity of $S$-deoxy leinamycin is approximately 150 fold less than that of leinamycin, it is surprising to know that it still retains substantial biological activity that is comparable to some of the clinically used anti cancer agents like mitomycin C$^{14}$ (1.8 µM) and bleomycin$^{15}$ (3.3 µM). For example, some clinically used drugs like cisplatin, melphalan and bleomycin have a 50% growth inhibitory concentration values of 3 µM, 17 µM and 1 µM respectively against HepG2 cell lines.$^{16}$ However, the molecular basis for the observed cytotoxicity of $S$-deoxy leinamycin is not known. This fact has motivated us to investigate the chemical mechanism responsible for the biological activity of the $S$-deoxy leinamycin. The following section will state the goals of this project.
6.2 Goals of this Chapter

The major goal of this project is to understand the chemical mechanism(s) responsible for the biological activity of the S-deoxy leinamycin. For that, we plan to examine the chemical properties of the 1,2-dithiolan 3-one heterocycle, which is the primary reactive sub-unit of the S-deoxy leinamycin. Scientists have often used structurally similar small molecules to understand the functional properties of the macromolecules/natural products. For example, a careful analysis of the chemical properties of the small molecule mimic of leinamycin’s triggering unit has led us to unearth a novel hydrodisulfide mediated oxidative DNA-damaging mechanism (Scheme 6.2). Knowing the chemical mechanism for the reaction of 1,2-dithiolan 3-one 1-oxide heterocycle and its analogs with thiol, has further helped us to clearly understand the mechanism for the biological activity of other polysulfide containing natural products, like varacin.

In a similar approach, we anticipated that the biological activity of S-deoxy leinamycin would involve the production of reactive oxygen species. With that in mind, the goals of this chapter are set as follows:

a) Synthesize a simple chemical model to mimic the crucial triggering unit of the S-deoxy leinamycin, 1,2-dithiolan 3-one heterocycle (S-deoxy core, 116).

b) Examine the thiol triggered production of reactive oxygen species.

c) Characterize the product(s) stemming from the thiol mediated decomposition of the S-deoxy core molecule.

d) Investigate the possible chemical mechanisms leading to the generation of reactive oxygen species.
We believe that understanding chemical mechanism underlying the biological activity of S-deoxy leinamycin may reveal some novel chemistry for cell killing by natural products. To begin with, we will discuss the synthesis of S-deoxy core molecule (116) in the following section.

6.3 Synthesis of 1,2-dithiolan 3-one heterocycle

Various methods are available for the synthesis of 1,2-dithiolan 3-one heterocycle of S-deoxy leinamycin.20, 21 One such simple and straightforward approach by Pattendon and coworkers involves stirring sodium glycidate 113, that was obtained in two steps from acetone and ethyl 2-chloropropionate, with sodium sulfide in methanol at room temperature to give 114. The mercapto acid 114 was activated using iso-butylchloroformate in presence of triethylamine, which undergoes ring closure by an intramolecular attack of thiol to yield thiolactone 115. The thiolactone was ring opened in presence of hydrogen sulfide and triethylamine at -78 °C to give an intermediate 3-methyl-3-mercapto butanethioic acid, which on in situ oxidation using ferric chloride was converted to 1,2-dithiolan 3-one heterocycle (116) as shown in Scheme 6.5.

![Scheme 6.5: Sodium glycidate approach for the synthesis of 1,2-dithiolan 3-one moiety](image)

In our synthesis, we employed Pattendon’s approach with some minor modifications. The first step of our approach involves a simple Darzen’s condensation to give the epoxy ester (118). Compound 118 was then subjected to base hydrolysis to convert the ester into a sodium salt of the acid (113). The sodium salt of the epoxy acid was then ring opened in the presence of a titanium tetraisopropoxide and benzyl...
mercaptan to give 119. Some difficulties were experienced initially in performing this reaction. It was later found that it is necessary to maintain this reaction under inert atmosphere at all times. Specifically, the sodium salt of benzyl mercaptan was getting oxidized leading to the formation of the benzyl disulfide, and hence the nucleophile was transferred to the reaction flask containing the compound 113 using a canula. Benzyl mercaptan was chosen as a nucleophile for this reaction as it can be viewed as a protected analog of NaSH.\textsuperscript{22} The benzyl group was then removed to give the 2-hydroxy-3-mercapto-2,3-dimethylbutanoic acid (114) using sodium and liquid ammonia.

The synthesis of compound 115 was achieved under mild conditions, using dicyclohexyl carbodiimide (DCC). Finally, thiolactone (115) was ring opened in presence of H\textsubscript{2}S to generate 2-hydroxy-3-mercapto-2,3-dimethylbutanethioic acid (120), which was then oxidized to obtain 1,2-dithiolan 3-one (116). The nucleophilic ring opening and the subsequent oxidation was carried out in one pot, in this step. Thus, the synthesis of S-deoxy leinamycin’s triggering unit was achieved in six steps as shown in Scheme 6.6.

\textbf{Scheme 6.6:} Synthesis of 1,2-dithiolan 3-one heterocycle
6.4 DNA damage by 1,2-dithiolan 3-one heterocycle

After the synthesis of the crucial triggering unit of S-deoxy leinamycin, we set out to explore its ability to generate reactive oxygen species. Based on our previous experience with related sulfur heterocycles in our lab,\textsuperscript{23,24,18,19} we expected that the reaction of thiol with dithiolanone heterocycle (116) might lead to the production of reactive oxygen species. We utilized plasmid based DNA cleaving assays to investigate the production of reactive oxygen species in the reaction of thiol with 116. It is well documented that the production of reactive oxygen species leads to strand breaks in plasmid DNA.\textsuperscript{25,26} Typically, a single event of strand scission would result in the conversion of supercoiled DNA (form I) into the open circular form (form II). The two forms of DNA can be separated based on the differences in their electrophoretic mobility in an agarose gel and can be visualized under UV light after staining with DNA binding dye such as ethidium bromide. Our assay revealed that compound 116 effects a thiol triggered DNA damage. In a typical assay, various concentrations of compound 116 was incubated with 15 equivalents of 2-mercaptoethanol in a 50 mM sodium phosphate buffer containing 10\% acetonitrile by final volume. The mixture was then incubated at 37 °C for 12 h followed by an agarose gel electrophoretic analysis of the reaction, as previously reported by our group.\textsuperscript{9}
Figure 6.4. Thiol dependent DNA cleavage by various concentrations of 1,2-dithiolan 3-one (116). Supercoiled pGL2 basic DNA (78 µM bp) was incubated for 12 h at 37°C with various concentrations of 116 and 15 equiv of 2-mercapto ethanol in sodium phosphate buffer (50 mM, pH 7) containing 10% acetonitrile (by volume). Solutions were prepared using glass distilled, deionized water and 99+% pure sodium phosphate salts (no transition metals were added to the reactions). Reactions were 20 µL final volumes and were conducted under a headspace of air in sealed 500 µL Eppendorf tubes. Agarose gel electrophoresis was performed as described previously. The values in parentheses following the description of each lane below indicates the S-value (mean number of strand breaks per plasmid molecule) for each lane and was calculated using the equation $S = -\ln f_i$, where $f_i$ is the fraction of plasmid in a given lane that is present as uncut, form I DNA. Values reported here represent the average of at least three experiments, and the standard error in these measurements is less than 2%. Lane 1, DNA alone (0.16); lane 2, 500 µM 116 alone (0.46); lane 3, 10 µM 116 + thiol (0.31); lane 4, 50 µM 116 + thiol (0.63); lane 5, 100 µM 116 + thiol (0.84); lane 6, 250 µM 116 + thiol (2.83); lane 7, 500 µM 116 + thiol (4.54); lane 8, 7.5 mM thiol alone (0.21).

It is clear from the data that S-deoxy core molecule is a thiol-dependent DNA damaging agent and the efficiency of damage increases with the increase in concentration of the drug (compound 116). A substantial increase in the % of form II DNA was observed when the concentration was increased from 100 µM to 250 µM (lanes 5 and 6). The fact that the drug alone or 7.5 mM of 2-mercapto ethanol alone failed to initiate significant strand breaks (Figure 6.4, lanes 2 and 8) demonstrates the importance of the presence of thiol in triggering the DNA damage by compound 116. The following section will be dedicated to understanding the mechanism of the DNA strand break.

6.5 Investigation of the mechanism of thiol triggered DNA damage by 116

It has been established that the 1,2-dithiolan 3-one molecule causes thiol triggered DNA damage. However, the exact mechanism leading to DNA damage was not known. Hence, our main goal was to understand the nature of the DNA damaging reactive intermediates generated in the reaction of thiol with compound 116. The thiol triggered DNA cleaving properties of 116 is consistent with the thiol-mediated DNA damage observed for similar dithiolanone heterocycles (102 and 103). This led us to speculate
the involvement of reactive oxygen species in the DNA damage. To further investigate the role of ROS in the thiol-mediated DNA strand breaks by 116, we performed cleavage assays in presence of additives that interact with various species shown in Scheme 6.7. In the following paragraphs, we will probe the generation of ROS in the reaction of thiol with 116.

Presence of 1 M concentration of classic hydroxyl radical scavengers such as methanol, ethanol and DMSO significantly reduced the thiol mediated DNA cleavage by 116 (Figure 6.5, lanes 4, 5 and 8). Methanol and ethanol are efficient hydroxyl radical scavengers with a rate constant for trapping reported to be $9.7 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$ and $1.9 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$ respectively. DMSO is another well established hydroxyl radical trapping agent. Thus, considerable decrease in DNA cleavage by all these hydroxyl radical scavengers clearly suggests the involvement of hydroxyl radical in the DNA damage.

![Figure 6.5](image)

**Figure 6.5.** Thiol dependent DNA cleavage by 116 in presence of various radical scavengers and ROS scavenging enzymes. Supercoiled pGL2 basic DNA (78 µM bp) was incubated for 12 h at 37°C with 150 µM 1 and 15 equiv of 2-mercapto ethanol and various additives as mentioned below, in sodium phosphate buffer (50 mM, pH 7.0) containing 10% acetonitrile (by volume). Solutions were prepared using glass distilled, deionized water and 99+% pure sodium phosphate salts (no transition metals were added to the reactions). Reactions were 20 µL final volumes and were conducted under a headspace of air in sealed 500 µL Eppendorf tubes. Agarose gel electrophoresis was performed as described previously. Values reported in table 6.1 represent the average of at least two experiments, and the standard error in these measurements is less than 2%.

The production of hydroxyl radical could arise from the superoxide anion. We envisioned a thiol triggered, compound 116 mediated reduction of molecular oxygen to superoxide radical anion (Eqn 5), that in turn generates hydrogen peroxide by disproportionation (Eqn. 1 & 2). Thus generated hydrogen peroxide undergoes a typical fenton reaction in presence of metal ions under our reaction conditions to produce highly
reactive, DNA damaging hydroxyl radicals as shown in Scheme 6.7 (Eqns. 3 & 4). Similar chemistry has been observed before at various instances in our laboratory.$^{29,9,11,19,13}$

In an attempt to probe other steps of the above mentioned scheme, we performed thiol triggered DNA cleavage reaction by compound 116 in the presence of metal chelating agents. It is well documented in the literature that metal chelators such as desferal, diethylenetriamine pentaacetic acid (DETAPAC) and ethylenediaminetetraacetic acid (EDTA) can sequester adventitious trace metal ions like iron and copper. The metal ions are typically involved in catalyzing the electron transfer process in the Fenton reaction to produce the reactive oxygen species (eqn 3).$^{30,31}$ We observed a significant decrease in the DNA damage when the reaction was conducted in presence of 10 mM desferal or DETAPAC (Figure 6.5, lanes 6 and 7). This observation further supports the generation of the reactive oxygen species in the reaction of thiol with compound 116.
Table 6.1. Effect of additives on thiol mediated DNA damage by compound 116

<table>
<thead>
<tr>
<th>Reaction/Additive</th>
<th>% Nicked, Form II DNA</th>
<th>S-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Alone</td>
<td>13.5</td>
<td>0.14</td>
</tr>
<tr>
<td>S-deoxy (116) alone</td>
<td>18.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Std rxn: 116 (150 µM) + thiol (2.25 mM)</td>
<td>82</td>
<td>1.7</td>
</tr>
<tr>
<td>Std rxn + additive:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>methanol (1 M)</td>
<td>27.5</td>
<td>0.32</td>
</tr>
<tr>
<td>ethanol (1 M)</td>
<td>19</td>
<td>0.21</td>
</tr>
<tr>
<td>desferal (10 mM)</td>
<td>26</td>
<td>0.30</td>
</tr>
<tr>
<td>DETAPAC (10 mM)</td>
<td>19</td>
<td>0.21</td>
</tr>
<tr>
<td>DMSO (1M)</td>
<td>15.5</td>
<td>0.17</td>
</tr>
<tr>
<td>catalase (100 µg/mL)</td>
<td>19</td>
<td>0.21</td>
</tr>
<tr>
<td>SOD (100 µg/mL)</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>SOD (50 µg/mL)</td>
<td>100</td>
<td>-</td>
</tr>
</tbody>
</table>

Catalase is a hydrogen peroxide destroying enzyme that is known to catalyze the decomposition of hydrogen peroxide <i>in vivo</i> to molecular oxygen and water.<sup>32</sup> It has been widely used to probe the production of hydrogen peroxide <i>in vitro</i>.<sup>9,11</sup> Addition of catalase in our reaction, effectively inhibits the thiol mediated DNA cleavage by compound 116 (lane 10). This strongly suggests an important role for hydrogen peroxide in the DNA cleavage, which could either be produced directly or by the rapid disproportionation of superoxide radical anion (Scheme 6.7).<sup>33</sup>

Finally, generation of superoxide radical anion was examined with the help of an enzyme, superoxide dismutase (SOD). This metalloenzyme containing Cu-Zn, catalyzes the rapid disproportionation of superoxide radical anions into hydrogen peroxide and oxygen.<sup>33</sup> Compound 116 mediated DNA strand breaks increased significantly in
presence of SOD. This is in good agreement with the known fact that SOD catalyzes the disproportionation of two molecules of superoxide radicals to generate one molecule of hydrogen peroxide, which can produce hydroxyl radicals through Fenton chemistry. In addition, SOD can also result in an increase in the concentration of oxygen, which can lead to the production of more superoxide radical. The enhancement of DNA cleavage in presence of SOD provides evidence for the intermediacy of the superoxide radical anion. Thus, the results presented here clearly suggest the production of reactive oxygen species in the thiol triggered DNA damage of compound 116. A careful analysis of the literature provided us with more reasoning for the observed SOD-mediated increase in strand breaks. We will discuss that in the following section.

6.6 Other possible reasons for the SOD mediated increase in strand breaks

It is generally accepted that the presence of SOD would decrease the reactive oxygen species mediated DNA damage. A good example for that would be superoxide radical dependent DNA cleaving molecule, quinocarcin and their analogs, in which the presence of SOD is known to effectively inhibit the DNA damage.\textsuperscript{34} This can be explained by the fact that the superoxide molecule, apart from generating hydrogen peroxide by its disproportionation, also serves as a reducing agent that converts the oxidized adventitious trace metal ions back to its reduced forms (Scheme 6.7, eqn 4). Though the presence of SOD leads to the generation of hydrogen peroxide via rapid dismutation of superoxide radical, it also decreases the concentration of superoxide radicals and thereby inhibiting the reduction of oxidized trace metals. So, in the absence of the reduced metal ions, the generated hydrogen peroxide cannot effectively undergo
the Fenton reaction to produce hydroxyl radical, which is responsible for the overall DNA damage.

A careful analysis of the literature revealed few other systems that show increase in DNA damage in presence of SOD. Adriamycin, a natural product, showed increased DNA damage in presence of SOD. Presence of SOD also seems to significantly increase phenazine di-N-oxide mediated DNA cleavage. In addition, cyclic polysulfides, hydrodisulfides and some 1,2-dithiolan 3-one 1-oxide heterocycles have also been reported to cleave DNA much better in presence of SOD. A closer look into these systems reveal that they all require thiol either for activation or in some stage for their DNA damaging activity unlike the quinocarcin analogs that require no thiol for cleaving DNA. This in fact tells us that the added thiol has a major role to play in the SOD mediated increase seen in these molecules. It can be better explained by the fact that though SOD quenches superoxide anions and thereby preventing the reduction of trace metal ions to push the Fenton chemistry forward, presence of excess thiol in these systems can in fact act as a reducing agent to effect the hydrogen peroxide mediated Fenton reaction. Thiols can either reduce the trace metal ions or can directly reduce hydrogen peroxide to produce the highly reactive hydroxyl radicals. So, there is an absolute need for the presence of reducing agents in order to drive the Fenton chemistry forward and effect significant DNA damage. This can explain the increase in DNA cleavage seen in presence of SOD in thiol mediated DNA damaging agents.

In a totally different context, SOD is known to catalyze the oxidation of hydrogen sulfide in presence of oxygen to give hydrogen peroxide and elemental sulfur. Searcy and co-workers report that Cu,Zn-SOD has affinity for HS\(^{-}\) and can effectively bind to
the hydrogen sulfide ion to catalyze the oxidation of HS\textsuperscript{−} to hydrogen peroxide, as shown in Scheme 6.8. The rate of HS\textsuperscript{−} oxidation at pH 7.8 and 20 °C was measured to be 0.2 mol min\textsuperscript{−1} (mol SOD)\textsuperscript{−1}. Although the overall catalytic process is complex, SOD was found to possess HS\textsuperscript{−}:O\textsubscript{2} oxidoreductase activity.

\[
\text{HS}^{-} + \text{O}_2 + \text{H}^{+} \xrightarrow{\text{SOD}} \text{S}_\text{0} + \text{H}_2\text{O}_2
\]

**Scheme 6.8.** Oxidation of hydrogen sulfide by superoxide dismutase.

This finding has prompted us to test the possible generation of hydrogen sulfide under our reaction conditions. We anticipated that generation of H\textsubscript{2}S in our assays may be responsible for an increase in concentration of ROS in presence of SOD. It is now clear that the thiol triggered DNA damage by compound 116 produces reactive oxygen species. But now we wanted to examine if it also leads to the generation of hydrogen sulfide, which can add to the overall cytotoxicity of this drug molecule. In the following section we will examine the production of hydrogen sulfide in the reaction of thiol with compound 116.

**6.7 Generation of hydrogen sulfide in the reaction of 116 + thiol**

The SOD-mediated increase in DNA cleavage combined with the reported generation of hydrogen sulfide in similar cyclic polysulfide systems,\cite{19} motivated us to explore the possible thiol triggered generation of hydrogen sulfide by compound 116. We will discuss the qualitative detection of the hydrogen sulfide in our assays, in the following section.
6.7.1 Qualitative detection of hydrogen sulfide in our assays

Addition of 2-mercaptoethanol to a solution containing compound 116 in a sodium phosphate buffer solution had immediately turned the lead acetate paper black, indicating the generation of hydrogen sulfide. A control reaction containing no 116 but with same amounts of 2-mercaptoethanol failed to generate the lead sulfide (PbS) precipitate, even after 24 h of stirring. Consistent with our expectation, authentic H₂S (purchased from Aldrich chemical company) turned the lead acetate paper black. This suggested the production of hydrogen sulfide in the reaction of thiol with 1,2-dithiolan 3-one heterocycle (116). The fact that a common thiol, like 2-mercaptoethanol, did not turn the lead acetate paper black under our reaction conditions reveals two things: a) it rules out the involvement of any thiol related species towards the generation of PbS and b) the important role of compound 116 in the production of hydrogen sulfide.

To further validate our findings, we took compound 116 in a sealed round bottom flask in a mixture of sodium phosphate buffer containing 20% acetonitrile by volume. The reaction was initiated by the addition of 5 equivalents of 2-mercapto ethanol. A stream of nitrogen was passed into the reaction and the effluent gas was bubbled into a solution of lead acetate in water. The lead acetate solution turned black due to the generation of hydrogen sulfide and the resulting black precipitate was collected and dried under vacuum. A control reaction involving all other components except compound 116 did not produce the black precipitate. To confirm the identity of the black precipitate obtained in our reaction, it was oxidized using concentrated nitric acid and 30% hydrogen peroxide. Accordingly, PbS was oxidized to its corresponding lead sulfate under these conditions to give a white solid. Consistent with our expectations, oxidation of the
black precipitate resulted in a white solid suggesting the conversion of PbS into PbSO₄. This reiterates the fact that the black precipitate obtained in the reaction of compound 116 with thiol was indeed lead sulfide (PbS). Thus, we confirmed the production of hydrogen sulfide in the reaction of thiol with the S-deoxy core molecule (116). In the following section we will characterize the products in the thiol-mediated reaction of compound 116 in aqueous buffer conditions.

6.8 Characterizing the products in the reaction of thiol with 116

Our next goal was to understand the nature of the chemical process that may be responsible for the production of reactive oxygen species in our assay conditions. For this, we isolated and characterized the major product(s) in the reaction of 2-mercapto ethanol with compound 116. 1,2-dithiolan 3-one (116) was stirred in a mixture of sodium phosphate buffer (50mM, pH 7.0) containing 20% acetonitrile and to this mixture was added 5 equivalent of 2-mercapto ethanol. The reaction was stirred at 25 °C for about 45 min and the disappearance of 116 was monitored by TLC. Isolation and characterization of the products revealed the presence of thioester (124) and the disulfide of 2-mercapto ethanol.

We envisioned a chemical mechanism involving an attack of thiol on the S-deoxy core molecule (116) to produce an intermediate dithioester derivative. This was followed by an intramolecular attack of thiol onto the carbonyl carbon to generate a four membered thiolactone with concomitant release of hydrosulfide. A similar intramolecular attack involving sulfenate ion to produce oxathiolanone and hydrosulfide was observed in case of leinamycin. The hydrosulfide thus generated, is a known DNA damaging agent, which in presence of thiol is capable of producing
reactive oxygen species. Furthermore, reaction of hydrodisulfide (persulfides) with thiol also leads to the generation of hydrogen sulfide as reported by Dr. Tonika Chatterji, in a separate context and observed by us earlier (see section 6.7) in the current molecular context. The four-membered thiolactone (115) undergoes further ring opening in presence of one molecule of thiol to produce the expected thioester product as shown in Scheme 6.9.

![Scheme 6.9. Proposed mechanism explaining the formation of observed product(s) in the reaction of thiol with compound 116](image)

We expected the intermediate thiolactone (115) to be very unstable under the reaction conditions of excess thiol. So, in order to observe the presence of thiolactone, we carried out the same reaction in presence of lesser equivalents of thiol (2 equ.). Consistent with our expectations, we indeed observed the presence of the intermediate thiolactone by thin layer chromatography (TLC). Treatment of an authentic thiolactone with thiol under the same conditions as our model reaction generated the expected thioester product (124). Furthermore, when the assay mixture was treated with excess methyl iodide after the reaction, we obtained S-methylated thioester (125), confirming the identity of the originally obtained thioester product 124 (Scheme 6.10).
After having detected one of the intermediates thiolactone in our assays, we set out to observe polysulfide products anticipated to arise from the ejection of persulfides in our reaction. The following section will describe our efforts in identifying the production of polysulfides.

6.9 Generation of polysulfides in the reaction of thiol with 116

Persulfides have been implicated in the thiol mediated generation of ROS in the reaction of leinamycin analogs (102, 103) with thiol. On the other hand, leinamycin itself is known to eject persulfide upon activation with thiols, which is responsible for the overall oxidative DNA damage. Typically, persulfides are more acidic than the corresponding thiols. As a result, larger fraction of RSSH will be in ionized form (RSS⁻) compared to RSH at physiological pH. Hence, analogous to the auto oxidation of thiols, persulfide anion is expected to undergo oxidation in presence of trace metal ions to produce a persulfide radical (RSS'). This will lead to the generation of superoxide radical anion as shown in Scheme 6.11. The superoxide radical can undergo rapid disproportionation to yield hydrogen peroxide, which can undergo a metal mediated fenton reaction to generate highly reactive hydroxyl radicals that can cause DNA strand breaks.
Scheme 6.11. Persulfide mediated production of ROS

In addition, the generated persulfide radical can undergo further reaction with the thiolate to generate a polysulfide radical anion analogous to the reactions reported for the oxidation of thiols.\textsuperscript{41,42,43} The polysulfide radical anion is a strong reducing agent that converts molecular oxygen to superoxide radical and produces polysulfides (Scheme, 6.12 Eqn 6). The generated polysulfide reacts further with another molecule of thiol to regenerate persulfide anion (Eqn. 7). Likewise, smaller amount of persulfide produced in a reaction can act as a catalyst to convert considerable amounts of thiol to disulfide and in turn generating superoxide radical anion. The persulfide produced is very unstable and cannot be detected directly. Polysulfides are the characteristic stable decomposition products stemming from the oxidation of persulfide.\textsuperscript{13} Hence, identification of polysulfide serves to confirm the formation of a persulfide intermediate in our model reaction.

Scheme 6.12. Reactions of persulfide leading to the production of ROS

Accordingly, incubation of compound 116 with 5 equiv. of 2-mercaptoethanol in sodium phosphate buffer (50mM, pH 7.0) containing 20% acetonitrile resulted in the production of polysulfides. The presence of polysulfides were identified using reverse phase HPLC, that revealed peaks corresponding to di, tri and tetra sulfides of 2-
mercaptoethanol. The retention times of the polysulfide produced matched exactly with an authentic spectrum generated from the reaction of $3H$-$1,2$-benzodithiol-$3$-one $1$-oxide (102) with thiol.$^{13,19,18}$

Furthermore, the reaction of persulfide with thiol can lead to the production of hydrogen sulfide (Scheme 6.12, Eqn 8). Accordingly, we observed the release of hydrogen sulfide in the thiol mediated ring opening of $1,2$-dithiolan 3-one (116), which may have resulted from the reaction of polysulfides with excess thiol. Thus our proposed chemical mechanism (Scheme 6.9) provides a possible explanation for the production of ROS and $H_2S$ in the thiol mediated DNA damage by $S$-deoxy core molecule (116). However, we also considered the possibility of other mechanisms that may explain the formation of the observed products. In the following section, we will throw some light on various evidences that helped us rule out some of the other mechanisms.

### 6.10 Possible chemical mechanisms for the reaction of 116 with thiol.

Several possible mechanisms can be formulated to explain the formation of the products seen in the reaction of thiol with the compound 116 that ultimately leads to the production of ROS. We would like to begin addressing the issue at the initial step of thiol attack on the $1,2$-dithiolan 3-one heterocycle. The reaction of compound 116 with thiol produced mercaptothioacid (121) via a thiolactone (115) intermediate, and hydrogen sulfide. Typically, the $1,2$-dithiolan 3-one heterocycle possess three possible electrophilic centers in carbonyl carbon and two sulfenyl sulfur atoms.$^{44}$ Hence, we expected the attack of a nucleophilic thiolate to occur at one of these electrophilic centers. We will look into the potential mechanisms based on the attack of thiol at various positions of the heterocycle towards generating the expected products.
6.10.1 Attack on S1 sulfur of 1,2-dithiolan 3-one heterocycle

Our first mechanism is based on the attack of the thiolate on the electrophilic sulfinyl sulfur S1 of the 1,2-dithiolan 3-one heterocycle. Initial attack of thiolate on S1 sulfur leads to the generation of a mixed disulfide (X) with a thiolacetic acid leaving group as shown in Scheme 6.13.

![Scheme 6.13. Initial attack of thiolate on S1 sulfur of 1,2-dithiolan 3-one heterocycle](image)

Mechanism I:

![Mechanism I](image)

Attack of thiolate on the carbonyl carbon of thiolacetic acid moiety can lead to the direct release of hydrogen sulfide followed by another exchange of thiol with the mixed disulfide (128) to generate the desired mercaptothioester product (124, Mechanism I). Though this mechanism explains the generation of the expected products, the step involving the thiolysis of thiolacetic acid does not seem to occur under our reaction conditions. Incubation of authentic thiolacetic acid under our assay conditions revealed that it was very stable to both hydrolysis and thiolysis. In addition, the fact that the reaction of thiolacetic acid with 2-mercapto ethanol failed to generate hydrogen sulfide gas clearly ruled out this mechanism.
As the direct intermolecular attack of thiol/water on thiolacetic acid seems unlikely, we anticipated an intramolecular assistance in the elimination of hydrogen sulfide. In the second mechanism (mechanism II), we expected the thiol exchange to occur first and the resulting thiolate anion (129) would cyclize onto the adjacent carbonyl carbon to release hydrogen sulfide as shown in Scheme 6.14.

Mechanism II:

![Scheme 6.14. Thiolate attack on mixed disulfide to generate the product 124](image)

Though this mechanism seems reasonable, the second step involving an intramolecular attack of thiolate on thiolacetic acid to produce 115, does not have precedence in the literature. Moreover, compounds with similar structures (3-mercapto thioacetic acid, 129) are stable and have been isolated before. Hence this mechanism also seems unlikely to generate our observed products.

### 6.10.2 Attack on S2 sulfur of 1,2-dithiolan 3-one heterocycle

The second possibility involves an attack of thiolate on the S2 sulfenyl sulfur of the heterocycle. Similar reaction has been known to occur in case of leinamycin and its analogous dithiolanone heterocycles. So we favored an initial reaction of thiol at the central sulfenyl sulfur to generate a 3-mercaptop dithioester derivative (130) as shown in Scheme 6.15.
Analogous to the thiol mediated activation reaction of leinamycin, we proposed an intramolecular thiolate attack on the carbonyl carbon to form a four membered thiolactone with the release of persulfide, as shown in Scheme 6.16. The persulfide generated can lead to the production of hydrogen sulfide (Scheme 6.12, Eqn.8). Furthermore, a subsequent thiol-mediated ring opening of X would yield the final mercaptothioester (124), as shown in mechanism III.

Mechanism III:

We favored this mechanism (Scheme 6.16) as this provides a logical explanation for the formation of observed products. Other possibilities involving direct hydrolysis at the carbonyl carbon of 130 or the thiol exchange at the disulfide sulfur of 130 to generate the product seems unlikely. However, a direct thiolysis at the carbonyl carbon ejecting persulfide and leading to the formation of the product 124 cannot be ruled out at this point.
6.10.3 Attack of thiol at the carbonyl carbon of the 1,2-dithiolan 3-one heterocycle

Herein we considered the possibility of a thiolate attack on an electrophilic carbonyl carbon. Initial attack of thiol at the carbonyl carbon leads to the generation of a mixed disulfide (131). Compound 131 can further undergo exchange with two molecules of thiol to release hydrogen sulfide and the product 124 as shown in Scheme 6.17.

Mechanism IV:

![Scheme 6.17. Attack of thiol at the carbonyl carbon of compound 116](image)

This scheme explains the formation of expected products. An attack of a soft thiolate anion at the hard carbonyl carbon seems unfavorable in presence of thiophilic sulfur atoms. Moreover, as this mechanism cannot explain the formation of thiolactone (115) observed in our assays, it can be ruled out.

6.11 Comparison of DNA damage by 1,2-dithiolan 3-one analogs (102, 116 and 133)

In this section, we will compare the DNA-damaging efficiency of two of the S-deoxy compounds, 3H-1,2-benzodithiolan 3-one (133) and 1,2-dithiolan 3-one (116). Earlier, we reported that 100 µM of S-deoxy-benzo model compound (133) with 5 equiv. 2-mercaptoethanol, effects significantly lesser DNA damage compared to the oxy-analog 102. Herein, we wanted to examine how well does the DNA damaging abilities of a S-deoxy-benzo model (133) compare to its more leinamycin like, aliphatic model compound (116). In addition, we will examin how well does the DNA damage by both
the S-deoxy model compounds (133 and 116) compare to the 1,2-dithiolan 3-one 1-oxide heterocycle (102).

Incubation of compounds 133 and 116 under similar conditions (150 µM with 15 equ. 2-mercaptoethanol and 50 mM sodium phosphate buffer containing 10% acetonitrile by final volume) revealed that the compound 133 cleaves DNA less efficiently than the aliphatic model (116). Compound 133 cleaves just 10% above the background DNA damage caused by the addition of same amounts of 2-mercaptoethanol alone, whereas, 116 results in ~70% nicks in the plasmid DNA. As expected, the leinamycin analog 102 is much superior in effecting DNA damage with 94% nicks when compared to both the deoxy analogs under similar conditions, as shown in Figure 6.6 (compare lanes 8, 9 and 10).

**Figure 6.6.** Thiol dependent DNA cleavage by compounds 116, 102 and 133. Supercoiled pGL2 basic DNA (76 µM bp) was incubated for 18 h at 37°C with various concentrations of 116, 102 and 133 and 10 and 15 equiv of 2-mercapto ethanol in sodium phosphate buffer (50 mM, pH 7) containing 10% acetonitrile by final volume. Solutions were prepared using glass distilled, deionized water and 99+% pure sodium phosphate salts (no transition metals were added to the reactions). Reactions were 20 µL final volumes and were conducted under a headspace of air in sealed 500 µL Eppendorf tubes. Agarose gel electrophoresis was performed as described previously. The values in parentheses following the description of each lane below indicates the S-value (mean number of strand breaks per plasmid molecule) for each lane and was calculated using the equation S = -ln f1, where f1 is the fraction of plasmid in a given lane that is present as uncut, form I DNA. Values reported here represent the average of at least three experiments, and the standard error in these measurements is less than 2%. Lane 1, DNA alone (0.19); lane 2, 150 µM 116 alone (0.32); lane 3, 150 µM 102 alone (0.39); lane 4, 150 µM 133 alone (0.21); lane 5, 100 µM 116 + 1.5 mM thiol (0.89); lane 6, 100 µM 102 + 1.5 mM thiol (2.04); lane 7, 100 µM 133 + 1.5 mM thiol (0.34); lane 8, 150 µM 116 + 2.25 mM thiol (1.17); lane 9, 150 µM 102 + 2.25 mM thiol (2.5); lane 10, 150 µM 133 + 2.25 mM thiol (0.40); lane 11, 150 µM 116 + 1.5 mM thiol (1.05); lane 12, 150 µM 102 + 1.5 mM thiol (3.0); lane 13, 150 µM 133 + 1.5 mM thiol (0.43); lane 14, 2.25 mM thiol alone (0.22).

The DNA damage increased with the increase in concentrations of 133, 116 and 102. On the other hand, increase in thiol from 10 to 15 equivalents did not have
significant effect on the DNA cleavage. In line with our earlier work, 100 and 150 µM of compound 133 did not effect significant DNA damage. We suspect that the ejection of DNA damaging persulfides is a slow process in benzo model (133) compared to the aliphatic model compound (116), under our assay conditions. A careful look at the mechanism of the thiol mediated ring opening of compound 133 in parallel to the proposed mechanism for the 1,2-dithiolan 3-one 1-oxide heterocycle (102) would lead to the formation of an energetically unfavorable intermediate, in which a four membered thiolactone is fused to the benzene ring as shown in Scheme 6.18.

![Scheme 6.18](image)

**Scheme 6.18.** Reaction of thiol with 3H-1,2-benzodithiolan 3-one

Formation of benzothietone (135) as a reactive intermediate has been cited before. However, 135 was very unstable and found to undergo spontaneous decomposition to generate the corresponding dimeric product (137). With this in mind, we attempted to observe the dimerized product in the reaction of 3H-1,2-benzodithiolan 3-one (133) with 2-mercaptoethanol, to see if it produces 137. However, when 133 was treated with thiol, we did not observe the desired dimerized product (137). Moreover, the reaction of 133 with thiol was very slow as the starting 133 was observed to be more stable than the 1,2-dithiolan 3-one (116).

![Scheme 6.19](image)

**Scheme 6.19.** Dimerization of a benzothietone (135)
Taken together, our results suggest that the reaction of thiol with compound 133 may not go through the same mechanism as 1,2-dithiolan 3-one compounds (102 and 116) to eject the DNA damaging persulfide. We speculate that the crucial cyclization step to generate persulfide may not be possible in case of compound 133.

6.12 Conclusions

In conclusion, we have provided a possible chemical mechanism for the biological activity of the S-deoxy leinamycin. We find that the reaction of thiol with the model compound 116 leads to the generation of DNA-damaging reactive oxygen species. We propose that the attack of thiol on the 1,2-dithiolan 3-one heterocycle results in the ejection of persulfide via a thiolactone intermediate. The persulfide generated in this manner will result in the production of a) reactive oxygen species through its reaction with excess thiol and b) highly cytotoxic hydrogen sulfide. The mechanism of the reaction is analogous to the previously characterized release of persulfides from leinamycin and its synthetic analogs, though the production of ROS in case of S-deoxy analog (116) is lesser compared to the oxidized leinamycin analog (104). This is not surprising given the fact that leinamycin is more potent than the S-deoxy leinamycin. The potency of leinamycin may, in part, stem from its ability to alkylate DNA (Scheme 6.4), whereas a similar mechanism leading to the generation of highly reactive episulfonium ion intermediate is not possible in S-deoxy leinamycin.

Indeed, the model reaction between S-deoxy core molecule and thiol can explain the molecular basis for the overall biological activity of the S-deoxy leinamycin. Cellular oxidative stress is responsible for various diseases including parkinsons, alzheimers, neurodegenerative disorders and many others. The oxidative stress is caused by
the production of ROS inside the cells. Thus, the cytotoxicity of the S-deoxy leinamycin may stem from the thiol mediated generation of ROS and toxic hydrogen sulfide. However, we are aware of the fact that the production of ROS may not entirely account for the observed cytotoxicity of S-deoxy leinamycin. For example, reaction of nucleophiles at the electrophilic α,β,γ,δ unsaturated carbonyl on the left half of leinamycin may also contribute to the overall cytotoxicity. Alternatively, it is also possible that S-deoxy leinamycin can undergo an enzymatic oxidation at the sulfenyl sulfur to generate leinamycin in cells analogous to the last step in the biosynthesis of leinamycin proposed by Dr. Ben Shen and coworkers.51

Finally, the remarkable hydrolytic stability and a facile reactivity in presence of thiols, make 1,2-dithiolan 3-one heterocycle (116) an interesting alternative to selectively target the thiol rich interior of the cells. Nonetheless, compound 116 may serve as a delivery vehicle to supply biologically active reactive oxygen species, hydrogen sulfide and polysulfides to the interior of cells.

6.13 Experimental procedures

Materials and Methods

Reagents were of highest purity available and were used without further purification unless otherwise noted. Materials were purchased from the following suppliers: HPLC grade solvents, Fisher; silica gel 60 (0.04-0.063 mm pore size) for column chromatography, Merck; all other chemicals were purchased from Aldrich Chemical Company. Thin layer chromatography (TLC) was performed on plates from Aldrich Chemical Company, coated with general purpose silica containing UV254 fluorophore and
compounds were visualized by illumination of the plates with short-wave (254 nm) UV light. Water was distilled, deionized and glass redistilled. The oxidizing agent, dimethyl dioxirane (DMD), was freshly synthesized according to literature methods and stored at -18 °C. High Resolution Mass Spectrometry was performed at The Ohio State University Mass Spectrometry facility and the University of Illinois-Urbana Champaign. Low resolution mass spec. were performed at the University of Missouri–Columbia. NMR spectra were recorded using Bruker DRX 500, DRX 300, or ARX 250 MHz instruments at the University of Missouri–Columbia. HPLC spectra of compounds were recorded directly without an internal standard. The standard error in the HPLC peak area for 3 injections was estimated to be 2 ± 0.5 %.

**Ethyl-2,3-epoxy-2,3-dimethylbutanoate, 118.** To a stirred solution of ethyl-2-chloropropionate, (20 g, 0.15 mol) and dry acetone (8.47 g, 0.145 mol) at 0 °C under nitrogen was added a solution of potassium t-butoxide (18 g, 0.16 mol), in 220 mL of dry THF slowly over 45 min. The resulting pale yellow solution was stirred for 1 h at 0 °C and for further 4 h at 25 °C. The reaction mixture was then diluted with distilled water (150 mL) and extracted with diethyl ether (3 x 100 mL). The combined organic extracts were dried over anhydrous sodium sulfate, filtered and evaporated to yield a pale yellow solution. The solution was then vacuum distilled (62-65 °C, 0.1mm Hg) to give the epoxy ester 118 as colorless oil (14.82 g, 64.5%). \(^1\)H-NMR (CDCl3, 250 MHz) δ 1.30 (6H, t, J=7.2), 1.31 (3H, s), 1.37 (3H, s), 1.54 (3H, s), 4.23 (2H, q, J=7.1); \(^1^3\)C-NMR (CDCl3, 62.9MHz) δ 170.81, 63.43, 61.84, 60.91, 20.37, 19.63, 15.94, 13.98. The \(^1\)H and \(^1^3\)C NMRs matched the reported ones.\(^{20}\)
Sodium salt of 2,3-Epoxy-2,3-dimethylbutanoic acid, 113. A solution of epoxy ester, 118 (13.5 g, 85 mmol) in 70 mL absolute ethanol was stirred at 0 °C. To this was added finely chopped sodium metal (1.95 g, 85 mmol) in portions. Distilled water (1.5 mL, 85 mmol) was then added after all sodium pieces have been dissolved and the mixture was allowed to stir at 25°C for 15 h. The resulting orange-yellow solution was evaporated to yield a red orange oily gum. The gum was stirred vigorously with large volumes of hexane-ethyl acetate (1:1, v/v, 120 mL) mixture for about 2-3 h to give a pale yellow powder. The powder was filtered off, finely grounded and washed with diethyl ether. The resulting pale yellow solid was dried under vacuum for 3 h and then lyophilized overnight to give the sodium salt of epoxy acid 113, (11.2 g, 87%) as an off-white powder. ¹H-NMR (DMSO-d₆, 300MHz) δ 1.14 (3H, s), 1.16 (3H, s), 1.28 (3H, s). The ¹H and ¹³C NMRs matched the reported ones.²⁰

2-Hydroxy-3-benzylthio-2,3-dimethylbutanoic acid, 119. Finely chopped sodium metal (1.8 g, 82 mmol) was added to a solution of benzyl mercaptan (11.5 mL, 82 mmol) in dry THF (100 mL) and the resulting milky solution was stirred under dry nitrogen for 12 h. In another flask was taken sodium salt of epoxy acid 113 (5 g, 32.8 mmol) in dry THF (100 mL) and the solution was stirred under nitrogen for 1 h. To this was added titanium (IV) isopropoxide (24.4 mL, 82 mmol) via syringe and the resulting mixture was stirred for 1 h and then transferred to the flask containing benzyl mercaptan using a canula under inert argon atmosphere. The resulting pale yellow solution was then stirred for 24 h at 25 °C and then refluxed for 3 h at 82 °C under nitrogen, to give a yellow solution. This was allowed to cool to 25 °C and then acidified with 5% sulfuric acid (250 mL) to pH 2. Diethyl ether (250 mL) was
added and the mixture was separated. The aqueous layer was extracted with diethyl ether 
(3 x 100 mL) and the combined organic extracts dried over anhydrous sodium sulfate, 
filtered and evaporated to yield a yellow oily gum. Hexane was added to this gum and 
the resulting yellow solid was separated by filtration and re-crystallized from hot hexane 
to give a yellow crystals. This was then dried under vacuum to yield 119, as pale yellow 
crystals (4.9 g, 50.5%). 1H-NMR (CDCl₃, 250 MHz) δ 1.50 (3H, s), 1.52 (3H, s), 1.54 
(3H, s), 3.89 (2H, s), 7.23 (5H, m); 13C-NMR (CDCl₃, 62.9 MHz) δ 177.26, 137.35, 129.03, 128.63, 127.23, 80.45, 53.14, 34.66, 25.09, 24.78, 22.11. The 1H and 13C NMRs 
matched the reported ones.20

2-hydroxy-3-mercapto-2,3-dimethylbutanoic acid , 114. Liquid ammonia (75 mL) was 
condensed into a flame dried flask with a dry ice/acetone condenser. Finely chopped sodium metal (2.2 g, 96 mmol) was added to the liquid 
ammonia followed by the addition of compound 119 (3.5 g, 13.8 
mmol). The resulting deep blue solution was allowed to stir for 1.5 h in presence of dry 
ice/acetone condenser. Solid ammonium chloride was then added to the reaction mixture 
until the hydrogen evolution ceased and the excess ammonia was evaporated by a stream 
of nitrogen gas. The resulting white residue was dissolved in distilled water and then 
acidified to pH 2 using concentrated hydrochloric acid. Ethyl acetate (75 mL) was added 
and the organic layer was separated. The aqueous layer was extracted with ethyl acetate 
(3 x 50 mL) and the combined organic extracts dried over anhydrous sodium sulfate. The 
solution was then filtered and evaporated to yield a pale yellow gum. The gum was 
stirred with hexane (15 mL) for 1 h and the resulting white powder was filtered and 
vacuum dried to yield 114 (1.81 g, 80%) as a fine white powder. 1H-NMR (300 MHz,
CDCl₃) δ 1.51 (3H, s), 1.53 (3H, s), 1.55 (3H, s), 2.09 (1H, s); ¹³C-NMR (CDCl₃, 75.47 MHz) δ 178.23, 79.73, 51.30, 28.05, 27.77, 21.52. The ¹H and ¹³C NMRs matched the reported ones.²⁰

3-hydroxy-3,4,4,-trimethylthietan-2-one, 115. A mixture of compound 114 (0.3 g, 1.82 mmol), DCC (0.41 g, 2 mmol), and DMAP (10 mg) was stirred in dry THF (40 mL) at 25 °C under dry nitrogen for 2 days. The DCU was filtered off and the crude product was purified by a flash column chromatography (11:1, Hexane-EtOAc) to yield 115 (170 mg, 64%) as a colorless oil. ¹H-NMR (CDCl₃, 250 MHz) δ 1.57 (3H, s), 1.63 (3H, s), 1.73 (3H, s), 3.71 (1H, s); ¹³C-NMR (CDCl₃, 62.9 MHz) δ 198.59, 94.97, 54.74, 27.48, 24.64, 21.21. The ¹H and ¹³C NMRs matched the reported ones.²⁰

4-hydroxy-4,5,5-trimethyl-1,2-dithiolan-3-one, 116. A stirred solution of 115 (100 mg, 0.685 mmol) in dichloromethane (12 mL) was maintained at –78 °C and hydrogen sulfide gas was constantly bubbled through the solution. Triethylamine (0.2 mL, 1.37 mmol) was added and the resulting solution was constantly saturated with H₂S gas for 5 h at –78 °C. After that the reaction mixture was allowed to warm to 25 °C and the excess hydrogen sulfide was removed by a stream of nitrogen gas to leave a pale yellow solid (this step must be carried out in a well ventilated hood as H₂S gas is highly toxic). The resulting yellow solid was stirred in methanol (12 mL) followed by a slow addition of aqueous iron (III) chloride (0.05 M, 10 mL) over 20 min. The resulting dark solution was allowed to stir for 1 h at 25 °C. Diethylether (15 mL) was added and the organic layer was separated. The aqueous layer was extracted with diethyl ether (3 x 15 mL) and the combined extracts dried over anhydrous Na₂SO₄,
filtered and evaporated to yield a yellow oil that was purified by a flash column chromatography (11:1 Hexane/EtOAc) to yield 116 as yellow liquid that turned to solid upon standing (77 mg, 63%). $^1$H-NMR (CDCl$_3$, 250 MHz) $\delta$ 1.40 (3H, s), 1.48 (6H, s), 3.0 (1H, s); $^{13}$C-NMR (CDCl$_3$, 62.9 MHz) 208.1, 83.2, 58.0, 22.2, 22.0, 18.9. The $^1$H and $^{13}$C NMRs matched the reported ones.$^{20}$

**Synthesis of 2-Hydroxy-3-mercapto-2,3-dimethyl (S-2-hydroxyethyl) butanethioate (124):** To a stirred solution of compound 116 (20 mg, 0.112 mmol) in water (410 µL) and acetonitrile (450 µL) was added sodium phosphate buffer (1350 µL, 500 mM, pH 7.5) and 2-mercapto ethanol (40 µL) and the resulting colorless solution was stirred at 25 °C (final concentrations: 116, 50 mM; buffer, 300 mM; thiol, 250 mM; acetonitrile, 20% by volume). The reaction was allowed to stir for 1 h as the disappearance of the starting material was monitored using TLC (1:1 hexane/EtOAc). The product was extracted into diethyl ether (3 x 5 mL) and the combined organic extracts dried over anhydrous sodium sulfate, filtered and evaporated to yield a yellow oil, which was then purified by a flash column chromatography (7:3 hexane/EtOAc) to yield compound 124 (13.4 mg, 54%) as colorless oil. Rf = 0.25 (7:3 hexane/EtOAc). $^1$H NMR (CDCl$_3$, 500 MHz) $\delta$ 1.46 (3H, s), 1.48 (3H, s), 1.53 (3H, s), 1.86 (1H, t, OH), 1.97 (1H, s), 3.05 (2H, m), 3.35 (1H, s), 3.77 (2H, m); $^{13}$C-NMR (CDCl$_3$, 125.7 MHz) 205.76, 84.12, 61.69, 52.37, 31.68, 28.54, 27.33, 21.90. MS (ESI) [M+H]$^+$ 224.90.

**Synthesis of 2-Hydroxy-3-methylthio-2,3-dimethyl (S-2-hydroxyethyl) butanethioate (125):** To a stirred solution of compound 116 (16 mg, 0.09 mmol) in water (328.5 µL) and acetonitrile (360 µL) was added sodium phosphate buffer (1080 µL, 500 mM, pH 7.5) and 2-mercapto ethanol (31.5 µL) and the resulting colorless solution was stirred at
25 °C (final concentrations: 116, 50 mM; buffer, 300 mM; thiol, 250 mM; acetonitrile, 20% by volume). The reaction was allowed to stir for 3 h to make sure that the entire starting S-deoxy molecule has reacted. Methyl iodide was added to this reaction mixture and the colorless reaction mixture was allowed to stir at 25 °C for 10 h. The resulting yellow solution was then extracted into diethyl ether (3 x 5 mL) and the combined organic extracts dried over anhydrous sodium sulfate, filtered and evaporated to yield a yellow oil, which was then purified by a flash column chromatography (1:1 hexane/EtOAc) to yield compound 125 (12.6 mg, 60%) as colorless oil. $^1$H NMR (CDCl$_3$, 500 MHz) δ 1.46 (3H, s), 1.51 (3H, s), 1.53 (3H, s), 1.97 (1H, s, broad), 2.27 (3H, s), 2.92 (2H, dt, J = 2 Hz, J = 6 Hz), 3.48 (1H, s), 3.89 (2H, s, broad); $^{13}$C-NMR (CDCl$_3$, 125.7 MHz) δ 205.76, 84.89, 60.41, 57.75, 42.91, 24.14, 24.04, 22.39, 12.10.

**HPLC detection of polysulfides:** Compound 116 was incubated (10 µL of a 10 mM stock in CH$_3$CN) at 25 °C in a mixture of sodium phosphate buffer (50 µL, 500 mM, pH 7), water (333 µL) and acetonitrile (90 µL). To this was added 2-mercaptoethanol (17 µL of a 30 mM stock) as the final component. The mixture (final concentrations: 116, 200 µM; buffer, 50 mM, pH 7; thiol, 1 mM; acetonitrile, 20% by volume) was vortex mixed and analyzed by reverse phase HPLC. The separation was effected using a C$_{18}$ reverse phase Microsorb column (100 Å sphere size, 5 µm pore size, 25 mm length, 10 mm id) eluted with a linear gradient of 100:0 to 61.5:38.5 water/acetonitrile over 40 min, followed by a linear gradient of 61.5:38.5 to 0:100 water/acetonitrile over 20 min and a final elution with 0:100 water/acetonitrile for 20 min. An authentic sample of polysulfides was generated under identical conditions by the reaction of thiol with 3H-1,2-benzodithiol-3-one 1-oxide (102) and separated using the same method as above.

247
Qualitative detection of hydrogen sulfide in the reaction of 116 with thiol: To a stirred solution of 116 (60 mg, 0.33 mmol) in a mixture of acetonitrile (1020 µL) and sodium phosphate buffer (2024 µL, 500 mM, pH 7.5) was added 2-mercaptoethanol (356 µL). Hydrogen sulfide was produced immediately and the generated gas swept from the reaction mixture by passing a stream of nitrogen gas through the solution (final concentrations: 116, 100 mM; buffer, 300 mM; thiol, 1.5 M, 15 equiv; acetonitrile, 30% by volume) and the effluent bubbled into a solution of lead acetate (350 mg in 5 mL water, 0.184 mM) to yield a black lead sulfide (PbS) precipitate. The black precipitate was collected, dried under vacuum and analyzed qualitatively to confirm the identity of lead sulfide. For example, the black precipitate was converted to a white solid (PbSO₄) upon boiling in concentrated nitric acid. Similarly, boiling in H₂O₂ (30%) converted the black precipitate into white PbSO₄; see: Vogel, A.I. *Vogel’s Textbook of Macro and Semimicro Quantitative Inorganic Analysis*; Orient Longman Ltd, London: 1985, 195. Control reactions were carried out in exactly the same manner with 116 alone or 2-mercapto ethanol alone.
References


250


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The ill spot from the column in the rxn of S-deoxy JDI w/ 5 eq. thiol under
(the major product)
VITA

Santhosh was born in Coimbatore, India and grew up in Trichy, India. He was initially educated at the E.R. Higher Secondary School (Trichy) followed by the St. Joseph’s College, Trichy, where he received his Bachelors degree in Chemistry. He obtained his Masters degree in Chemistry with a specialization in Organic chemistry from the Indian Institute of Technology, Bombay (I.I.T.-Bombay) in Mumbai, India. He then joined Prof. Kent S. Gates to pursue his Ph.D. at the University of Missouri–Columbia in Bio-organic chemistry. He completed his MS and Ph.D in March 2008 under the guidance of Prof. Kent Gates. He is currently working in the laboratory of Prof. Ortiz de Montellano at the department of pharmaceutical chemistry in the University of California–San Francisco (UCSF), towards gaining some postdoctoral research experience. Besides research, Santhosh enjoys listening to music, outdoor activities and spending quality time with friends and family.