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# Thiol Specific Fluorogenic Agents for Live Cell Subcellular Organelle Thiol Imaging

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# THIOL SPECIFIC FLUOROGENIC AGENTS FOR LIVE CELL SUBCELLULAR ORGANELLE THIOL IMAGING

BY

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy in Pharmaceutical Sciences degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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# ABSTRACT

THIOL SPECIFIC FLUOROGENIC AGENTS FOR LIVE CELL SUBCELLULAR ORGANELLE THIOL IMAGING

YAHYA ALQAHTANI

2019

Thiols or sulfhydryl groups (-SH) play a significant role in protein structures and functions. The unique properties of a thiol group are the basis for its roles in cellular functions. Structurally, thiols are divided into protein thiols (PSH) and non-protein thiols (NPSH). NPSH include glutathione, cysteine, and homocysteine. Thiols are distributed in an uneven manner in the cell and subcellular organelles. Disturbance of thiol levels has been associated with serious diseases such as cancer, aging, cardiovascular diseases, Alzheimer disease, liver damage, and edema.

The significant roles of thiols in the biological system have stimulated the development of various approaches to monitor and detect thiols. These approaches include enzymatic assays, colorimetric assays, gel electrophoresis, LC/MS, and HPLC. The major drawback of these conventional analytical approaches is that these methods require the cell to be homogenized before analysis. The breakage of cells is time-consuming and can cause a loss of information. Thus, developing an approach to determine thiol status in intact live cells will provide a unique advantage over the conventional analytical methods.

Fluorescence microscopy has been successfully used in determining analytes in intact live cells. The main challenge for using fluorescence microscopy in detecting thiols

is to turn thiols into fluorescent molecules for detection. This challenge results in the necessity to develop fluorescent/fluorogenic agents that exhibit selectivity and specificity for thiols and are capable of turning thiols to fluorescent molecules to facilitate detection and quantification. Most of our current knowledge on thiols' functions/dysfunctions at cellular or subcellular levels were derived from the data obtained from conventional analytical methods that involve breakage of cells. A fluorescence probe that can be used to detect subcellular thiols in live cells will be a valuable tool to provide better understanding of thiols' roles in the function and dysfunction of the subcellular organelles.

In this dissertation, we will present our work related to the development of thiol specific fluorogenic agents for cell surface thiol imaging and lysosomal thiol imaging in live cells. The rational design, synthesis, and determination of TBONES and TBOZEN as thiol specific fluorogenic reagents for cell surface thiol imaging in live cells will be presented. However, TBONES and TBOZEN failed to image cell surface thiols due to their inability to react with PSH. Interestingly, TBONES and TBOZEN turned out to be able to image thiols in lysosomes in live cells.

Based on the work of TBONES and TBOZEN, we designed TIMBOS as a thiol specific and fluorogenic agent for lysosomal thiol imaging in live cells. TIMBOS was successfully synthesized and characterized to be thiol specific and fluorogenic. TIMBOS was demonstrated to effectively image NPSH in lysosomes in live cells and to detect NPSH change in lysosomes in a quantitative manner.

In summary, we have synthesized and characterized three rationally designed thiol specific fluorogenic reagents. These three reagents were able to image NPSH and to detect NPSH change in a quantitative manner in lysosomes in live cells. These reagents will be useful tools in exploring the roles of lysosomal thiols in cellular function/dysfunction.

# CHAPTER 1. GENERAL INTRODUCTION AND DISSERTATION OVERVIEW

## 1.1 Thiols and the roles of thiols in the biological system

Thiol molecules are organic compounds that contain the sulfhydryl (-SH) group. A thiol group or thiolate plays a crucial role in the biological system due to its unique chemical properties that cannot be found with any other organic functional groups. These chemical properties include being a strong nucleophilic group, an excellent reducing group, and an exceptional chelating group that contribute to detoxify heavy metals. These unique properties are the basis for the role of thiols in a variety of cellular functions including enzyme catalytic reactions, termination of toxic reactive electrophiles, termination of reactive oxygen or nitrogen species, chelation of heavy metals, involvement in signal transduction, and cell division[1, 2]. In addition, the pair of thiols and disulfides, the oxidized from of thiols, is one of the major redox buffers in the cell to reflect cellular redox status. The cell maintains its highly reducing status through maintaining a high level of thiols.[3] Moreover, the disulfide bond contributes to the stabilizing and folding of proteins' secondary, tertiary, and quaternary structures[4].

## 1.2 Thiol distribution in the biological system

Thiols are present throughout the cell. They are also present in different extracellular compartments[5]. Intracellularly, thiols are unevenly distributed in various subcellular organelles such as mitochondria, nuclei, cytosol, and cell surface[6]. Structurally, cellular thiols are divided into protein thiols (PSH) and non-protein thiols (NPSH). NPSH include cysteine, homocysteine, thiol-containing small peptides such as glutathione which is the most abundant thiol molecule in cells (Figure 1.1).

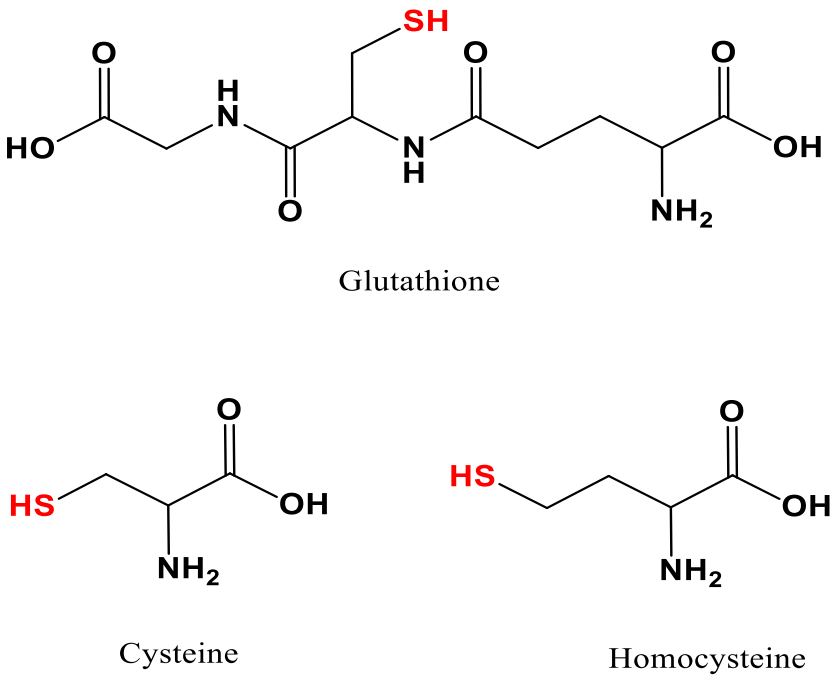



**Figure 1.1** Chemical structures of glutathione, cysteine and homocysteine.

### 1.3 Alteration of thiol levels

Thiol concentrations in the biological system can be easily changed by various factors such as cellular oxidative stress that formed by reactive oxygen species, reactive nitrogen species, or presence of reactive electrophiles[7]. Numerous reports have showed that disturbance of thiol levels is associated with various diseases and disorders such as aging, neurodegenerative disorders, cardiovascular disease, and cancer[8]. In particular, a decrease in GSH level has been linked with cancer, aging and heart diseases. Also a deficiency in cysteine concentration has been found to lead to neurodegenerative diseases and liver damage while an increase in homocysteine level is a potential reason for cardiovascular diseases and Alzheimer disease[8-16].

## 1.4 Detection methods of biological thiols

The significant roles of thiols in the biological system have stimulated the development of various approaches to monitor and detect thiols. The conventional analytical methods for thiol assays include enzymatic assays[17], colorimetric assays[18], gel electrophoresis[19], liquid chromatography-mass spectrometry (LC/MS)[20], and high-performance liquid chromatography (HPLC) [13, 21]. The previous methods have been used to determine thiol levels at the tissue, cellular, and subcellular levels. The methods require breakage of biological samples prior to the analysis. Most of our knowledge on thiols' roles at the subcellular levels was derived from isolation of thiols from the subcellular structure through breaking the subcellular structure followed with one of these conventional analytical methods. These methods have several limitations including being time-consuming and the potential for loss of information caused by cell breakage and sample preparation[22]. Thus, developing an approach to determine thiol status in intact live cells will provide unique advantages over the conventional analytical methods. Fluorescence microscopy has been successfully used in determining an analyte in intact live cells and therefore is a potential analytical method for detection of thiols in live cells.

## 1.5 Fluorescence microscopy for thiol imaging.

In comparison with the conventional analytical methods, detecting thiols in live cells through fluorescence microscopy has the advantage of allowing us not only to quantify thiols but also to visualize them in their intact and natural physiological environment and expose information that cannot be obtained by the conventional methods[23]. However, the major challenge in detecting thiols in live cells through fluorescence microscopy is a lack of a reagent that can selectively, ideally specifically,

turn thiols into fluorescent molecules for fluorescence imaging at the cellular or subcellular level.

### 1.6 Fluorescent/fluorogenic reagents for thiols

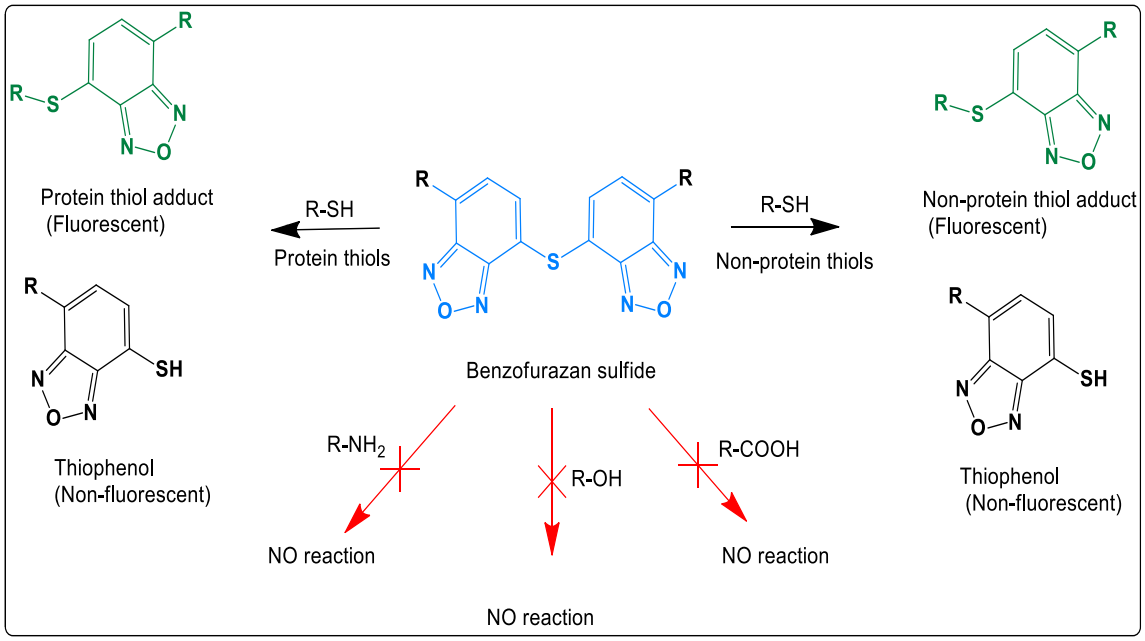
A fluorescent reagent is an agent that itself exhibits fluorescence. A fluorogenic reagent is a reagent that itself exhibits no fluorescence and turns into a fluorescent molecule after reacting with the analyte of interest. A fluorogenic reagent is usually preferred for imaging an analyte in live cells due to less fluorescence interference by the reagent.

There are various reagents reported for imaging thiols in cellular and subcellular organelles in live cells through fluorescence microscopy. These reagents include monochlorobimane, chloromethyl fluorescein, *O*-phthaldialdehyde, rosamine-based, rhodamine-based, naphthalimide-based, maleimide, and iodoacetamide dyes[24-28]. These reagents have been designed to exploit the strong nucleophilicity of the thiol group. The reaction between these reagents and thiol molecules involves different mechanisms such as Michael addition, cyclization with aldehyde, nucleophilic substitution, cleavage of disulfide by thiol and others[29-31]. Limitations associated with these reagents have been reported to include a lack of selectivity or a slow reaction rate[6, 24, 26, 32].

Therefore, developing reagents that can effectively and specifically image and quantify thiols at the cellular or subcellular level in intact live cells through fluorescence microscopy is still of great interest. These fluorescence probes can be a valuable tool in exploring thiols' roles in the function and dysfunction in the biological system.

## 1.7 Benzofurazan sulfide-based thiol specific fluorogenic agents

4-Chloro-7-nitrobenzofurazan is a commercially available fluorogenic agent that has been used to detect different nucleophilic functional groups such as $–NH_2$, -SH, -OH, -COOH, and generate their corresponding fluorescent compounds[33-37]. Our research group has used 4-chloro-7-nitrobenzofurazan to develop thiol specific fluorogenic agents for thiol imaging in live cells through fluorescence microcopy. We have developed a symmetric benzofurazan sulfide (Scheme 1.1) that exhibits minimum fluorescence and reacts specifically with thiols through a **thiol specific thiol-sulfide exchange reaction** to produce corresponding fluorescent thiol adducts (Scheme1.1)[38]. Based on this, we have successfully developed thiol specific fluorogenic agents that can be used to image total cellular thiols [38], total cellular NPSH [39], and NPSH in mitochondria [40] in live cells through fluorescence microcopy (Figure 1.2).




**Scheme 1.1** Symmetric benzofurazan sulfide as thiol specific and fluorogenic agents.

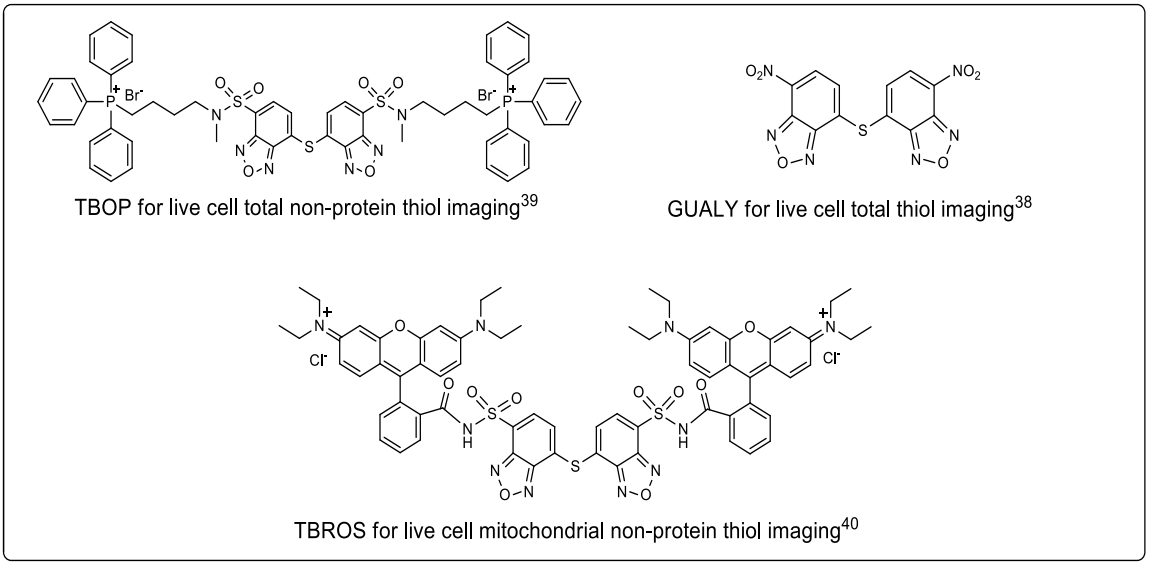



**Figure 1.2** Chemical structures of our early reported thiol specific fluorogenic symmetric benzofurazan sulfides.

The aim of this dissertation was to develop thiol specific fluorogenic agents for live cell subcellular thiol imaging by adding a subcellular targeting moiety to the basic symmetric benzofurazan sulfide structure (Figure 1.3). Specifically, we would like to develop thiol specific fluorogenic agents for cell surface thiol imaging and lysosome thiol imaging.

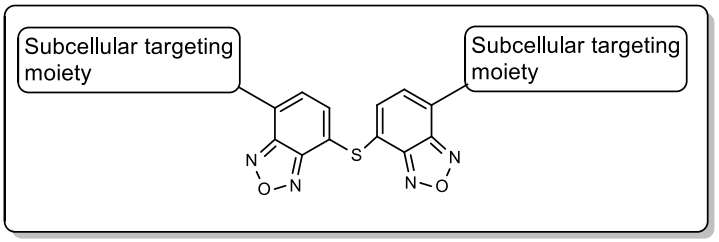



**Figure 1.3** Designed subcellular targeting thiol specific fluorogenic reagents.

Therefore, in this dissertation, Chapter 2 describes the rational design, synthesis, and determination of TBONES and TBOZEN as thiol specific fluorogenic reagents for cell surface thiol imaging in live cells. We have successfully synthesized TBONES and TBOZEN and demonstrated that they were thiol specific and fluorogenic. However, TBONES and TBOZEN failed to image cell surface thiols due to their inability to react with PSH. Interestingly, TBONES and TBOZEN turned out to be able to image thiols in lysosomes in live cells.

Chapter 3 is devoted to the full characterization of TBONES and TBOZEN as effective reagents to image NPSH in lysosomes in live cells through fluorescence microscopy.

Chapter 4 describes the rational design, synthesis, and validation of TIMBOS as a thiol specific fluorogenic reagent for imaging NPSH in lysosomes in live cells through fluorescence microscopy.

In summary, we have synthesized and characterized the rationally designed reagents for cell surface thiol imaging. These reagents failed to image cell surface thiols due to their inability to react with PSH. We have developed three thiol specific and fluorogenic reagents for NPSH imaging in lysosomes in live cells. These three reagents will be useful tools in exploring the roles of lysosomal thiols in cellular function/dysfunction.

# CHAPTER 2. DESIGN, SYNTHESIS, AND DETERMINATION OF NOVEL THIOL SPECIFIC FLUOROGENIC REAGENTS FOR IMAGING CELL SURFACE THIOLS IN LIVE CELLS

## 2.1 Introduction

### 2.1.1 Significance of cell surface thiols

Thiols have a significant role in the structure and the function of the cells. They are involved in many crucial physiological/pathophysiological events. Thiols are present throughout the cell: on the cell surface, intracellularly and extracellularly. Thiols on the cell surface are very significant for the cell function by serving as part of proteins structures of transporters, receptors, and enzymes[5].

Thiols on the cell surface are in dynamic movement between the two different forms of thiols: the reduced form as most thiols in intracellular parts [41] or the oxidized form as most thiols in extracellular parts[42]. Thiols on the cell surface are susceptible to be modified by exterior oxidants or reductants. Consequently, cell surface thiols have been denoted as probes of the environmental redox state[43, 44]. The ratio of thiols: disulfides is a main indicator to demonstrate the status of cellular oxidative stress[45].

Thiols on the cell surface are easily affected by oxidative stress which can influence both health and disease statuses[5]. A probe that can image thiol distribution and determine thiol concentration on the cell surface in live cells will be a useful tool in exploring how cells respond to oxidative stress factors and facilitate the development of treatment for the diseases that rise from thiol disturbance on the cell surfaces. Unfortunately, limited reagents are available to detect thiols on the cell surface[46]. The

limitation of an analytical method to determine cell surface thiols hampers our understanding of the roles of cell surface thiols in cellular function and dysfunction.

### 2.1.2 Design of thiol specific fluorogenic agents for cell surface thiols

In order to image cell surface thiols, we need to design a molecule that can turn cell surface thiols into a fluorescent molecule, but the molecule itself should not enter the cell. In other words, it is cell membrane impermeable. It is known that a molecule with more than one acidic function group will have difficulty penetrating the cell membrane. Based on this, the design of thiol specific fluorogenic agents for cell surface thiol imaging involves symmetric addition of four sulfonic acid functional groups to the benzofurazan sulfide structure (Figure 2.1). TBONES and TBOZEN were designed based on this principle (Figure 2.1). These molecules were expected to exhibit no fluorescence themselves and react with a thiol molecule to form fluorescent thiol adducts. Figure 2.2 uses TBOZEN to demonstrate the proposed reaction of these compounds with thiols on the cell surface resulting in a fluorescent thiol adduct for fluorescence detection.

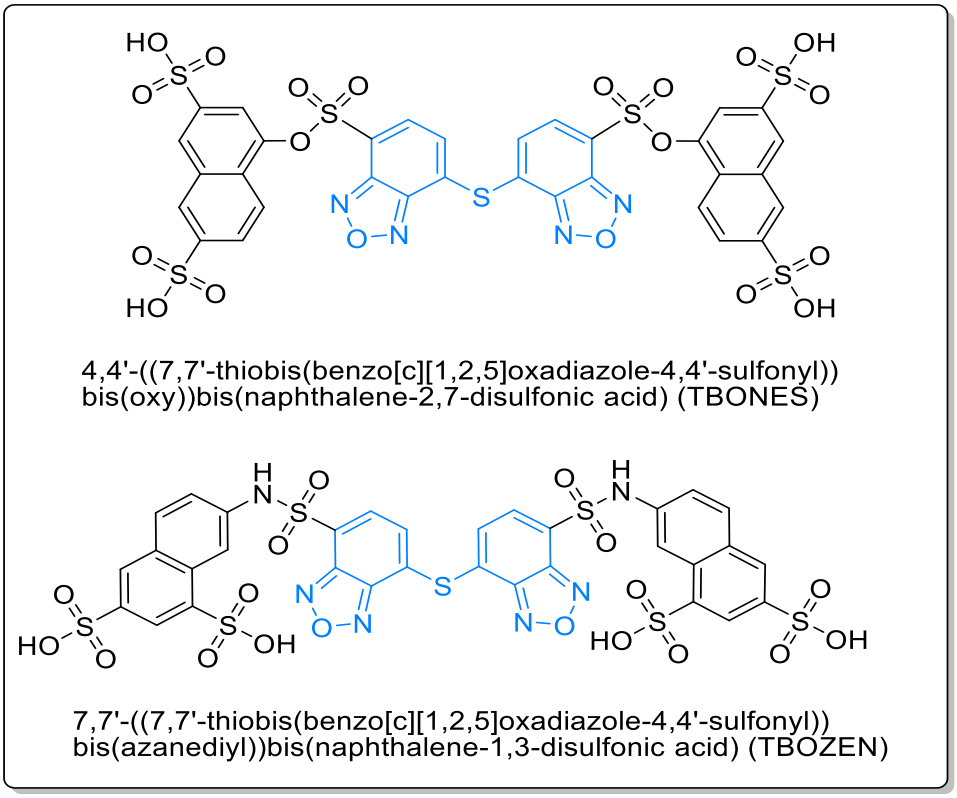



**Figure 2.1** Chemical structures of rationally designed thiol specific fluorogenic agents for cell surface thiol imaging.

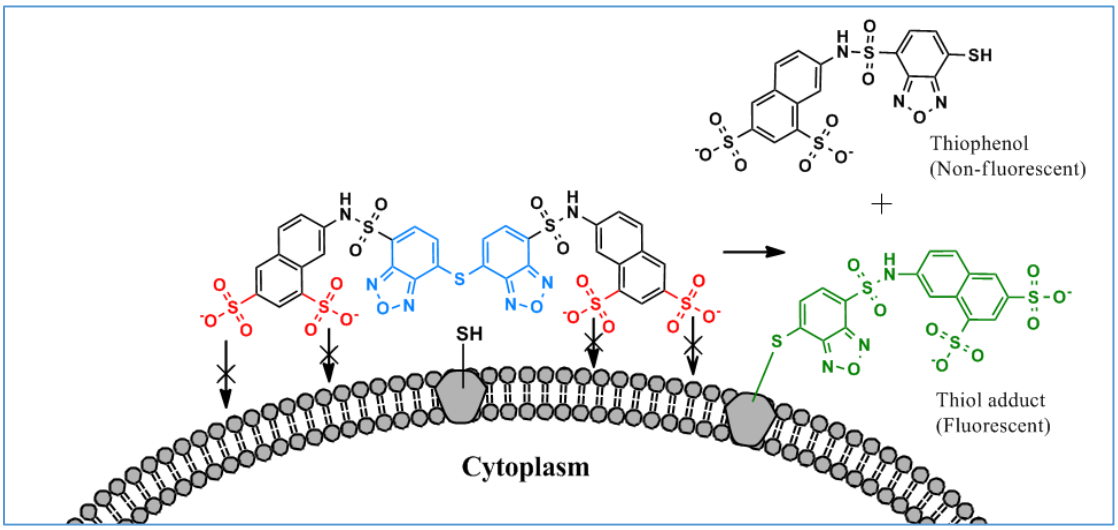



**Figure 2.2** Schematic drawing illustrating TBOZEN's reaction with thiols on cell surface to form fluorescent thiol adducts

## 2.2 Experimental section

### 2.2.1 Materials and Instruments

Unless otherwise stated, all chemical reagents and solvents were obtained from commercial sources and used without further purification. 4-Hydroxy-2,7-naphthalenedisulfonic acid disodium salt, 7-amino-1,3-naphthalenedisulfonic acid, sodium hydrosulfide hydrate, and sodium carbonate were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-Chloro-7-chlorosulfonyl-2,1,3-benzoxadiazole was purchased from TCI (Portland, OR, USA). Dulbecco's phosphate-buffered saline (Gibco™DPBS, no calcium, no magnesium,1x) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Sulfosalicylic acid (SSA) cell lysis solution was prepared as a 3% (w/v) solution in deionized water containing 0.1% (v/v) Triton X-100.

Flash column chromatography was carried out on a W-Prep 2XY Yamazen Dual Channel Flush Chromatography System (San Bruno, California). $^{1}$HNMR spectra were recorded on a Bruker Varian 600 MHZ spectrometer in a deuterated solvent as indicated. All NMR peaks were given as chemical shift in parts per million relative to DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) as the internal standard. Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and brs (broad singlet). *J* values are given in Hz. Low resolution mass spectra (LRMS) were obtained on a Thermoquest Finnigan LCQ Deca Mass Spectrometer (Waltham, MA, USA), and high-resolution mass spectra (HRMS) were acquired on a Bruker Daltonics SolariX 12 tesla Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (Department of Chemistry, University at Buffalo, NY).

Fluorescence properties were determined on a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, California). Fluorescence microscopy images were obtained on an upright fluorescence microscope (Zeiss AXIO Imager A1) equipped with a camera (AxioCam MRc5) (Zeiss, United States) or on Olympus FV1200 Scanning Confocal Microscope.

HPLC analysis was achieved on an Agilent HPLC system connected with Agilent 1100 fluorescent detector and a Diode Array Detector. The HPLC analysis condition utilized an Apollo C18 column (250 mm x 4.6 mm i.d., 5 μm) with a mobile phase system consisting of solvent A (aqueous solvent) and solvent B (acetonitrile) with a flow rate of either 1 mL/min or 0.5 mL/min. The mobile phase started with 10% of solvent B for 10 min and then increased to 50% of solvent B in 10 min for TBONES or 10% of solvent B and then increased to 60% of solvent B in 25 min for TBOZEN. The injection volume was 5 μL or 10 μL. The wavelength of 254 nm was used for UV detection and the wavelengths of 400 nm and 540 nm were used as the excitation and emission wavelengths for fluorescence detection of TBONES, or 350 nm and 450 nm for TBOZEN.

### 2.2.2 Cell culture

Human lung cancer cells NCI-H226 ( National Cancer Institute) were cultured in the RPMI 1640 growth medium provided with 10% FBS, 100 units/mL of penicillin (mediatech, Inc., Herndon, VA), and 100 μg/mL of streptomycin (Mediatech, Inc., Herndon,VA) in a humidified atmosphere containing 5% $CO_2$ at 37 ℃. Cell numbers were determined on a Nexcelom Cellometer Auto T4 Automated Cell Counter (Lawrence, MA, USA). Cell viability was determined by a Trypan blue assay.

### 2.2.3 Synthesis of TBONES

***4-(((7-Chlorobenzo[c][1,2,5] oxadiazol-4-yl)sulfonyl)oxy)naphthalene-2,7-disulfonic acid*** **(3**). To a stirred solution of 4-chloro-7-chlorosulfonyl-2,1,3-benzoxadiazole (**1**) (0.727 g, 2.86 mmol) in acetonitrile (20 mL) was added dropwise a mixture of 4-hydroxy-2,7-naphthalenedisulfonic acid disodium salt (**2**) (0.500 g, 1.43 mmol) and $Na_2CO_3$ (0.212g, 2 mmol) in deionized water (5 mL). The mixture was kept stirring in an ice bath for 2 h before the solvents were removed by a rotary evaporator under reduced pressure. The resulting residue was purified through a C18 column (water/acetonitrile/trimethylamine) to produce the desired compound (**3**) as an oily yellow product (0.580 g, 78%). The product was characterized by $^1$H NMR and MS. $^1$H NMR (600 MHz, $D_2O$) δ 8.30 (s, 1H), 8.25 (s, 1H), 7.97 (d, *J* = 7.4 Hz, 1H), 7.78 (q, *J* = 9.1 Hz, 2H), 7.53 (d, *J* = 7.4 Hz, 1H), 7.50 (s, 1H). MS (ESI) of $C_{16}H_8N_2O_{10}S_3Cl$ (M-H): 518.92.

***4,4'-((7,7'-Thiobis(benzo[c][1,2,5]oxadiazole-4,4'-sulfonyl))bis(azanediyl))bis (naphthalene-2,7-disulfonic acid)(TBONES).*** An aqueous solution (1 mL) of sodium hydrosulfide (0.112 g, 2 mmol) was added dropwise to a stirred solution of **3** (0.580 g, 1.11 mmol) in water (5 mL) under argon at room temperature. The mixture was stirred for 2 h before the water was removed under reduced pressure by a rotary evaporator to produce a yellow residue. The residue was purified through a C18 column (water/acetonitrile/trimethylamine) to yield TBONES as an oily yellow product (0.320 g, 29%). The product was characterized by $^1$H NMR and HRMS. The purity of the product was confirmed by HPLC to be > 97%. $^1$H NMR (600 MHz, $D_2O$) δ= 8.44 (s, 2H), 8.39 (s, 2H), 8.27 (d, *J* = 7.2 Hz, 2H), 8.01 (d, *J* = 8.8 Hz, 2H), 7.96 – 7.91 (m, 2H), 7.87 (d, *J* =

7.2 Hz, 2H), 7.62 (d, *J* = 1.2 Hz, 2H). HRMS calculated for $C_{32}H_{17}N_4O_{20}S_7$ (M-H) 1000.8487; found 1000.8488.

### 2.2.4 Synthesis of TBOZEN

***7-(7-Chlorobenzo[c] [1,2,5] oxadiazole-4-sulfonamido) naphthalene-1,3-disulfonic acid (3).*** To a stirred solution of 4-chloro-7chlorosulfonyl-2,1,3-benzoxadiazole (**1**) (0.830 g, 3.3 mmol) in acetonitrile (20 mL) was added dropwise a mixture of 7-Amino-1,3-naphthalenedisulfonic acid (2) (0.500 g, 1.65 mmol) and $Na_2CO_3$ (0.173g, 1.64 mmol) in deionized water (5 mL). The mixture was stirred in an ice bath for 8 h before the solvents were removed by a rotary evaporator under reduced pressure. The resulting residue was purified through a C18 column (water/acetonitrile/trimethylamine) to obtain the desired compound (**3**) as an oily yellow product (0.640 g, 75%). The product was characterized by $^1$H NMR and HRMS. $^1$H NMR (600 MHz, $D_2O$) δ 8.46 (s, 1H), 8.40 (s, 1H), 8.27 (d, *J* = 7.4 Hz, 1H), 8.21 (s, 1H), 7.67 (d, *J* = 8.9 Hz, 1H), 7.56 (d, *J* = 7.4 Hz, 1H), 7.31 (d, *J* = 8.9 Hz, 1H). Calculated HRMS for $CH_9ClN_3O_9S_3^-$ (M-H) is 517.9195; found 517.9192.

***7,7'-((7,7'-Thiobis(benzo[c] [1,2,5] oxadiazole-4,4'-sulfonyl)) bis(azanediyl)) bis (naphthalene-1,3-disulfonic acid) (TBOZEN).*** An aqueous solution (1 mL) of sodium hydrosulfide (0.100g, 1.78 mmol) was added dropwise to a stirred solution of **3** (0.500 g, 0.96 mmol) in water (5 mL) under an inert atmosphere of argon gas at room temperature. The mixture was stirred for 2.5 h before the water was removed under reduced pressure by a rotary evaporator to produce a yellow residue. The resulting residue was purified through a C18 column (water/acetonitrile/trimethylamine) to obtain the desired product (TBOZEN) as an oily yellow product (0.354 g, 37%). The product was characterized by

$^1$H NMR and HRMS. The purity of the product was confirmed by HPLC to be > 97%. $^1$H NMR (600 MHz, $D_2O$) δ 8.52 (d, *J* = 1.4 Hz, 2H), 8.36 (d, *J* = 1.7 Hz, 2H), 8.20 (d, *J* = 7.3 Hz, 2H), 7.94 (s, 2H), 7.28 (d, *J* = 6.5 Hz, 4H), 7.18 (d, *J* = 8.2 Hz, 2H). HRMS calculated for $C_{32}H_{19}N_6O_{18}S_7^-$ $(M-H)^-$, 998.8806; found 998.8804.

### 2.2.5 Chemical stability determination

TBONES or TBOZEN (100 μM) was dissolved in Dulbecco's phosphate-buffered saline (DBPS) (pH 7, 1x) at 37 ℃. Aliquots (10 μL) were withdrawn for HPLC analysis every 2 h for 12 h.

### 2.2.6 Thiol selectivity determination

TBONES or TBOZEN (100 μM) was mixed with a thiol molecule [GSH, cysteine, homocysteine, N-acetylcysteine (NAC), N-acetylcysteine methyl ester (NAC methyl ester) in (ratio=1:1 or 1:5)], a non-thiol molecule [ valine, glycine, lysine, arginine, glutamic acid, tryptophan, tyrosine, and serine (ratio = 1:1, 1:5, 1:10, or 1:50)], or bovine serum albumin (BSA) (500 μM for TBONES and 1 mM for TBOZEN) in a DPBS buffer (pH 7.0 or 5.0, 1x) at 37 ℃. Aliquots were taken for fluorescence detection (100 μL) or HPLC/UV (10 μL) analysis. For fluorescence detection, aliquots were transferred to a 96-well plate and measured on a SpectraMax M2 microplate reader. HPLC conditions were presented in the Materials and Instruments section.

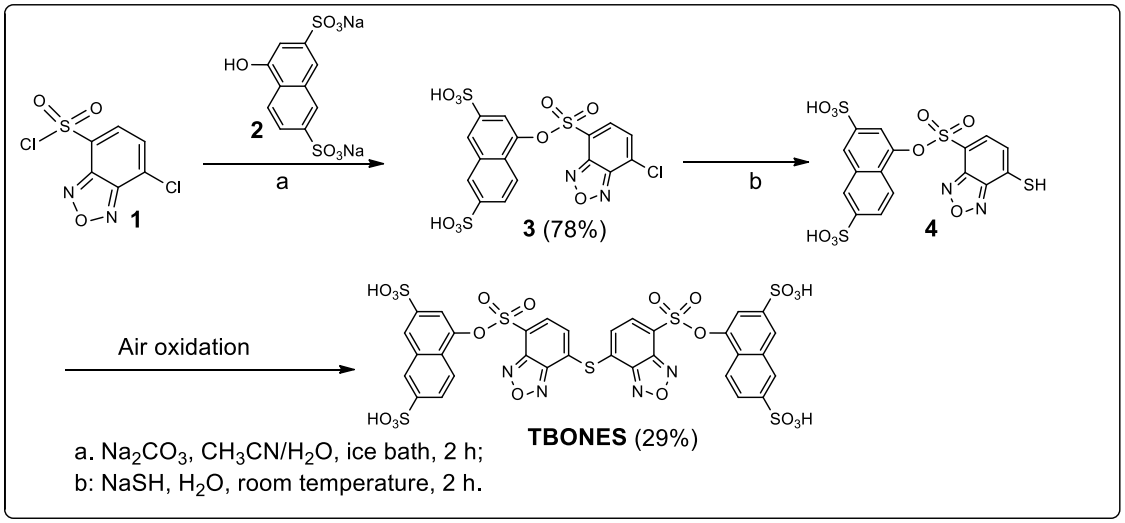
For the reaction with PSHs from cell homogenates, NCI-H226 cells ($8.6\times10^6$ cells for TBONES and $11.5\times10^6$ for TBOZEN) were washed once with DPBS and lysed by ultrasonication with 1 mL of 3% SSA for 10 min followed by centrifugation (14000 rpm, 5 min) at 4 ℃ to remove NPSH. Protein precipitates were washed with 1 mL of 3% of SSA five times before being re-suspended in a DPBS buffer (280 μL, pH 7 or pH 5, 1x)

and treated with TBONES (20 μL, 0.75 mM) or TBOZEN (20 μL, 1 mM) at 37 ℃. Aliquots (50 μL) were withdrawn at different time points (0 h, 1 h, 4 h, 12 h), added with methanol (150 μL), followed by centrifugation (14000 rpm, 5 min) to remove proteins before 20 μL was injected for HPLC analysis.

## 2.3 Results and Discussion

### 2.3.1 Synthesis of TBONES

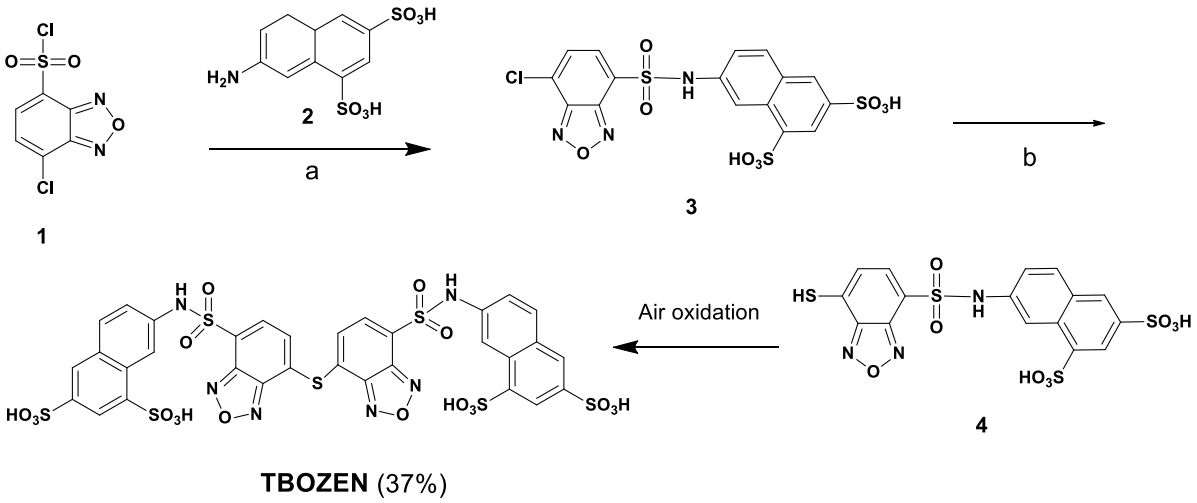
Synthesis of TBONES started with esterification of the commercially available 4-hydroxy-2,7-naphthalenedisulfonic acid disodium salt (**2**) with 4-chloro-7-chlorosulfonyl-2,1,3-benzoxadiazole (**1**) in the presence of sodium carbonate ($Na_2CO_3$) to produce compound **3** in 78% yield (Scheme 2.1). Treatment of compound **3** with sodium sulfhydride produced a thiophenol (**4**) which was air-oxidized in a one-pot reaction to complete the synthesis of TBONES in 29% yield (Scheme 2.1). The reaction conditions for the synthesis were not optimized. TBONES was characterized by $^1H$ NMR and HRMS. The purity of TBONES was confirmed to be >97% by HPLC.




**Scheme 2.1** Synthesis of TBONES

### 2.3.2 Synthesis of TBOZEN

The synthesis of TBOZEN was achieved in two steps. In the first step, 7-amino-1,3-naphthalenedisulfonic acid was coupled with 4-chloro-7chlorosulfonyl-2,1,3-benzoxadiazole through a sulfonamide bond to produce the intermediate **3** in 75% yield (Scheme 2.2). In the second step, sodium hydrosulfide hydrate was added to the intermediate **3** to produce first thiophenol in order to obtain the final product (TBOZEN) in 37% yield (Scheme 2.2). TBOZEN was characterized by $^{1}H$ NMR and HRMS. The purity of TBOZEN was confirmed by HPLC to be more than 97%.




a. $Na_2CO_3$, acetonitrile/water, ice bath, 8 h
b. NaSH, water, room temperature, 2.5 h.

**Scheme 2.2** Synthesis scheme of TBOZEN

### 2.3.3 Chemical stability

The chemical stabilities of both TBONES and TBOZEN were checked in a DBPS buffer (pH 7, 1x) at 37 ℃ for at least 12 h. Both were stable for at least 12 h (Figure 2.3, A and B).

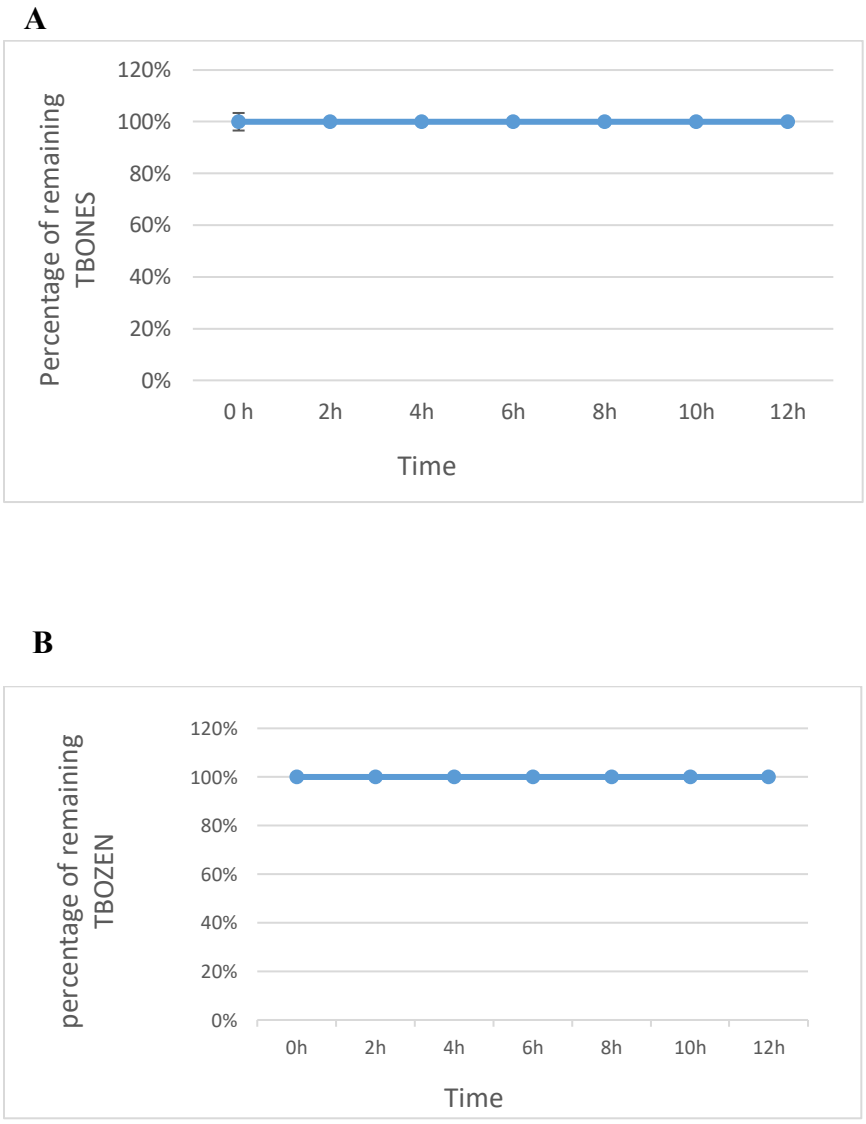



**Figure 2.3** Chemical stability of TBONES (**A**) and TBOZEN (**B**). TBONES or TBOZEN (100 µM) was dissolved in a DBPS buffer (pH 7, 1x) at 37 ℃. Aliquots (10 µL) were withdrawn for HPLC analysis every 2 h. The results were expressed as a percentage of

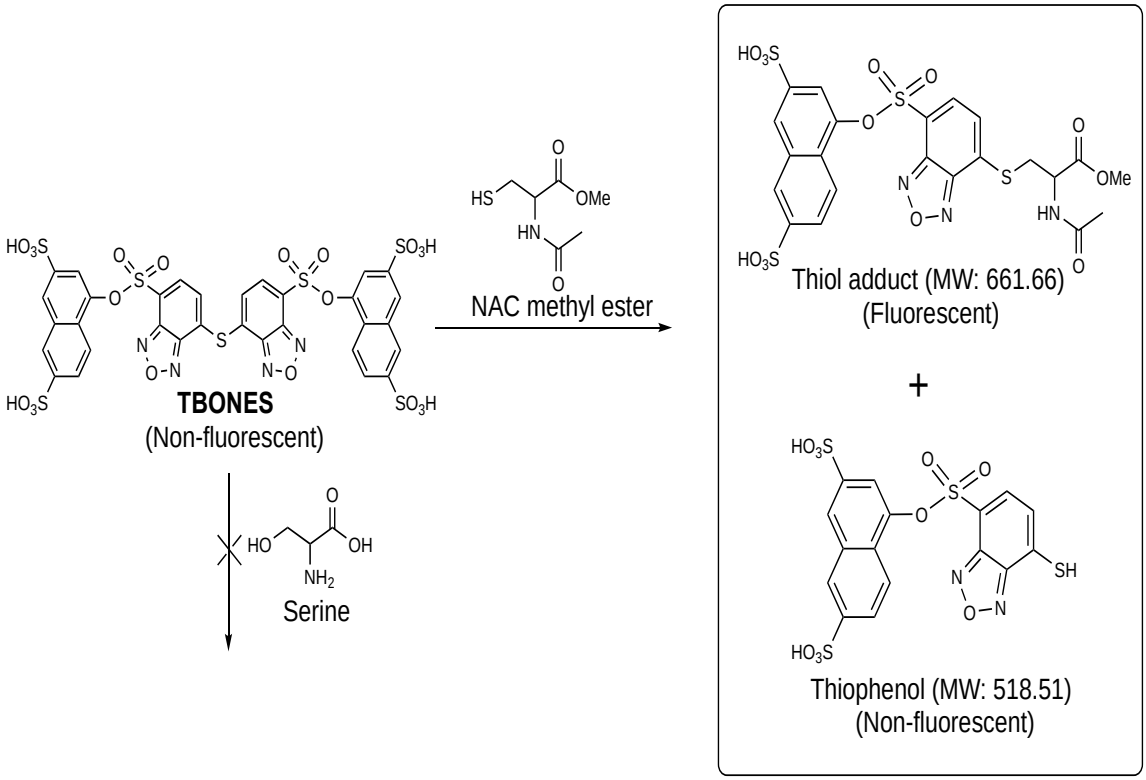
the remaining TBONES or TBOZEN and representing an average of three injection of the same sample. The experiments were repeated, and the same results were obtained.

### 2.3.4 Thiol specificity and fluorogenicity

Determination of TBONES as a thiol specific reagent was carried out through a reaction of TBONES with various NPSH [GSH, cysteine, homocysteine, *N*-acetylcysteine (NAC), *N*-acetylcysteine methyl ester (NAC methyl ester)] *vs* a reaction with serine which is an amino acid containing -$NH_2$, -OH, and -COOH but no thiol group. The functional groups -$NH_2$, -OH, and -COOH are the most commonly seen nucleophilic functional groups other than a thiol in biological systems. Our data demonstrate that TBONES reacted with a thiol molecule readily but not with serine. Scheme 2.3 shows the reaction scheme of TBONES with NAC methyl ester *vs* a reaction with serine.

The HPLC results from the reaction of TBONES with NAC methyl ester are presented in Figure 2.4. At t = 0 min, only the TBONES peak (4.7 min) was observed when the HPLC was monitored by a diode array detector at 254 nm (Figure 2.4a), and no peak was observed when the HPLC was monitored by a fluorescence detector ($\lambda_{ex}$ = 400 nm and $\lambda_{em}$ = 540 nm) (Figure 2.4c) suggesting that TBONES exhibited no fluorescence at the excitation and emission wavelengths. After 30 min, the peak of TBONES disappeared, and two new peaks (5.9 min and 16.5 min) formed when the HPLC was monitored by a diode array detector at 254 nm (Figure 2.4b). The two new peaks were characterized by mass spectroscopy as the thiol adduct and the corresponding released thiophenol (Scheme 2.3 and Figure 2.4b). However, only the thiol adduct peak was observed when the HPLC was monitored by a fluorescence detector (Figure 2.4d)

suggesting that the thiophenol was not fluorescent. These results were consistent with our early reports that the symmetric benzofurazan sulfide is not fluorescent. It reacts with a thiol to form a corresponding fluorescent thiol adduct and non-fluorescent thiophenol[39, 40].




**Scheme 2.3** Reaction of TBONES with NAC methyl ester (a thiol representative molecule) or serine (a non-thiol amino acid).

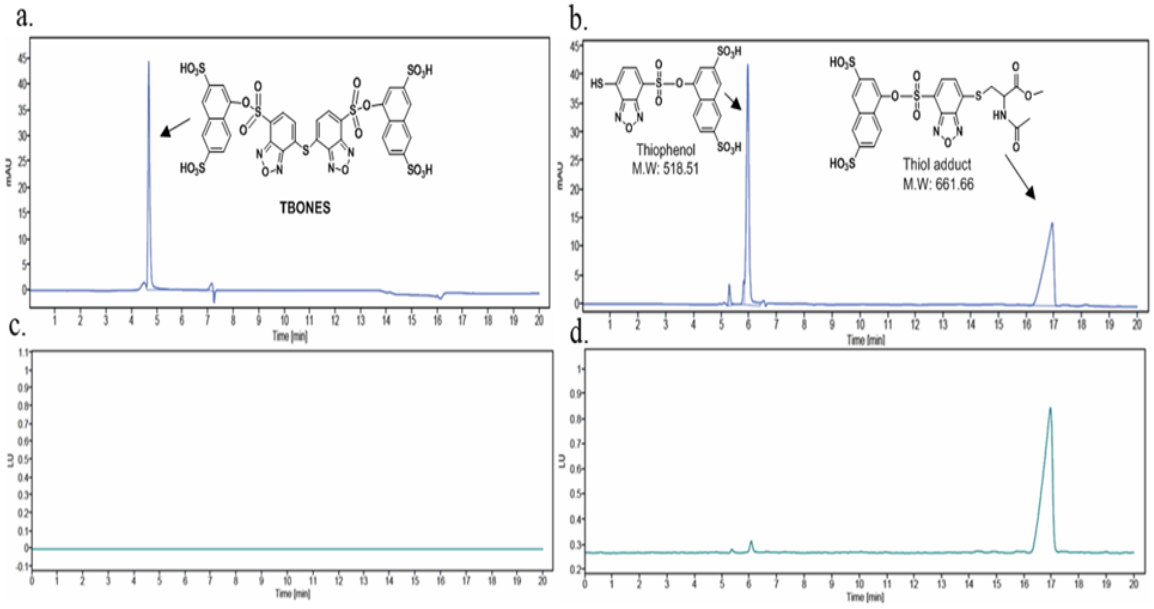



**Figure 2.4** HPLC chromatograms derived from a reaction of TBONES with NAC methyl ester. TBONES (100 μM) reacted with NAC methyl ester (500 μM) in a DPBS buffer (pH 7,1x) at 37 °C. Aliquots were withdrawn for HPLC analysis. The chromatograms were obtained by using a diode array detector (254 nm, a and b) or a fluorescence detector ($\lambda_{ex}$ = 400 nm, $\lambda_{em}$ = 540 nm, c and d) at 0 min (a and c) and 30 min (b and d).

When TBONES was mixed with serine in a ratio of 1 : 50, no reaction was observed based on the data from the HPLC analysis suggesting that TBONES did not react with $–NH_2$, -OH, and –COOH at the condition.

The selectivity of TBONES toward only thiols was further confirmed through a reaction of TBONES with other thiol-containing molecules and non-thiol amino acids. The examined other thiol containing molecules included GSH, cysteine, homocysteine, and *N*-acetylcysteine (NAC) while the examined non-thiol amino acids included two neutral amino acids (valine, glycine), two basic amino acids (lysine, arginine), one acidic amino acid (glutamic acid), one aromatic amino acid (tryptophan), and one amino acid with phenolic group (tyrosine). These data are presented in Figure 2.5. When TBONES was mixed with these non-thiol amino acids in a ratio of 1 : 5 at 37 ℃ for 12 h, no fluorescence change was observed while addition of a thiol-containing molecule in the same ratio led to the observation of strong fluorescence in 5 min (Figure 2.5) confirming further that TBONES only reacts with thiol molecules.

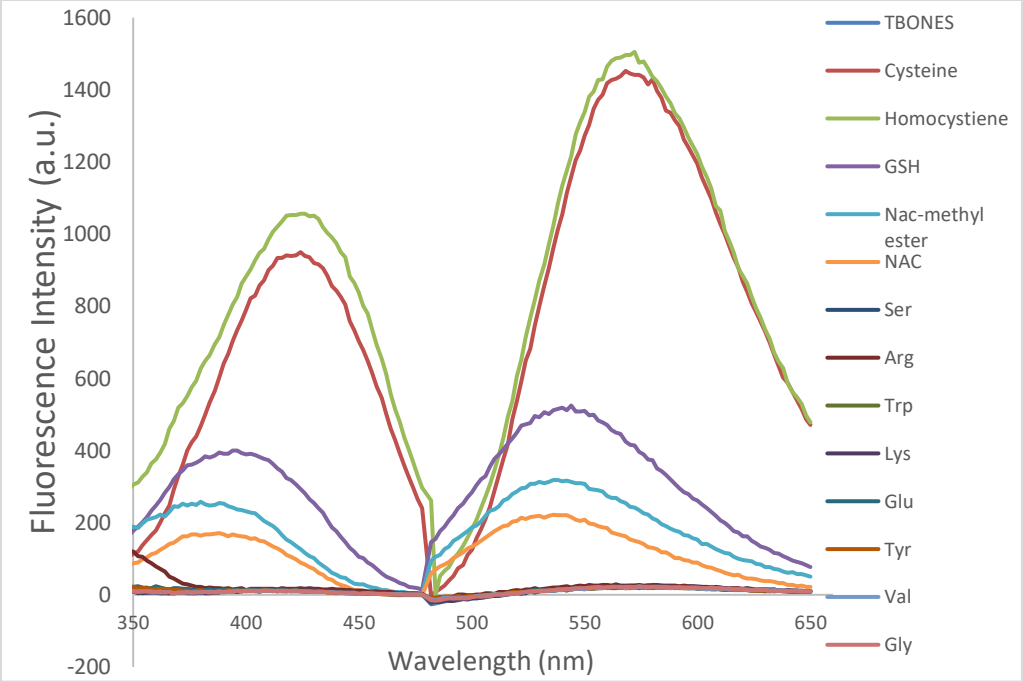
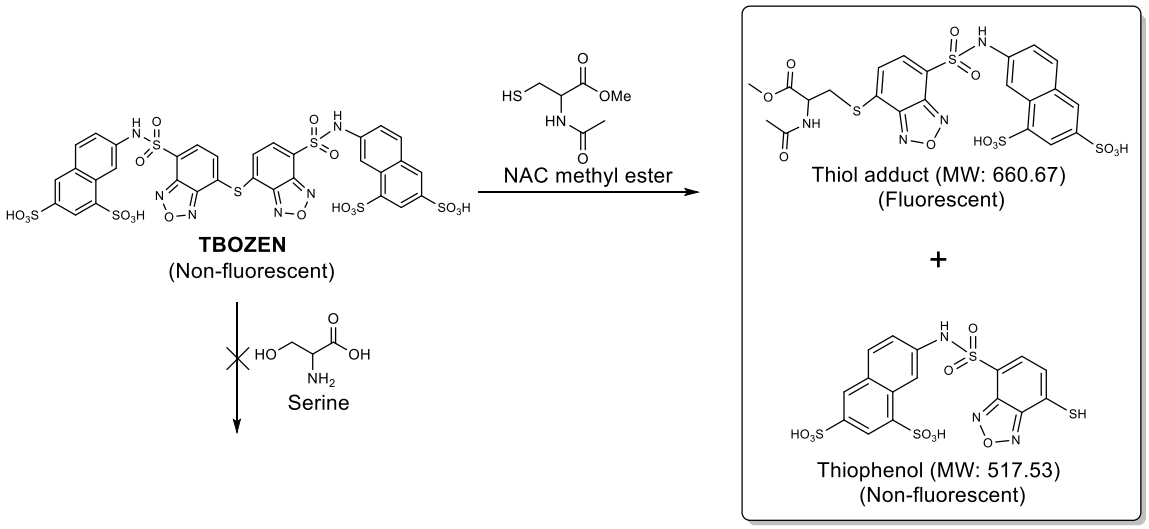



**Figure 2.5** Excitation and emission spectra of TBONES in the presence and absence of a NPSH molecule or a non-thiol amino acid. TBONES was mixed with a molecule in a ratio of 1:5 in a DPBS buffer (pH 7, 1x) at 37 ℃. An aliquot (150 µL) was withdrawn and transferred to a 96-well plate for fluorescence detection on a SpectraMax M2 microplate reader.

Similarly, our data demonstrate that TBOZEN reacted with a thiol molecule readily but not with serine. Scheme 2.4 shows the reaction scheme of TBOZEN with NAC methyl ester *vs* a reaction with serine. The HPLC results from the reaction of TBOZEN with NAC methyl ester are presented in Figure 2.6, At t = 0 min, only the TBOZEN peak was observed when the HPLC was monitored by a diode array detector at 254 nm (Figure 2.6A) and no peak was observed when the HPLC was monitored by a fluorescence detector ($\lambda_{ex}$ = 350 nm and $\lambda_{em}$ = 450 nm) (Figure 2.6B) suggesting that TBOZEN exhibited no fluorescence at the excitation and emission wavelengths. After 30 min, the peak of TBOZEN disappeared and two new peaks (7.9 min and 23.8 min) formed when the HPLC was monitored by a diode array detector at 254 nm (Figure 2.6C). The two new peaks were characterized by mass spectroscopy as the thiol adduct and the corresponding released thiophenol (Scheme 2.4). However, only the thiol adduct peak was observed when the HPLC was monitored by a fluorescence detector (Figure 2.6D) suggesting that the thiophenol was not fluorescent.




**Scheme 2.4** Reaction of TBOZEN with NAC methyl ester (a thiol representative molecule) or serine (a non-thiol amino acid).

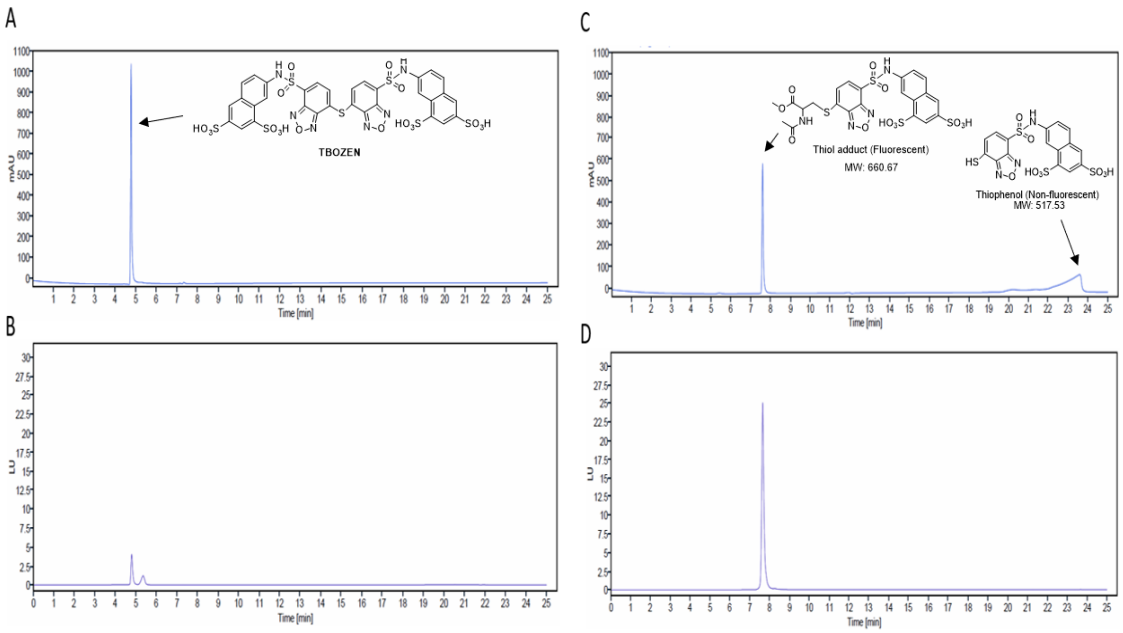



**Figure 2.6** HPLC chromatograms derived from a reaction of TBOZEN with NAC methyl ester. TBOZEN (100 µM) reacted with NAC methyl ester (1 mM) in a DPBS buffer (pH 7,1x) at 37 °C. Aliquots were withdrawn for HPLC analysis. The chromatograms were obtained by using a diode array detector (254 nm, A and C) or a fluorescence detector ($\lambda_{ex}$ = 350 nm, $\lambda_{em}$ = 450 nm, B and D) at 0 min (A and B) and 30 min C and D).

When TBOZEN was mixed with serine in a ratio of 1 : 50, no reaction was observed by HPLC (Figure 2.7).

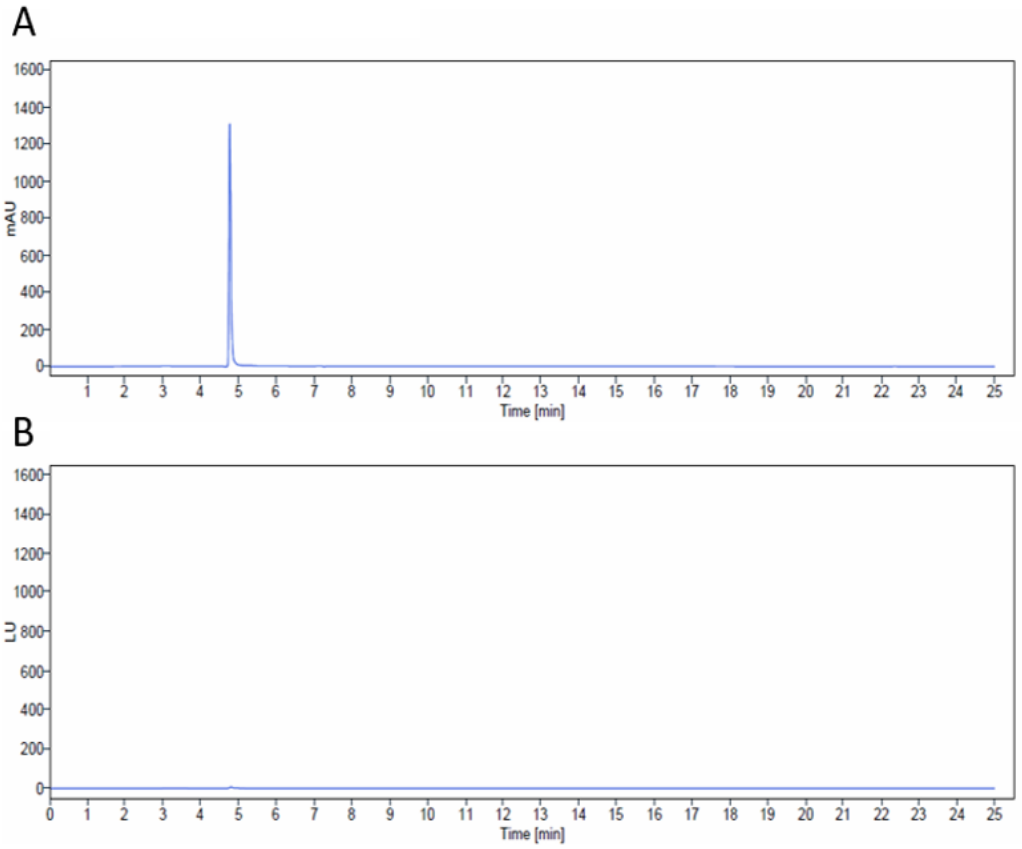



**Figure 2.7** HPLC chromatograms for the reaction of TBOZEN with Serine for 12h. TBOZEN (0.1mM) was mixed with serine (5 mM) in a DPBS buffer (pH 7,1x) at 37 ℃ for 12 h. UV−vis (254 nm) (A) and fluorescence detectors (λex = 350 nm, λem = 450 nm) (B) were employed for detection. Only TBOZEN peak was observed at 4.9 min indicating no reaction occurred between TBOZEN and serine.

Like TBONES, thiol specific reaction of TBOZEN was further confirmed through a reaction of TBONES with other thiol-containing molecules and non-thiol amino acids. The examined other thiol containing molecules included GSH, cysteine, homocysteine, and NAC while the examined non-thiol amino acids included two neutral amino acids (valine, glycine), two basic amino acids (lysine, arginine), one acidic amino acid (glutamic acid), one aromatic amino acid (tryptophan), and one amino acid with phenolic group (tyrosine). These data are presented in Figure 2.8, when TBOZEN was mixed with these non-thiol amino acids in a ratio of 1 : 5 at 37 ℃ for 12 h, no fluorescence change was observed while addition of a thiol-containing molecule in the same ratio led to the observation of strong fluorescence in 5 min (Figure 2.8) confirming further that TBOZEN only reacts with thiol molecules.

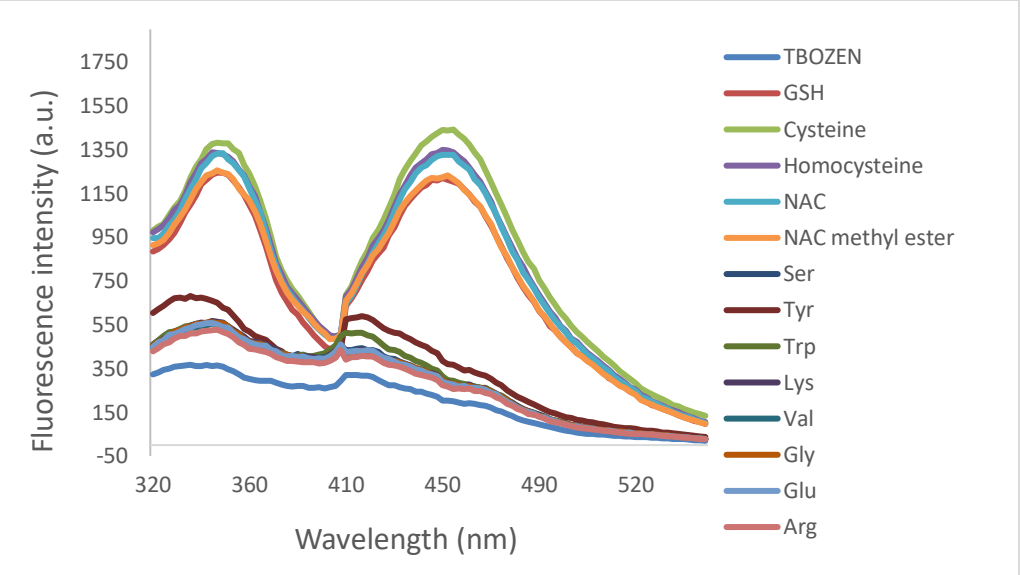



**Figure 2.8** Excitation and emission spectra of TBOZEN in the presence and absence of a NPSH molecule or a non-thiol amino acid. TBOZEN was mixed with a molecule in a ratio of 1:5 in a DPBS buffer (pH 7, 1x) at 37 ℃. An aliquot (150 µL) was withdrawn and transferred to a 96-well plate for fluorescence detection on a SpectraMax M2 microplate reader.

Our data demonstrate that both TBONES and TBOZEN were thiol specific and fluorogenic. The excitation and emission wavelengths are $\lambda_{ex}$ = 400 nm, $\lambda_{em}$ = 540 nm for TBONES and $\lambda_{ex}$ = 350 nm and $\lambda_{em}$ = 450 nm for TBOZEN respectively.

### 2.3.5 Imaging of cell surface thiols in live cells

After determining that TBONES and TBOZEN were thiol specific and fluorogenic, these two reagents were tested for their ability to image cell surface thiols in live cells. Unfortunately, both failed to image cell surface thiols (data not presented). Interestingly, both TBONES and TBOZEN were found to effectively image thiols in lysosomes. The data of TBONES and TBOZEN imaging lysosomal thiols will be presented in the next chapter.

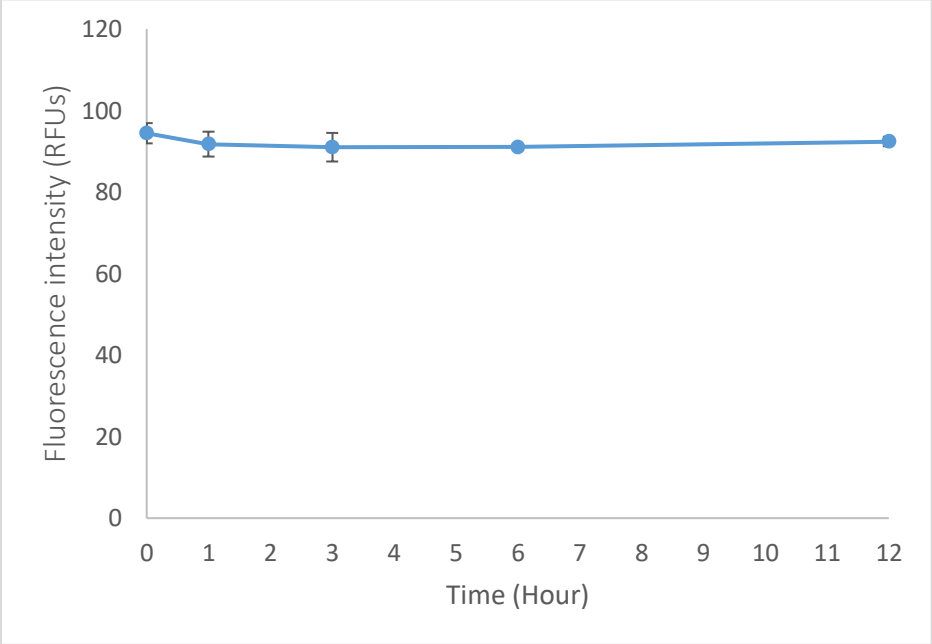
### 2.3.6 The ability to react with PSH

To check the reason for the failure of TBONES and TBOZEN to image cell surface thiols, the ability of TBONES and TBOZEN to react with PSH was determined. We found in our previous work that when a benzofurazan sulfide was too bulky, it failed to react with PSH.[39, 40] Obviously if TBONES and TBOZEN could not react with PSH, it would fail to image cell surface thiols since cell surface thiols are PSH. The results of TBONES and TBOZEN reacted with PSH from BSA and PSH from cell homogenates are presented in Figure 2.9 (TBONES) and Figures 2.10 and 2.11 (TBOZEN).

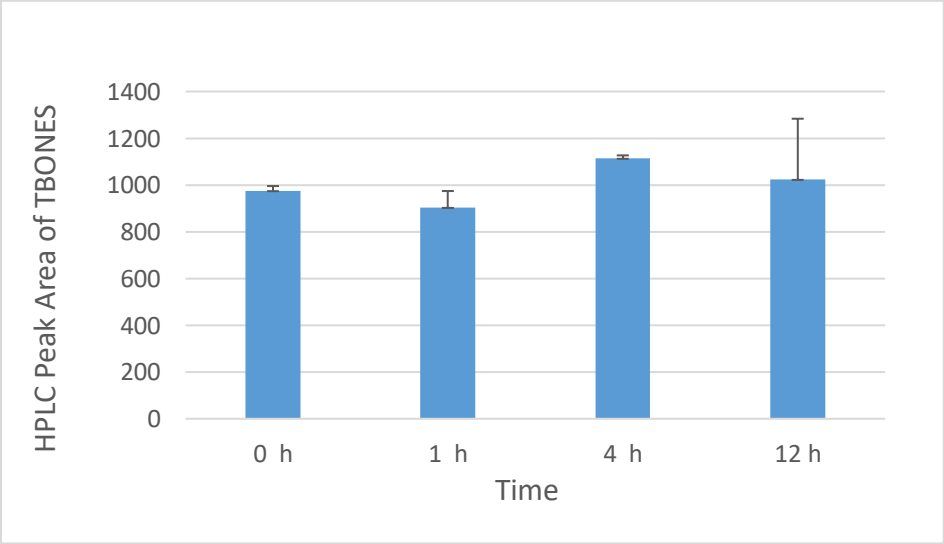
When TBONES (100 μM) was mixed with BSA (500 μM) in a DPBS buffer (pH 5, 1x) at 37 ℃, no fluorescence change was observed for 12 h (Figure 2.9A) suggesting that no fluorescent thiol adduct was formed and the inability of TBONES to react with PSH from BSA. To further confirm TBONES' inability to react with PSH, PSH from cell homogenates were employed. When TBONES (20 μL) was mixed with protein precipitates (280 μL) in a DPBS buffer (pH 5 and pH 7, 1x), the aliquots of the reaction mixture showed that the HPLC peak area of TBONES remained the same (Figure 2.9B-i) over a 12 h period and no new peak was observed (Figure 2.9B-ii). It is believed that the

steric hindrance or the bulkiness of the TBONES structure may contribute to this inability [39, 40].

A.




B-i.



B-ii.

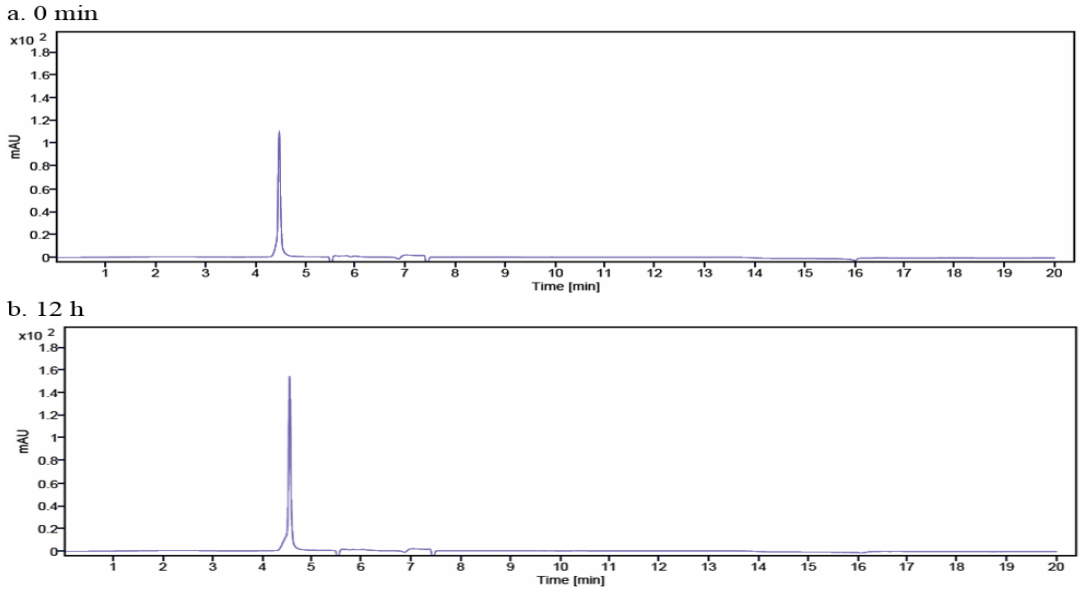



**Figure 2.9** Inability of TBONS to react with PSH. **A.** *The reactivity of TBONES toward PSH from BSA.* TBONES (100 μM) was mixed with BSA (500 μM) in a DPBS buffer (pH 5, 1x) at 37 ℃. An aliquot (100 μL) was withdrawn at different time points and transferred to a 96 well plate for fluorescence detection on a SpectraMax M2 microplate reader. The results were expressed as the observed fluorescence intensity and expressed as the mean value ± SD of three different experiments. **B.** *The reactivity of TBONES towards PSH from cell homogenates.* TBONES (20 μL, 0.75 μM) was mixed with protein precipitates in a DPBS buffer (280 μL, pH 5, 1 x) at 37 ℃. An aliquot (50 μL) was withdrawn and processed for HPLC analysis. B-i: HPLC peak area obtained from a reaction of TBONES with PSH from cell homogenate at pH 5 and 37 ℃. The results were expressed as peak area of TBONES and representing an average of three injection of the same sample. The experiment was repeated, and the same results were obtained. B-

ii: Representative HPLC chromatograms derived from a reaction of TBONES with cell homogenates at t = 0 h (a) and t = 12 h (b).

Similar results were obtained for TBOZEN. When TBOZEN was mixed with BSA (Figure 2.10) or proteins from cell homogenates, not reaction was observed (Figure 2.11 and table 2.1).

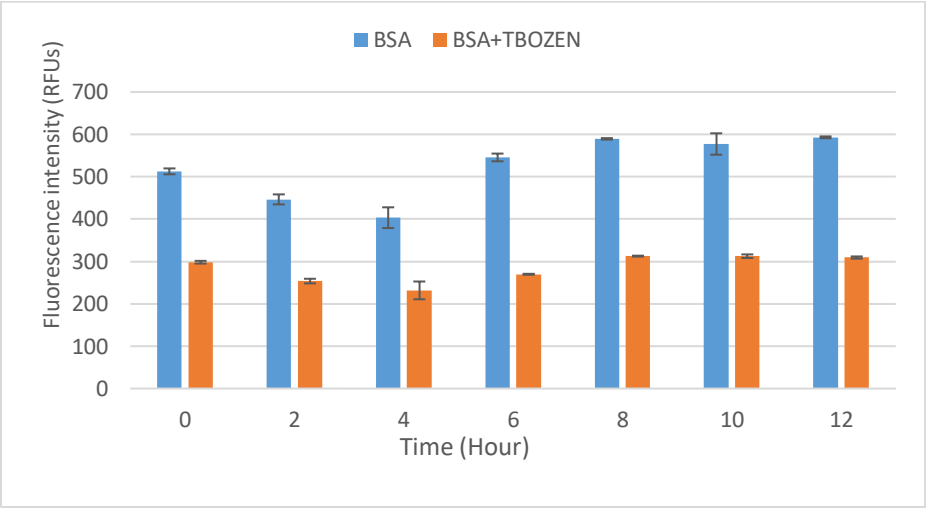
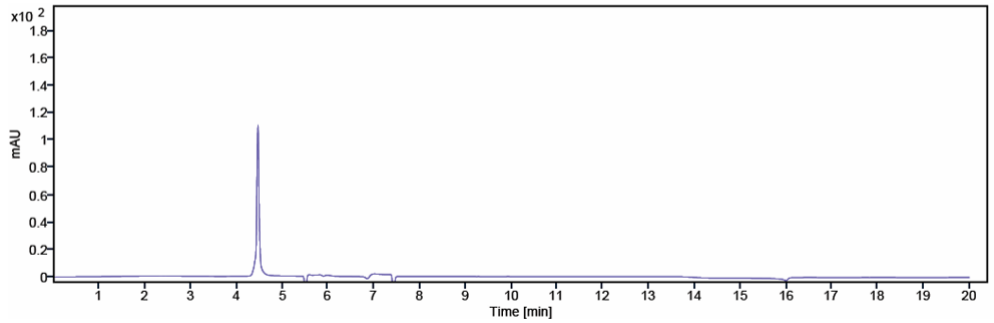



**Figure 2.10** The reactivity of TBOZEN towered PSH from BSA. TBOZEN (0.1 mM) was mixed with BSA (1 mM) in a DPBS (pH 5,1x) at 37 ℃. An aliquot (100 µl) was withdrawn at different time points and transferred to a 96 well plate for monitoring fluorescence change through SpectraMax M2 microplate reader. The results were expressed as the observed fluorescence intensity and expressed as the mean value ± SD of three different experiments.

a. 0 min




b. 12 h

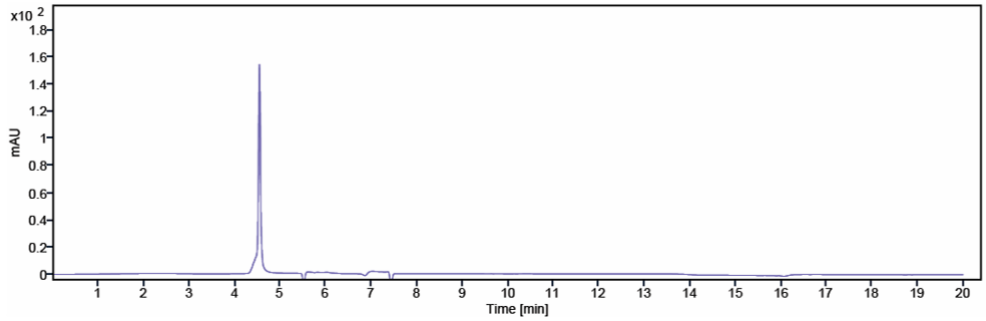



**Figure 2.11** The reactivity of TBOZEN towered PSH from cell homogenates. TBOZEN (20 µl, 1mM) was mixed with protein precipitates (280 µl) in DPBS buffer (pH 5, 1x) at 37 ℃. An aliquot (50 µl) was withdrawn for HPLC analysis at different time points (0 min and 12 h). Proteins were removed from the aliquot by addition of methanol (150 µl) followed by centrifugation (14000 rpm, 5 min). Sample (10 µl) was injected to HPLC.

Table 2.1 HPLC peak area obtained from a reaction mixture of TBOZEN with PSH from cell homogenates (pH 5) over 12 h time period.

| HPLC peak area of TBOZEN mixed with protein from cell homogenate at (pH5) | | | |
|---|---|---|---|
| Time in hour | Injection 1 | Injection 2 | Injection 3 |
| 0 h | 1319.88 | 1369.45 | 1363.80 |
| 1 h | 1290.60 | 1331.25 | 1365.22 |
| 4 h | 1429.02 | 1406.36 | 1363.10 |
| 12 h | 1421.23 | 1366.92 | 1360.24 |

The data from the reaction of TBONES or TBOZEN with BSA and cell homogenates demonstrated that both TBONES and TBOZEN were not able to react with PSH. This explains why TBONES and TBOZEN could not image cell surface thiols since cell surface thiols are PSH.

In summary, TBONES and TBOZEN were designed to image cell surface thiols. These two compounds were successfully synthesized. Both were found to be thiol specific and fluorogenic for NPSH. However, both failed to image cell surface thiols due to their inability to react with PSH. Both were found to effectively image NPSH in lysosomes in live cells through fluorescence microscopy. The data of TBONES and TBOZEN imaging thiols in lysosomes in live cells are presented in the next chapter.

# CHAPTER 3. CHARACTERIZATION OF TBONES AND TBOZEN FOR NPSH IMAGING IN LYSOSOMES IN LIVE CELLS

## 3.1 Introduction

Lysosomes are subcellular organelles that are distributed throughout the cytoplasmic matrix of the cell. They are separated from the surrounding cytoplasm by an impermeable membrane to ensure the lysosomal enzymes are maintained within the lysosomal acidic environment[47, 48]. Lysosomes were initially termed as the garbage disposal of the cell and maintain cell homeostasis through degradation and recycling of unwanted or dysfunctional macromolecules such as proteins, DNA, RNA, carbohydrates, and lipid membranes using approximately 60 different hydrolytic enzymes at acidic pH (~5)[49-51]. Current knowledge has extended the roles of lysosomes from simply a garbage disposal to a subcellular organelle that regulates a variety of cellular functions such as autophagy, endocytosis, and phagocytosis through the degradation of macromolecules[52]. In addition, the degradation also plays a role in numerous cellular processes such as metabolic signaling, antigen presentation, tumor invasion, and detoxification[53, 54]. Dysfunction of lysosomes has been associated with neurodegenerative disorders, cardiovascular diseases, cancer and cancer drug resistance[8, 55].

In lysosomes, thiols were reported to be involved in the activation of the lysosomal degradation process by reducing a protein disulfide bond[56, 57]. For instance, cysteine was found to activate the degradation of albumin in lysosomes and glutathione is thought to be involved in stabilizing lysosomal membrane against the internal acidic environment[57]. Moreover, lysosomal cysteine was found to induce cell death for cancer cells through degradation of macromolecules formed due to an increase in ROS[55].

Therefore, lysosomal thiols, like thiols in other parts of the cell, play a significant role in lysosomes' function.

Although there are a few reagents available for live cell imaging of lysosomal thiols[58-64], the thiol selectivity of these reagents was based on the strong nucleophilicity of thiols in a Michael addition or an aromatic nucleophilic substitution reaction. Since a Michael addition reaction or an aromatic nucleophilic substitution reaction can also occur with a nucleophilic group other than thiols such as -OH, $-NH_2$, and -COOH, the thiol selectivity of these reagents can be compromised and interfered by the presence of those nucleophilic functional groups in the biological system.

In the previous chapter, TBONES and TBOZEN have been demonstrated to be thiol specific fluorogenic agents and able to turn NPSH into fluorescent thiol adducts. The preliminary data showed that TBONES and TBOZEN were able to image thiols in lysosomes. This chapter presents the full investigation of the ability of TBONES and TBOZEN for imaging thiols in lysosomes in live cells through fluorescence microscopy.

## 3.2 Experimental Section

### 3.2.1 Materials and Instruments

Unless otherwise stated, all chemical reagents and solvents were obtained from commercial sources and used without further purification. TBONES and TBOZEN were prepared based on the procedures reported in the previous chapter. *N*-Ethylmaleimide (NEM) was purchased from Sigma-Aldrich (St. louis, MO, USA). LysoTracker™ Blue DND-22, Gibco™ Trypan Blue Stain (0.4%) and Dulbecco's phosphate-buffered saline (Gibco™DPBS, no calcium, no magnesium,1x) were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Fluorescence microscopy images were obtained on an upright fluorescence microscope (Zeiss AXIO Imager A1) equipped with a camera (AxioCam MRc5) (Zeiss, United States) or on Olympus FV1200 Scanning Confocal Microscope. Cell numbers were determined on a Nexcelom Cellometer Auto T4 Automated Cell Counter (Lawrence, MA, USA).

### 3.2.2 Cell culture

Human lung cancer cells NCI-H226 ( National Cancer Institute) were cultured in the RPMI 1640 growth medium provided with 10% FBS, 100 units/mL of penicillin (mediatech, Inc., Herndon, VA), and 100 µg/mL of streptomycin (Mediatech, Inc., Herndon,VA) in a humidified atmosphere containing 5% $CO_2$ at 37 ℃.

### 3.2.3 Cell viability study

A trypan blue assay was conducted to check the cell viability of cells after treating with 400 µM TBONES or TBOZEN. Briefly, NCI-H226 cells were seeded in a 12-well plate at a concentration of 100000 cells/well and placed in a 37 C and 5% CO2 incubator.

When an 80% confluence was reached, the cells were treated with 1000 μM TBONES or TBOZEN for 12 h. After treatment, the medium was removed and the cells was washed 3 times with DPBS to remove any residual old medium. The trypan blue assay was conducted according to the manufacture's protocol.

### 3.2.4 Imaging and confirmation of lysosomal thiol in live cells

Cells were seeded on a 15 mm diameter microscope cover glass in a 12-well plate at a concentration of 100,000 cells/well. After reaching to about 70% confluence, the cells were washed 3 times with DPBS, and then treated with TBONES or TBOZEN for 12 h at 37 ℃. After treatment, cells were washed 3 times with DPBS before staining with LysoTracker™ Blue DND-22 (75 nM) for 2 h at 37 ℃. The microscope cover glass with the cells was transferred to a microscope slide with DPBS as the mounting medium and placed on a confocal microscope (Olympus FV1200 Scanning Confocal Microscope) for imaging. The FITC filter was set for the detection of thiol adducts derived from TBONES or TBOZEN, and the DAPI filter was set for the detection of LysoTracker™ Blue DND-22.

### 3.2.5 Detection of lysosomal thiol changes in live cells

NCI-H226 cells were seeded on a 15 mm diameter microscope cover glass in a 12-well plate at a concentration of 30,000 cells/well. When the growth reached to about 80% confluence, the medium was removed, and the cells were washed 3 times with DPBS. The cells were treated with various concentrations of NEM for 3 h. The medium containing NEM was removed and the cells were washed 3 times with DPBS before treated with TBONES for 12h. After treated with TBONES, the cells were washed 3 times with DPBS and placed on a microscope slide with DPBS as the mounting medium.

The slide was placed on an upright fluorescence microscope (Zeiss AXIO Imager A1). Images were taken with the connected camera (AxioCam MRc5). FITC channel filter was set for thiol imaging by TBONES. The fluorescence intensity of the obtained images was quantified by Image J (https://imagej.nih.gov/ij/).

## 3.3 Results and Discussion

### 3.3.1 Effect of pH on the reactivity

Thiol is an acidic functional group. The ionization status of a thiol group is affected by the pH of the environment. The nucleophilicity of thiol is different when it is in its ionized form *vs* the unionized form. Most thiols react faster as a nucleophile at an alkaline condition at which thiols will be predominantly in the ionized form. Since lysosomes are the most acidic subcellular organelle in the cell with a pH of 4.5 to 6, the reaction of TBONES with a thiol at pH 5 and 7 was compared using GSH as a model thiol compound. Figure 3.1 showed that when TBONES (100 µM) was mixed with GSH (500 µM) in a DPBS buffer (pH 7 and pH 5) at 37 ℃, the reaction was completed in 30 min at both pH values suggesting no reaction rate difference at these two different pH environments.

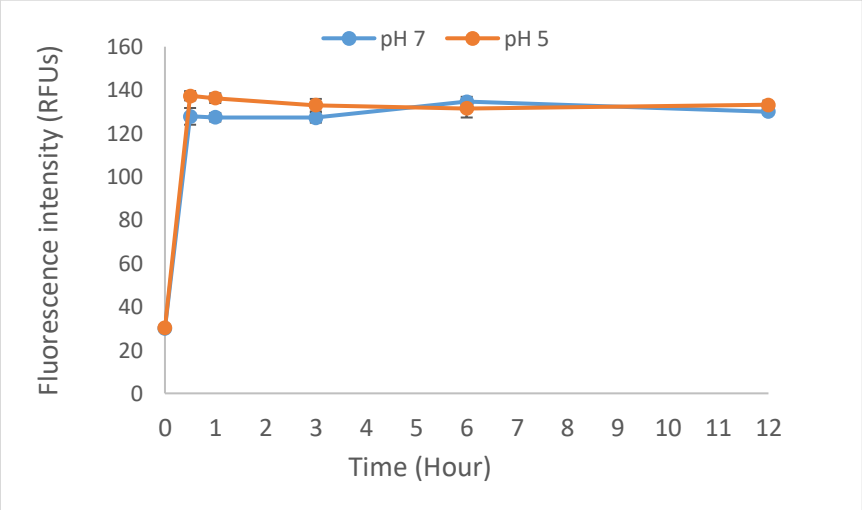



**Figure 3.1** The effect of pH on the reaction of TBONES. TBONES was mixed with GSH in a ratio of 1 : 5 in a DPBS buffer (pH 7 or 5, 1x) at 37 ℃. An aliquot (150 µL) was withdrawn and transferred to a 96-well plate for fluorescence detection on a SpectraMax

M2 microplate reader. The results were expressed as the observed fluorescence intensity and expressed as the mean value ± SD of three different experiments.

Similar results were obtained for TBOZEN, though a slight increase in the reaction rate was observed for TBOZEN at pH 7 (Figure 3.2).

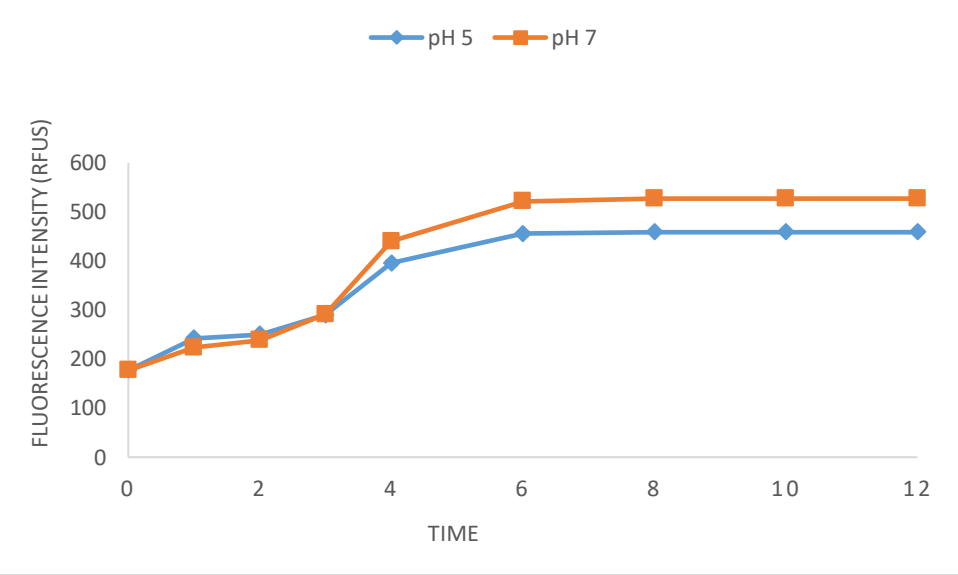


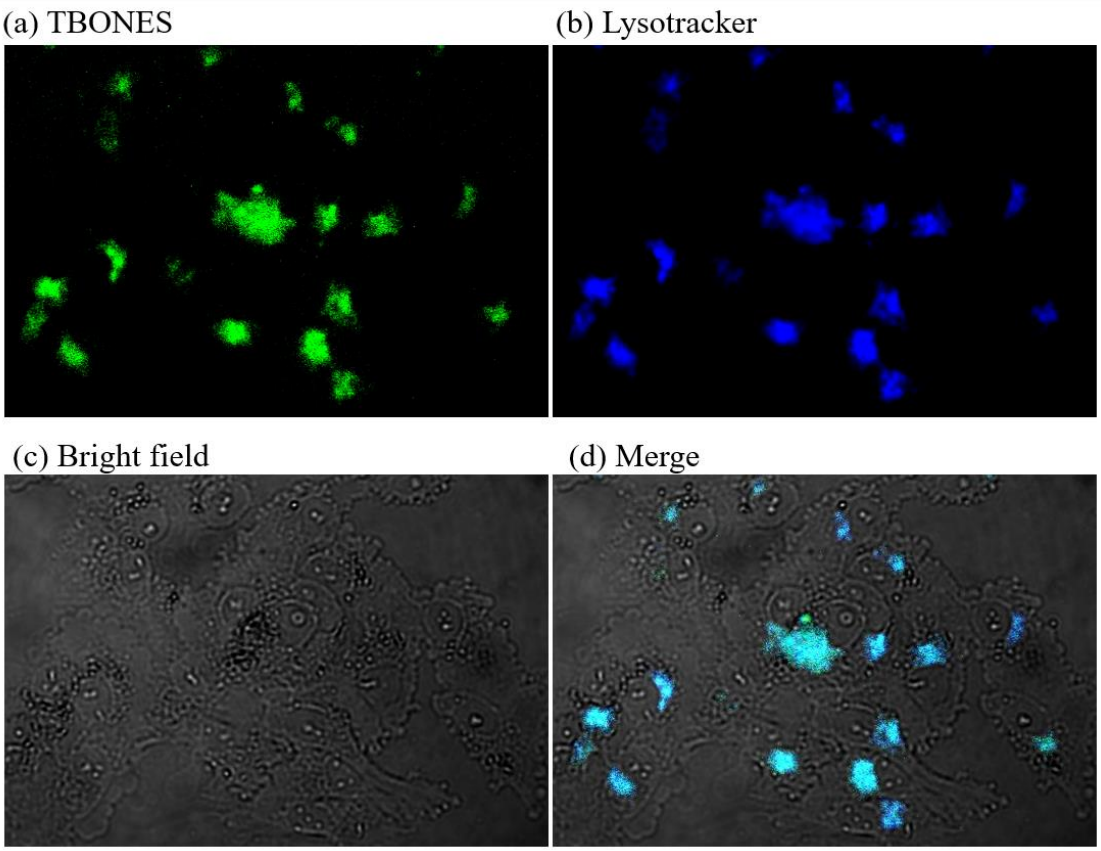

**Figure 3.2** The effect of pH on the reaction of TBOZEN. TBOZEN was mixed with GSH in a ratio of 1 : 5 in a DPBS buffer (pH 7 or 5, 1x) at 37 ℃. An aliquot (150 µL) was withdrawn and transferred to a 96-well plate for fluorescence detection on a SpectraMax M2 microplate reader. The results were expressed as the observed fluorescence intensity and expressed as the mean value ± SD of three different experiments.

These data showed that both TBONES and TBOZEN are able to react with NPSH at the acidic pH, which is similar to the pH of the lysosomal environment.

### 3.3.2 Imaging of lysosomal thiols in live cells by TBONES

NCI-H226 cells were treated with various concentrations of TBONES in order to determine the minimum required concentration for imaging. When cells were incubated with different concentration of TBONES for 12 h, fluorescence intensity increased with an increase in TBONES' concentration until 400 μM. No further increase in fluorescence was observed when the TBONES's concentration exceeded 400 μM. A Trypan blue assay revealed that the cell viability was >95% when the cells were treated with 400 μM TBONES for 12 h. Therefore, TBONES at 400 μM was selected for imaging. To confirm the organelles imaged by TBONES were lysosomes, cells were first treated with TBONES followed with a treatment of a lysosome tracking agent LysoTracker Blue DND-22 (75 nM). Images were obtained on a confocal microscope with FITC filter (green) set for detecting the green fluorescence of thiol adducts derived from TBONES, and DAPI filter (blue) set for detecting the blue fluorescence of LysoTracker™ Blue DND-22. Figure 3.3A presents representative images derived from thiol adducts (image a), LysoTracker™ (image b), and the merged image (d) of image a with image b. Image d (co-localization of image a with image b) confirmed that the fluorescent thiol adducts detected by TBONES were localized in lysosomes. Image c is the bright field image showing the shape of the cell and helps demonstrate the relative location of lysosomes in the cell.

A.



B.

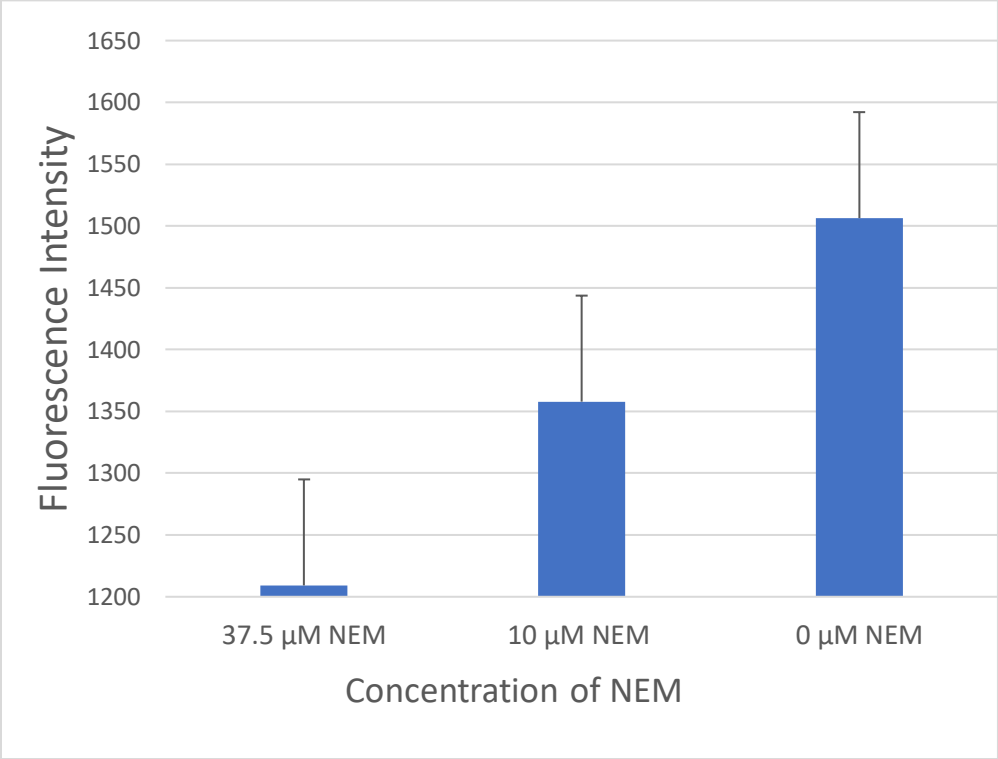



**Figure 3.3 A:** Fluorescence images obtained from NCI-H226 cells treated first with TBONES followed by lysotracker. (a): image from thiol adducts; (b): image from Lysotracker; (c): bright field image to show the cells; (d): image derived from merging of image a with image b. **B:** Ability of TBONES to detect the thiol change in lysosomes. NCI-H226 cells were seeded on a 15 mm diameter microscope cover glass in a 12-well plate. Cells were treated with NEM followed by imaging using TBONES. The fluorescence intensity of the obtained images was quantified by Image J (https://imagej.nih.gov/ij/).

### 3.3.3 Detection of thiol density change by TBONES

To check if TBONES could detect thiol density changes in lysosomes, NEM was employed. NEM was used in the literature to deplete thiols through a covalent binding with a thiol[38-40]. As shown in Figure 3.3B, the fluorescence decreased with an increase in NEM concentration. When NEM reached to 37.5 μM, minimal fluorescence was observed. These results demonstrate that TBONES could detect the change of thiols in lysosomes in live cells through fluorescence microscopy. The results further confirm that fluorescence derived from the cells treated with TBONES were from thiols since minimal fluorescence was observed when thiols were depleted by NEM.

### 3.3.4 Imaging of lysosomal thiols in live cells by TBOZEN

Similar experiments were conducted with TBOZEN and similar results were obtained for TBOZEN. As demonstrated in image c (co-localized image) in Figure 3.4, the image generated by TBOZEN (image a) co-localized well with the image generated from the LysoTracker (image b) confirming that the thiols imaged by TBOZEN were NPSH localized in lysosomes.

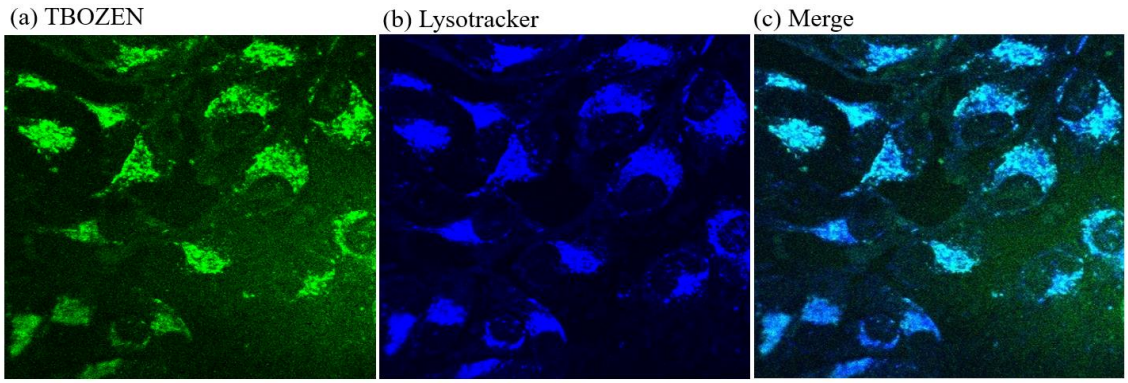



**Figure 3.4** Fluorescence images obtained from NCI-H226 cells treated with TBOZEN and lysotracker. (a) NCI-H226 cells treated with TBOZEN, (b) NCI-H226 cells stained with LysoTracker blue DND-22, and (c) co-localized image of image a (TBOZEN) and image b (LysoTracker blue DND-22). Data were from one of three representative experiments.

In summary, both TBONES and TBOZEN have been demonstrated to image NPSH in lysosomes. To our knowledge, these are the first two reagents that can specifically image thiols with high selectivity for lysosomes. These two reagents will be valuable tools in exploring the roles of lysosomal thiols in the cellular function and dysfunction.

# CHAPTER 4. DESIGN, SYNTHESIS, AND CHARACTERIZATION OF 7,7'-THIOBIS(N-(2-MORPHOLINOETHYL) BENZO[C][1,2,5] OXADIAZOLE-4-SULFONAMIDE) (TIMBOS) FOR NON-PROTEIN THIOL IMAGING IN LYSOSOMES IN LIVE CELLS

## 4.1 Introduction

As indicated in the last chapter, TBONES and TBOZEN were the first agents that were capable of imaging NPSH in lysosomes based on a thiol specific reaction: thiol-sulfide exchange reaction. It would be desirable to develop thiol specific fluorogenic agents that can image both NPSH and PSH. In this chapter, 7,7'-thiobis(*N*-(2-morpholinoethyl) benzo[c][1,2,5] oxadiazole-4-sulfonamide) (TIMBOS) ( Figure 4.1) was designed as a thiol specific fluorogenic agent that capable of imaging both NPSH and PSH. The design of the molecule involves addition of a morpholine structure symmetrically to the basic benzofurazan structure. The morpholine is known to be lysosome-targeting[65-69]. It is believed that basic amines with low ionic strength provide the targeting for lysosomes since lysosomes are internally acidic (pH 4 -6)[70].

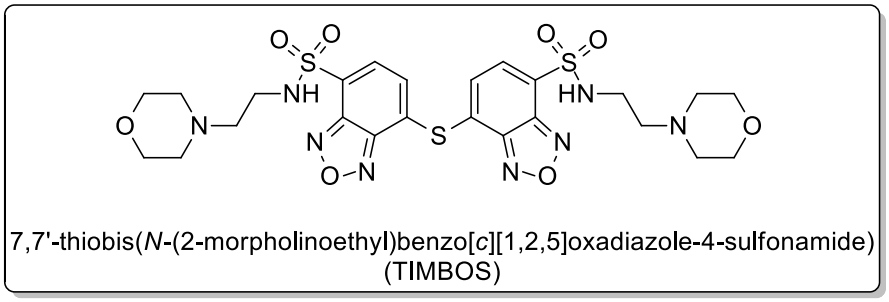



**Figure 4.1** Chemical structure of TIMBOS designed as a thiol specific lysosome-selective fluorogenic reagent

## 4.2 Experimental section

### 4.2.1 Materials and instruments

Unless otherwise stated, all chemical reagents and solvents were obtained from commercial sources and used without further purification. 4-(2-Aminoethyl)-morpholine, sodium hydrosulfide hydrate and *N*-ethylmaleimide (NEM) were purchased from Sigma-Aldrich (St. louis, MO, USA). 4-Chloro-7chlorosulfonyl-2,1,3-benzoxadiazole was purchased from TCI (Portland, OR, USA). LysoTracker® Red DND-99, Gibco™ Trypan Blue Stain (0.4%) and Dulbecco's phosphate-buffered saline (Gibco™DPBS, no calcium, no magnesium,1x) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Sulfosalicylic acid (SSA) cell lysis solution was prepared as a 3% (w/v) solution in deionized water containing 0.1% (v/v) Triton X-100.

Flash column chromatography was carried out on a W-Prep 2XY Yamazen Dual Channel Flush Chromatography System (San Bruno, California). $^{1}$HNMR spectra were recorded on a Bruker Varian 600 MHZ spectrometer in deuterated solvent as indicated. All NMR peaks were given as chemical shift in part per million relative to TMS (Tetramethylsilane) as the internal standard. Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and brs (broad singlet). *J* values are given in Hz. Low resolution mass spectra (LRMS) were obtained on a Thermoquest Finnigan LCQ Deca Mass Spectrometer (Waltham, MA, USA) and high resolution mass spectrometry (HRMS) was acquired on a Bruker Daltonics SolariX 12 tesla Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (Department of Chemistry, University at Buffalo, NY).

Fluorescence properties were carried out on a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, California). Fluorescence microscopy images were obtained on an upright fluorescence microscope (Zeiss AXIO Imager A1) equipped with a camera (AxioCam MRc5) (Zeiss, United States) or on Olympus FV1200 Scanning Confocal Microscope. Cell numbers were determined on a Nexcelom Cellometer Auto T4 Automated Cell Counter (Lawrence, MA, USA). Cell viability was determined by a Trypan blue assay.

HPLC analysis was achieved on an Agilent HPLC system connected with Agilent 1100 fluorescent detector and a Diode Array Detector. The HPLC analysis condition utilized an Apollo C8 column (100 mm x 4.60 mm i.d., 3 μm) with a mobile phase system consist of solvent A [aqueous solvent/ ammonium phosphate] and solvent B [acetonitrile] with a flow rate of 0.5 mL/min. The mobile phase started with 5% of solvent B and then increased to 90% of solvent B in 20 min. The injection volume was 10 μL. The wavelength of 254 nm was used for UV detection and the wavelengths of 380 nm and 540 nm were used as the excitation and emission wavelengths for fluorescent detection.

### 4.2.2 Synthesis of TIMBOS

***7-Chloro-N-(2-morpholinoethyl) benzo[c][1,2,5]oxadiazole-4-sulfonamide (3).*** To a stirred solution of 4-chloro-7chlorosulfonyl-2,1,3-benzoxadiazole (**1**) (0.500 g, 1.98 mmol) in acetonitrile (30 mL) was added dropwise 4-(2-aminoethyl)-morpholine (**2**) (0.257 g, 1.98 mmol). The mixture was stirring in an ice bath for 0.5 h before the solvents were removed by a rotary evaporator under reduced pressure. The resulting residue was purified through a silica gel column (dichloromethane/methanol) to obtain the desired

compound (**3**) as a solid yellow product (0.566 g, 82%). The product was characterized by $^1$H NMR and MS. $^1$H NMR (600 MHz, acetone) δ 8.07 (s, 1H), 7.99 (d, *J* = 93.0 Hz, 1H), 3.39 (d, *J* = 98.0 Hz, 6H), 3.06 (m, 6H). MS for $C_{12}H_{15}ClN_4O_4S$ (M+H) is 347.10

***7,7'-Thiobis(N-(2-morpholinoethyl) benzo[c][1,2,5] oxadiazole-4-sulfonamide) (TIMBOS).*** An aqueous solution (3 mL) of sodium hydrosulfide (0.024 g, 0.43 mmol) was added dropwise to a stirred solution of (**3**) (0.150 g, 0.43 mmol) in acetonitrile (10 mL) at room temperature. The mixture was stirred for 1 h before the solvents were removed under reduced pressure by a rotary evaporator to produce a yellow residue. The resulting residue was purified through a silica gel column (dichloromethane/methanol) to obtain the desired product (TIMBOS) as a solid yellow product (146 mg, 51%). The product was characterized by $^1$H NMR and HRMS. The purity of the product was confirmed by HPLC to be > 97%. $^1$H NMR (600 MHz, deuterated acetone) δ 8.21 (dd, *J* = 7.1, 3.4 Hz, 2H), 7.91 (dd, *J* = 7.2, 3.4 Hz, 2H), 4.29 (s, 4H), 4.02 (s, 8H), 3.71 – 3.56 (m, 4H), 3.48 – 3.36 (m, 8H). HRMS calculated for $C_{24}H_{31}N_8O_8S_3$ (M+1) is 655.1417; found 655.1417.

### 4.2.3 Chemical stability of TIMBOS

TIMBOS (100 µM) was dissolved in a DBPS (Dulbecco's phosphate-buffered saline) buffer (pH 7, 1x) at 37 ℃. Aliquot (10 µL) was withdrawn for HPLC analysis over a 28 hour period.

### 4.2.4 Reaction selectivity of TIMBOS toward NPSH and non-thiol amino acids

TIMBOS (100 µM) was mixed with NPSH [GSH, cysteine, homocysteine, *N*-acetylcysteine (NAC), *N*-acetylcysteine methyl ester (NAC methyl ester) (ratio=1:1 or 1:5)] or with non-thiol amino acids (TIMBOS to non-thiols amino acids ratio = 1:1, 1:5,

1:10, or 1:50) in a DPBS buffer (1x, pH 7) at 37 ℃. Aliquots were withdrawn for fluorescence detection (100 μL) or for HPLC /UV (10 μL) analysis. For fluorescence detection, aliquots were transferred to a 96 well and measured on a SpectraMax M2 microplate reader. HPLC conditions were presented in the Materials and Instruments section.

### 4.2.5 Reactivity of TIMBOS toward PSH

***Reactivity of TIMBOS towered PSH from BSA.*** TIMBOS (100 μM) was mixed with BSA (500 μM) in a DPBS (pH 5, 1x) at 37 ℃. An aliquot (100 μL) was withdrawn at different time points and transferred to a 96 well plate for monitoring fluorescence change using a SpectraMax M2 microplate reader.

***Reactivity of TIMBOS toward PSH in cell homogenates.*** NCI-H226 cells ($6 \times 10^6$) were washed once with DPBS then lysed by ultrasonication with 1 mL of 3% sulfosalicylic acid (SSA) for 10 min followed by centrifugation (14000 rpm, 5 min) at 4 ℃ to remove NPSH. Protein precipitates were washed five times with 1 mL of 3% of SSA before being re-suspended in DPBS buffer (pH 5, 280 μL) and treated with TIMBOS (0.5 mM, 20 μL) at 37 ℃. Aliquots (50 μL) were withdrawn at different time points (0 h, 1 h, 4 h), added with acetonitrile (150 μl), followed by centrifugation (14000 rpm, 5 min) to remove proteins before 20 μL being used for HPLC analysis.

### 4.2.6 Cell culture

Human lung cancer cells NCI-H226 ( National Cancer Institute) were cultured in the RPMI 1640 growth medium provided with 10% FBS, 100 units/mL of penicillin (Mediatech, Inc., Herndon, VA) and 100 μg/mL of streptomycin (Mediatech, Inc., Herndon,VA) in a humidified atmosphere containing 5% $CO_2$ at 37 ℃.

### 4.2.7 Cell viability study

A trypan blue assay was conducted to check the viability of cells. Briefly, NCI-H226 cells were seeded in a 12-well plate at a concentration of 75000 cells/well and placed in a 37 ℃ and 5% CO2 incubator. After reaching to about 80% confluence, the cells were treated with different concentration (100 μM, 200 μM, 400 μM) of TIMBOS for 6 h. After treatment, the medium was removed, and the cells were washed 3 times with DPBS to remove any residual from the old medium. The trypan blue assay was applied according to the manufacture protocol.

### 4.2.8 Imaging and confirmation of lysosomal thiols in live cells by TIMBOS.

Cells were seeded on a 15 mm diameter microscope cover glass in a 12-well plate at a concentration of 75000 cells/well. After reaching to about 80% confluence, the cells were washed 3 times with DPBS, then treated with TIMBOS (100 μM) for 4 h at 37 ℃. After treatment, cells were washed 3 times with DPBS before staining with LysoTracker® Red DND-99 (100 nM) for 0.5 h at 37 ℃. The microscope cover glass with the cells was transferred to a microscope slide with DPBS as the mounting medium and placed on a confocal microscope (Olympus FV1200 Scanning Confocal Microscope) for imaging. DAPI filter was set for the detection of thiol adducts derived from TIMBOS and rhodamine filter was set for the detection of LysoTracker® Red DND-99.

### 4.2.9 Detection of lysosomal thiol changes in live cells

NCI-H226 cells were seeded on a 15 mm diameter microscope cover glass in a 12-well plate at a concentration of 75000 cells/well. When the growth reached to about 80% confluence, the medium was removed and the cells were washed 2 times with DPBS. The cells were treated with various concentrations of NEM (400 μM, 100 μM, 50

μM, 25 μM, 0 μM) for 0.5 h. The medium containing NEM was removed and the cells were washed 3 times with DPBS before treated with TIMBOS (100 μM) for 4 h. After treated with TIMBOS, the cells were washed 3 times with DPBS and placed on a microscope slide with DPBS as the mounting medium. The slide was placed on an upright fluorescence microscope (Zeiss AXIO Imager A1). Images were taken with the connecting camera (AxioCam MRc5). DAPI channel filter was set for thiol imaging by TIMBOS. The fluorescence intensity of the obtained images was quantified by Image J (https://imagej.nih.gov/ij/).

## 4.3 Results and Discussion

### 4.3.1 Design, synthesis and chemical stability of TIMBOS agent

TIMBOS was designed as a thiol specific lysosome-selective fluorogenic reagent based on a symmetric benzofurazan sulfide (a) linked to a morpholine moiety (b) through a sulfonamide bond (Figure 4.2). The benzofurazan sulfide structure provides thiol specificity through the thiol-sulfide exchange reaction to react specifically with a thiol and form a fluorescent thiol adduct. The morpholine moiety is known to be lysosome targeting since it has basic amine with low ionic strength that provides the targeting for the weak acidic lysosome organelles. The symmetric nature of the TIMBOS was expected to make the structure non-fluorescent but to form a fluorescent thiol adduct after reacting with a thiol.

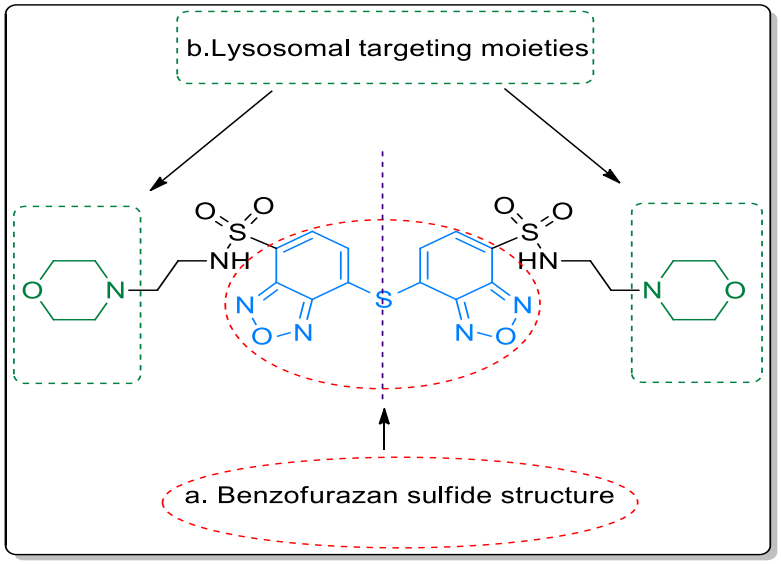
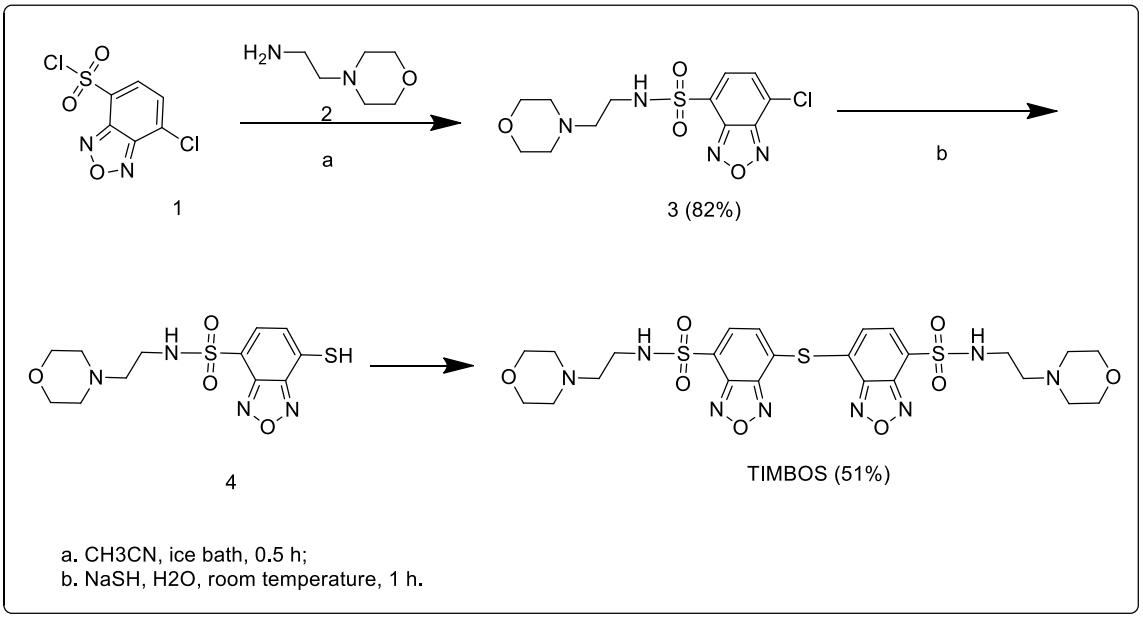



**Figure 4.2** Design of TIMBOS as a thiol specific lysosome-selective fluorogenic reagent.

The synthesis of TIMBOS started with addition of a commercially available 4-(2-aminoethyl)-morpholine to 4-chloro-7-chlorosulfonyl-2,1,3-benzoxadiazole to produce the intermediate **3** in 82% yield (scheme 4.1). In order to obtain TIMBOS, sodium hydrosulfide hydrate was added to the intermediate **3** to produce a thiophenol (**4**) which was oxidized by air during the reaction to produce the final product (**TIMBOS**) in 51% yield (scheme 4.1). TIMBOS was characterized by $^{1}$HNMR and HRMS. The purity of TIMBOS was confirmed by HPLC to be > 97%.




**Scheme 4.1** Synthesis of TIMBOS

The chemical stability of TIMBOS was evaluated by HPLC through observing the remaining TIMBOS over 28 h. The results from HPLC analysis showed no decrease in the remaining TIMBOS over the 28 h period indicating TIMBOS is stable at this condition (Figure 4.3).

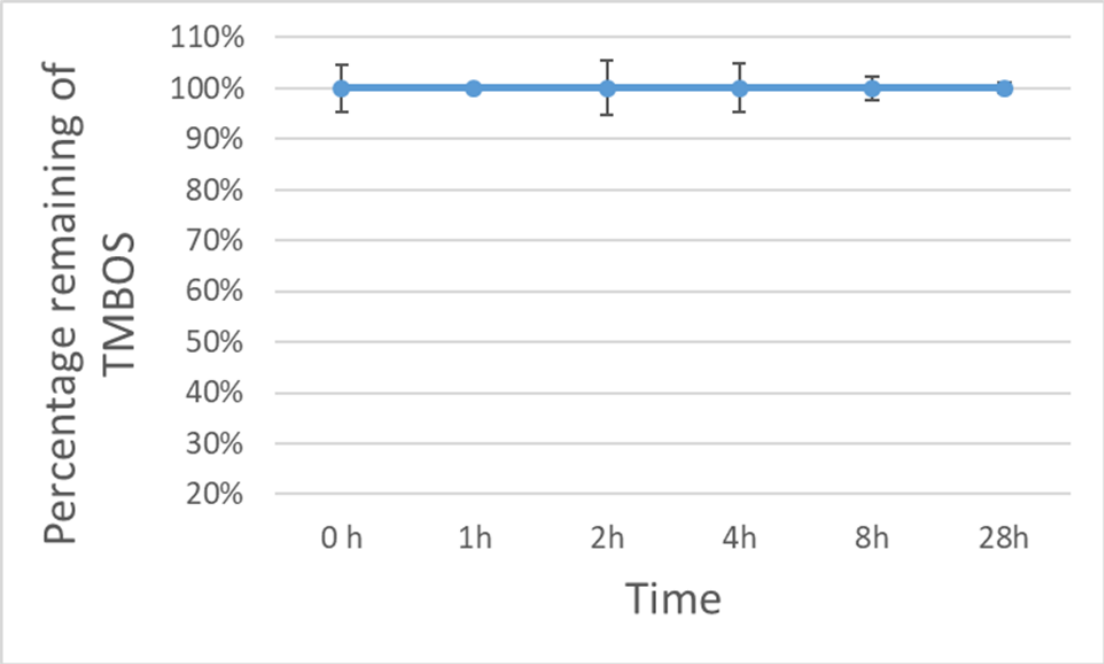
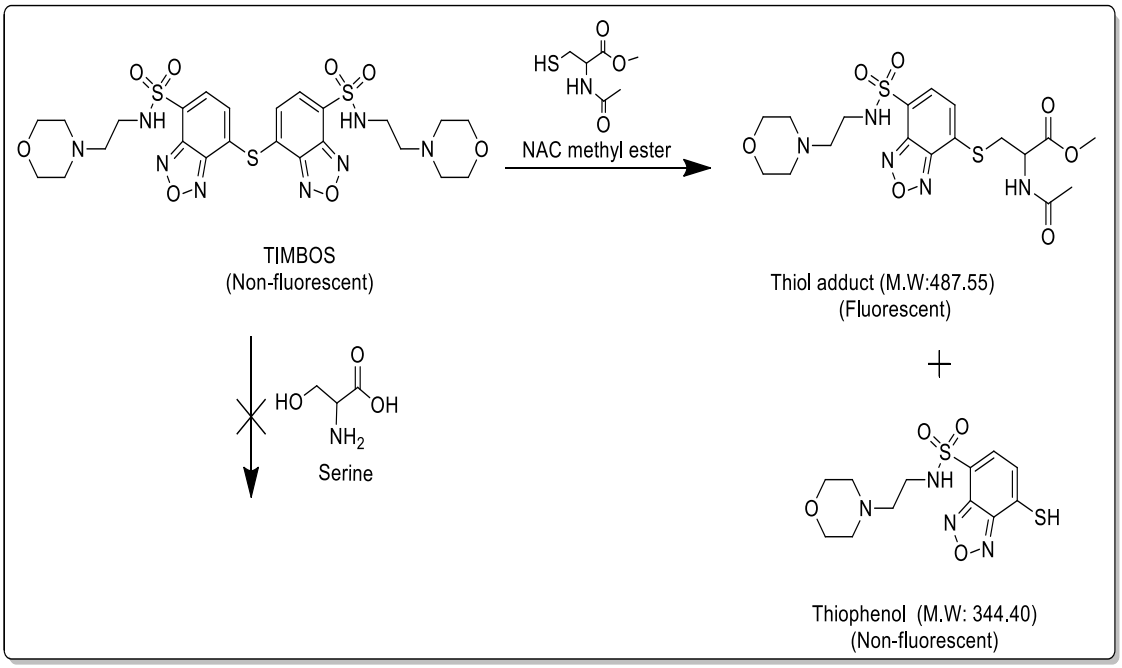



**Figure 4.3** Stability of TIMBOS. TIMBOS (100 µM) was dissolved in a DBPS buffer at 37 ℃. An aliquot (10 µL) was withdrawn for HPLC analysis for 28 h. The results were expressed as a percentage of the remaining TIMBOS and representing an average of three injection of the same sample. The experiment was repeated, and the same results were obtained.

### 4.3.2 Reaction selectivity of TIMBOS toward NPSH

*N*-Acetylcysteine methyl ester (NAC methyl ester) and serine were employed to determine the reaction selectivity of TIMBOS. NAC methyl ester was selected since it has only one thiol group (-SH) as a nucleophilic functional group while the amino acid serine was selected since it contains three different nucleophilic groups ($–NH_2$, -OH, and –COOH). These three functional groups are the most common nucleophilic groups that exist in the biological system other than the thiol (-SH) functional group. At a ratio of 1:5, TIMBOS was found to readily react with NAC methyl ester to form a fluorescent thiol adduct (Scheme 4.2). Figure 4.4 shows the HPLC chromatograms derived from the reaction of TIMBOS with NAC methyl ester. Chromatograms a and c were obtained from the reaction mixture and detected by a diode array detector at 254 nm. Chromatograms b and d were obtained from the same sample but detected by a fluorescence detector ($\lambda ex$ = 380 nm, $\lambda em$ = 540 nm). Chromatogram a (at time zero) shows only TIMBOS peak (15.5 min) and chromatogram c from the same sample shows the TIMBOS peak with minimum fluorescence. After 30 min of the reaction, the TIMBOS peak disappeared and two new products peaks formed at 4.0 min and 13.1 min respectively (chromatogram b). These two peaks were confirmed by mass spectra to be the corresponding thiophenol (4 min) and the thiol adduct (13.1 min). On the other hand, only thiol adduct peak was observed from the same sample when the HPLC was monitored by a fluorescence detector confirming that the thiol adduct is fluorescent while the thiophenol was not fluorescent (chromatogram d).




**Scheme 4.2** Reaction of TIMBOS with NAC methyl ester (a representative thiol molecule) or serine (a non-thiol amino acid).

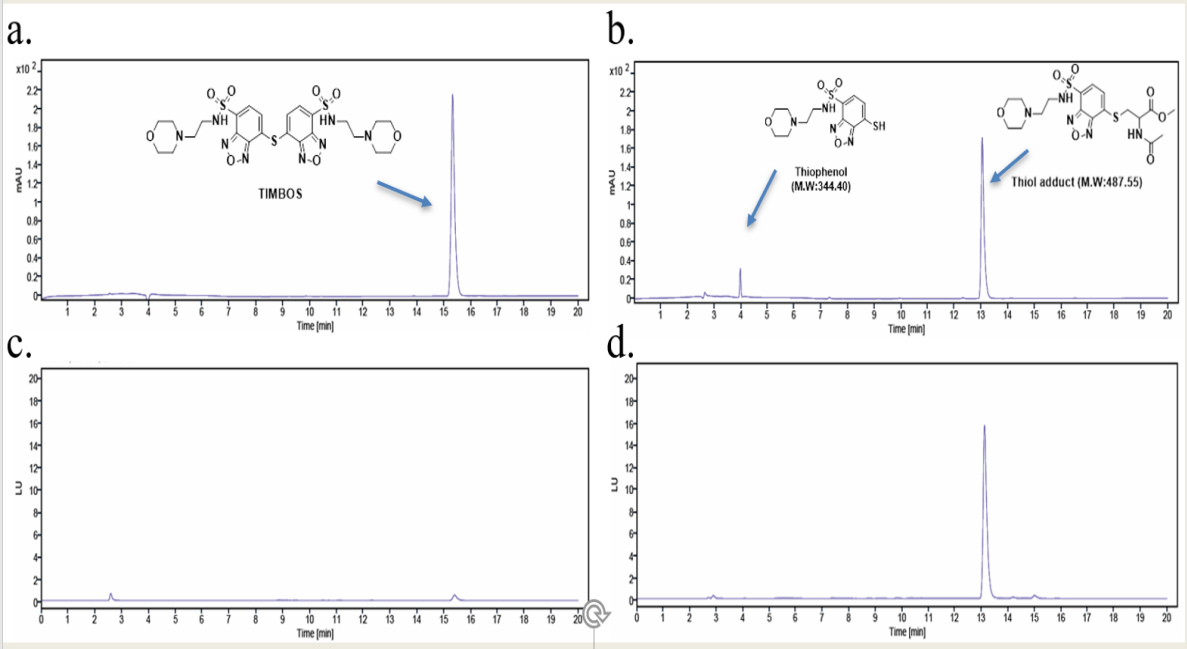



**Figure 4.4** HPLC chromatograms from the reaction of TIMBOS with NAC methyl ester. TIMBOS (100 µM) mixed with NAC methyl ester (500 µM) in a DPBS buffer (pH 7,1x) at 37 °C for 0 min (a and c) and 30 min (b and d). The chromatograms were obtained by using a diode array detector (254 nm, a and b) or a fluorescence detector (λex = 380 nm, λem = 540 nm, c and d).

In contrary to the reaction with NAC methyl ester, no reaction was observed when TIMBOS was mixed with serine even at a ratio of 1:50 of TIMBOS : serine for 6 h. Figure 4.5 shows that only TIMBOS peak was observed in the HPLC chromatograms (a. time = zero, and b. time = 6 h) obtained from the sample of the reaction of TIMBOS with serine. The HPLC was monitored by a diode array detector at 254 nm. The data suggest that TIMBOS does not react with the nucleophilic functional groups $–NH_2$, -OH, and –COOH.

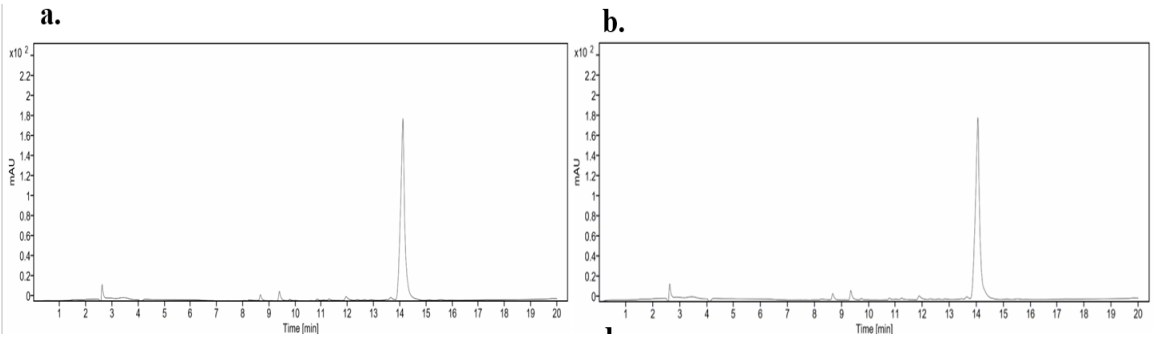



**Figure 4.5** HPLC chromatograms from the reaction of TIMBOS with serine. TIMBOS (100 μM) mixed with serine (5 mM) in a DPBS buffer (pH 7,1x) at 37 ℃ for 0 min (a) and 6 h (b). The chromatograms were obtained by using a diode array detector 254 nm.

To further confirm that TIMBOS does not react with non-thiol molecules, various non-thiol amino acids were employed. These non-thiol amino acids include two neutral amino acids (valine, glycine), two basic amino acids (lysine, arginine), one acidic amino acid (glutamic acid), one aromatic amino acid (tryptophan), and one amino acid with phenolic group (tyrosine). Figure 4.6 showed the inability of TIMBOS to react with these non-thiol amino acids at a ratio of 1:5 of TIMBOS : non thiol amino acids for 4 h at 37 °C. However, when TIMBOS was mixed with a NPSH at the same condition, strong fluorescence was observed further confirming that TIMBOS only reacts with thiol molecules.

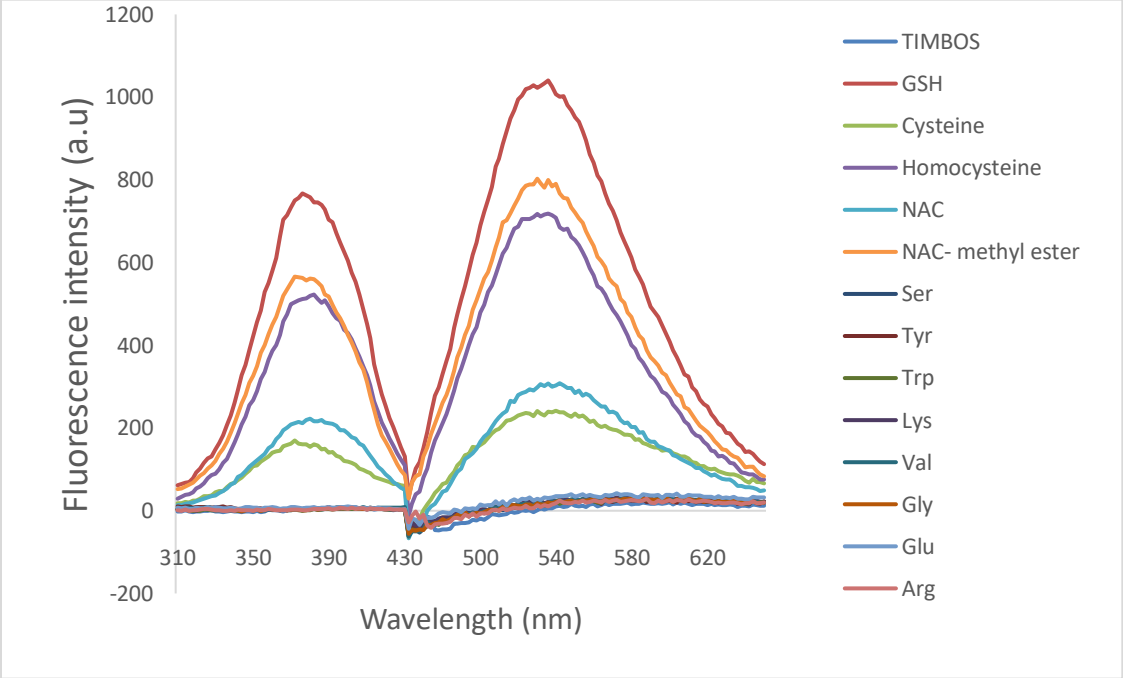



**Figure 4.6** Excitation and emission spectra of TIMBOS in the presence and absence of NPSH and various non-thiol amino acids. TIMBOS was mixed with a molecule in a ratio of 1:5 in a DPBS buffer (pH 7, 1x) at 37 °C for 4 h. An aliquot (150 µL) was withdrawn

and transferred to a 96-well plate for fluorescence detection on a SpectraMax M2 microplate reader.

### 4.3.3 Fluorescence property of TIMBOS and its thiol adducts.

As shown in Figure 4.6, TIMBOS alone exhibited very minimum fluorescence while strong fluorescence occurred when TIMBOS was mixed with a NPSH. These results are consistent with the data obtained from HPLC analysis (Figure 4.4 c & d) and further confirm that TIMBOS is a fluorogenic agent and can react with NPSH to form a fluorescent adduct. Figure 4.6 also demonstrates that although the excitation and emission wavelengths for the thiol adducts formed from different NPSH remain the same ($\lambda_{ex}$ = 380 nm, $\lambda_{em}$ = 540 nm), the fluorescence intensity was different with different thiols with the strongest intensity for GSH followed by NAC methyl ester, homocysteine, NAC, and cysteine (Figure 4.6).

### 4.3.4 Effect of pH on the reactivity of TIMBOS

Since TIMBOS was designed to detect thiols in lysosomes which have an acidic pH (4.5-6), TIMBOS' reactivity toward NPSH was checked at acidic pH (pH 5), and compared with a neutral pH (pH 7) to ensure that TIMBOS will also be able to react with NPSH at acidic pH. TIMBOS (100 μM) was mixed with NAC methyl ester (500 μM) in DPBS buffer (pH 7 and pH 5, 1x) at 37 ℃ for 4 h. The reaction rates at different pH (7 and 5) were evaluating through monitoring the increase in fluorescence intensity. Figure 4.7 shows that TIMBOS' reaction with NAC methyl ester completed in 2 h at both pH values though the fluorescence intensity was stronger at pH 5.

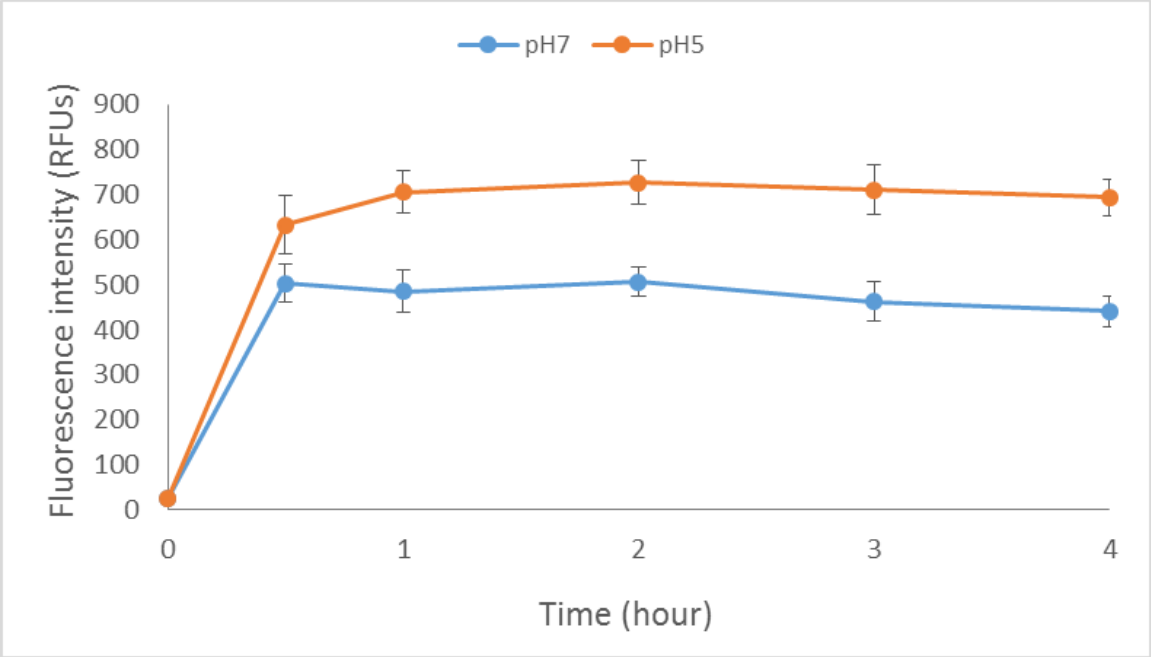



**Figure 4.7** Effect of pH on the reaction rate of TIMBOS with NAC methyl ester – a representative NPSH. TIMBOS was mixed with NAC methyl ester in a ratio of 1 : 5 in a DPBS buffer (pH 7 or pH 5,1x) at 37 ℃. An aliquot (150 µL) was withdrawn and transferred to a 96-well plate for fluorescence detection on a SpectraMax M2 microplate reader. The results were expressed as the observed fluorescence intensity and expressed as the mean value ± SD of three different experiments.

### 4.3.5 Reactivity of TIMBOS toward PSH.

In order to determine the TIMBOS' reactivity toward PSH, PSH from BSA and PSH from cell homogenates were employed. TIMBOS (100 µM) was mixed with BSA (500 µM) in a DPBS (pH 5, 1x) at 37 ℃. An aliquot (100 µL) was withdrawn at different time points and transferred to a 96 well plate to monitor fluorescence change through a SpectraMax M2 microplate reader. No increase in fluorescence intensity was observed (Figure 4.8) suggesting that TIMBOS is unable to react with PSH in BSA.

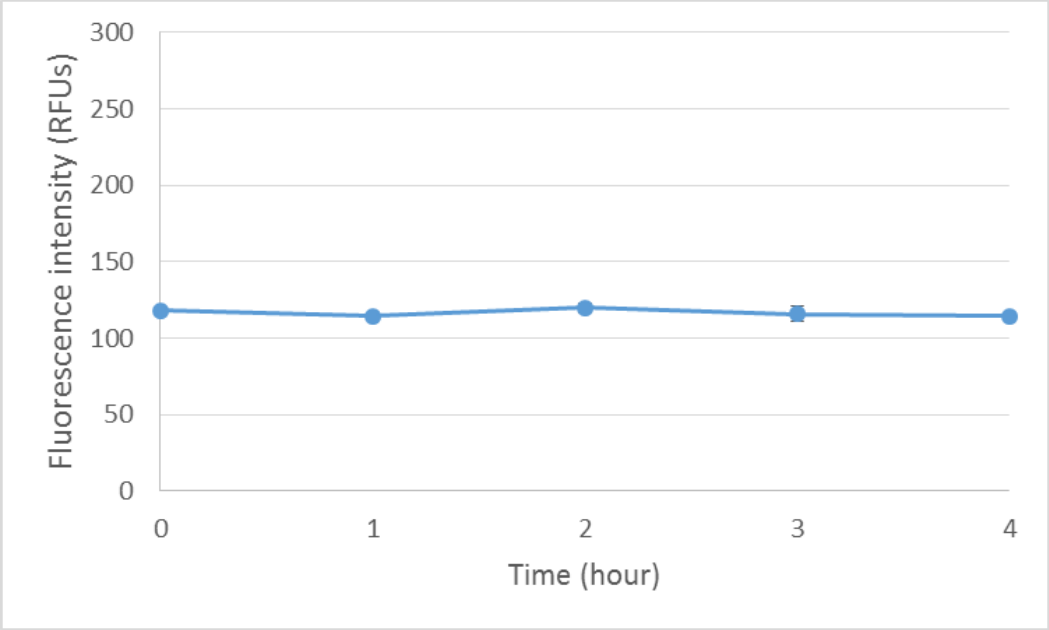



**Figure 4.8** The reactivity of TIMBOS toward PSH in BSA. TIMBOS (100 μM) was mixed with BSA (500 μM) in a DPBS buffer (pH 5, 1x) for 4 h at 37 ℃. An aliquot (100 μL) was withdrawn at different time points and transferred to a 96 well plate to monitor fluorescence on a SpectraMax M2 microplate reader. The results were expressed as the observed fluorescence intensity and expressed as the mean value ± SD of three different experiments.

To further confirm the inability of TIMBOS to react with PSH, PSH from cell homogenates were employed. When cell homogenates were mixed with TIMBOS in DPBS buffer (pH 5) followed by HPLC monitoring of the aliquots of the reaction mixture over 4 h, no new peak was observed (Figure 4.9 A), and no change in TIMBOS HPLC peak area was observed either (Figure 4.9 B). These results confirm that no reaction occurred between TIMBOS and PSH. It is believed that the inability of TIMBOS to react with PSH is due to most likely a steric hindrance of the reagent as observed with TBOP [39], TBROS [40], TBONES, and TBOZEN.

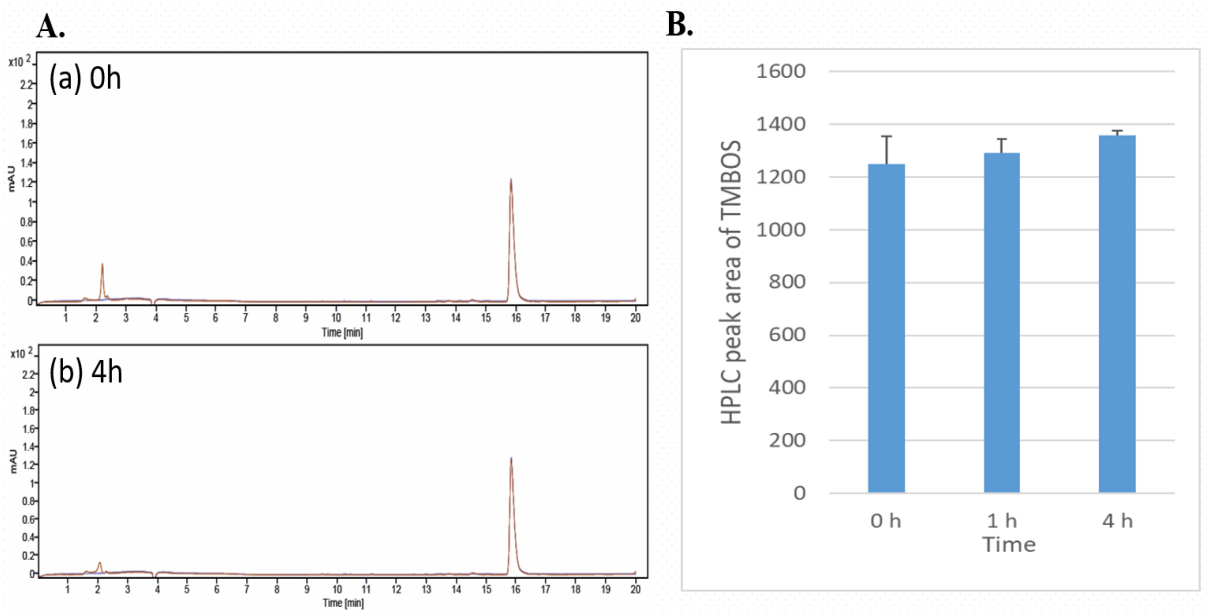



**Figure 4.9** Reactivity of TIMBOS towards PSH from cell homogenates. TIMBOS (20 µL, 0.5 µM) was mixed with protein precipitates in a DPBS buffer (280 µL, pH 5, 1 x) at 37 ℃. An aliquot (20 µL) was withdrawn for HPLC analysis. A: Representative HPLC chromatograms derived from a reaction of TIMBOS with cell homogenates at t = 0 h (a) and t = 4 h (b). B: HPLC peak area obtained from a reaction of TMBOS with PSH from cell homogenates at pH 5 and 37 ℃. The results were expressed as peak area of TBONES and representing an average of three injection of the same sample. The experiment was repeated, and the same results were obtained.

### 4.3.6 Imaging of lysosomal NPSH in live cells

#### 4.3.6.1 Cytotoxicity determination

After TIMBOS was confirmed to be a thiol specific and fluorogenic agent, the cytotoxicity of TIMBOS was determined in NCI-H226 cells using the Trypan blue assay to ensure that TIMBOS is not cytotoxic for live cell imaging. NCI-H226 cells were treated with different concentrations (100 μM, 200 μM, 400 μM) of TIMBOS for 6 h. It was found that cells remained alive (>95%) when treated with 100 μM of TIMBOS. However, 50% and 80% of the cells died when cells were treated with 200 μM and 400 μM respectively. Therefore, 100 μM of TIMBOS was chosen as the concentration for live cell imaging.

#### 4.3.6.2 Imaging of live cell lysosomal NPSH

Imaging of lysosomal thiols in live cells was achieved by cells treated with TIMBOS (100 μM) for 4 h. To ensure the fluorescence was derived from lysosomes, a co-localization experiment with a lysosome tracker was conducted. In the co-localization experiment, NCI-H226 cells were treated first with TIMBOS (100 μM, 4 h) followed by LysoTracker® Red DND-99 (100 nM), a commercially available lysosome tracker, for 0.5 hr. Figure 4.10 presents representative fluorescence images obtained from NCI-H226 cells treated with TIMBOS and LysoTracker. The blue image (image a) was the fluorescence derived from the cells treated with TIMBOS. The red image (image b) was the fluorescence derived from the cells stained with LysoTracker® Red DND-99. The image c is a co-localized image obtained by merging of TIMBOS image (image a) with LysoTracker® Red DND-99 image (image b). Image c demonstrates the images a and b

co-localized well with each other confirming that TIMBOS successfully imaged thiols in lysosomes.

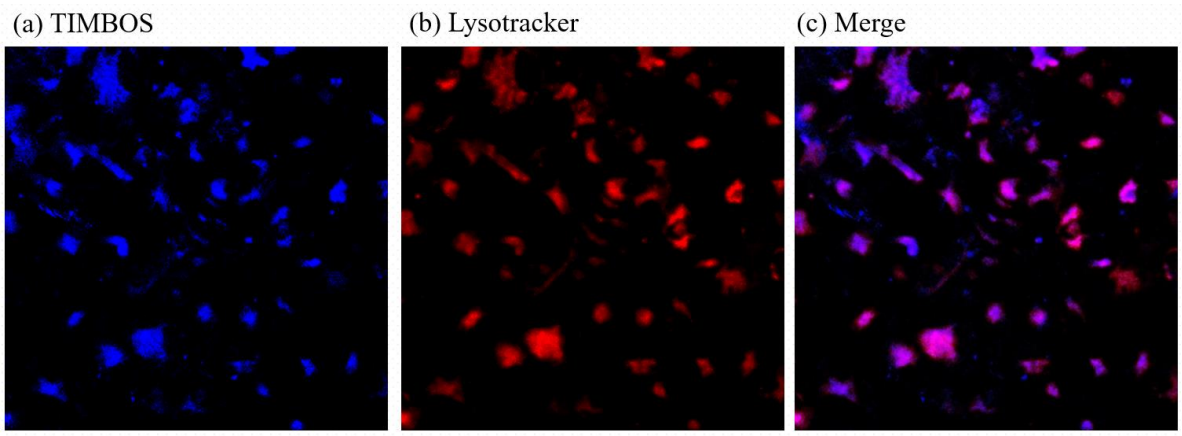



**Figure 4.10** Fluorescence images obtained from NCI-H226 cells treated with TIMBOS and LysoTracker. (a) NCI-H226 cells treated with TIMBOS, (b) NCI-H226 cells stained with LysoTracker blue DND-22, and (c) the co-localized image of image a and image b.

#### 4.3.6.3 TIMBOS' ability to detect the thiol change in lysosomes in live cells

Once TIMBOS was confirmed to image the lysosomal NPSH, the ability of TIMBOS to detect the change in thiol density in lysosomes was investigated through the use of NEM. NEM was used in the literature to deplete thiols through a covalent binding with a thiol.[38-40] Cells were first treated with different concentrations of NEM for 0.5 h to reduce cellular thiol concentration, followed by treatment of TIMBOS (100 μM, 4 h). Figure 4.11 showed the change in fluorescence intensity of thiols that obtained from treating NCI-H226 cells with different concentration of NEM followed by TIMBOS. This figure shows the relative percentage of the imaging intensity compared with the control where cells were treated with no NEM. As shown in Figure 4.11 the fluorescence

generated from the cells treated with TIMBOS decreased with an increase in NEM concentration. When NEM reached to 100 μM, only 4% of the fluorescence intensity was observed relative to the control group. These results demonstrated the ability of TIMBOS to detect the change of thiols in lysosomes in live cells through fluorescence microscopy. In addition, these results also confirm that fluorescence was generated from thiol molecules.

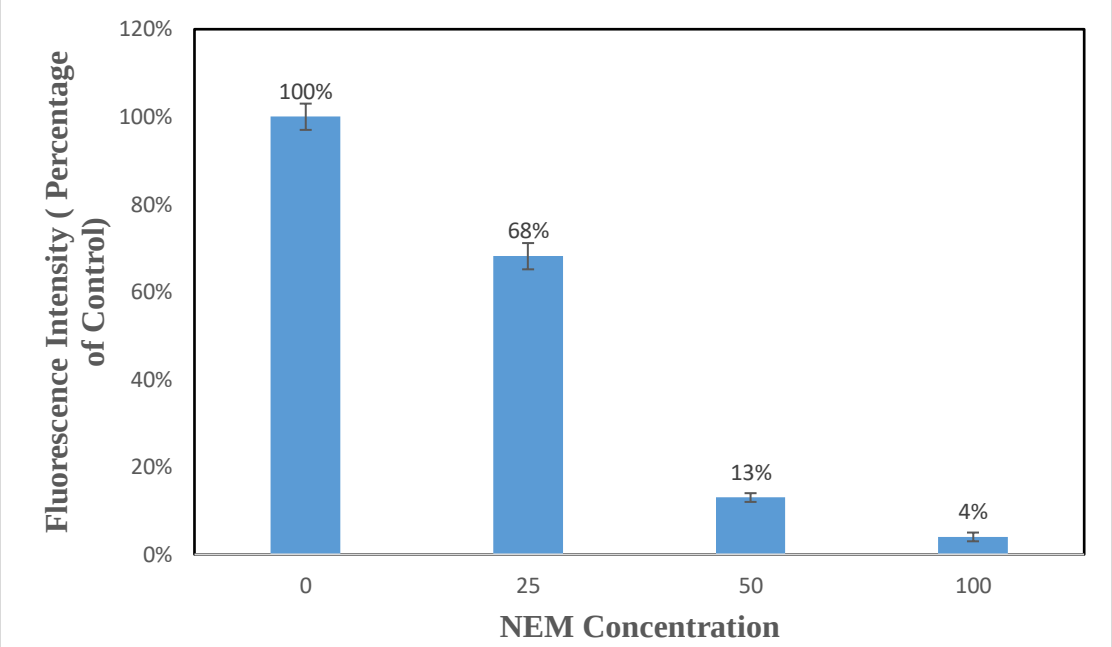



**Figure 4.11** Detection of lysosomal thiols change by TIMBOS**.** Fluorescence intensity obtained from NCI-H226 cells treated first with different concentrations of NEM followed by TIMBOS. NCI-H226 cells were seeded on a 15 mm diameter microscope cover glass in a 12-well plate. Cells were treated with different concentration of NEM for 0.5 h followed by treatment of TIMBOS (100 μM). The fluorescence intensity of obtained images was quantified by Image J.

## 4.4 Summary

We have rationally designed TIMBOS as a thiol specific lysosome-selective fluorogenic reagent. TIMBOS was synthesized and characterized as thiol specific lysosome-selective fluorogenic reagent for NPSH. TIMBOS was able to image lysosomal NPSH in live cells through fluorescence microscopy and to detect the change in NPSH in lysosomes. Unfortunately, like TBONES and TBOZEN, TIMBOS also only reacted with NPSH not PSH. TIMBOS will be a valuable tool in exploring the role of NPSH in the function and dysfunction of lysosomal thiols in live cells.

# CHAPTER 5. CONCLUSION, SIGNIFICANCE, AND FUTURE DIRECTION

## 5.1 Conclusion

Thiols play a significant role in the biological system. The unique properties of a thiol group are the basis of thiols in playing their roles in cellular functions. Thiols are distributed in an uneven manner in subcellular organelles. Disturbance of thiol levels has been associated with various diseases. Several analytical approaches were established for detection of thiols. These approaches require isolation of thiols from the cell through homogenization followed by a conventional analytical method. The drawbacks related to these methods include time-consuming and loss of information caused by cell breakage and sample preparation.

Fluorescence microscopy has been successfully used in determining an analyte in intact live cells. The main challenge for using fluorescence microscopy in detecting thiols is that thiols are not fluorescent. Fluorescence probe that can turn subcellular thiols in live cells into fluorescent molecules for fluorescence detection will be a valuable tool to provide better understanding the roles of thiols in the subcellular organelles' function and pathogenesis.

This dissertation presented the rational design of TBONES and TBOZEN as thiol specific fluorogenic agents for cell surface thiol imaging. TBONES and TBOZEN were synthesized and characterized as thiol specific and fluorogenic. However, TBONES and TBOZEN failed to image cell surface thiols due to the inability to react with PSH. Unexpectedly, TBONES and TBOZEN were found and validated to effectively image NPSH in lysosomes in live cells.

TBONES and TBOZEN were not expected to image any intracellular thiols since they were designed cell membrane impermeable due to its multiple sulfonic acid groups. One possibility for these two reagents to react with lysosomal thiols could be that these two acids might be the substrates of an organic anion transporter and were able to bind to an organic anion transporter. However, the anion transporter was not able to transport them into cells due to the bulkiness of these two acids. It is known that when a transporter cannot transport a substrate into cells, the transporter and the bound substrate would undergo endocytosis to end up in lysosomes. In other words, it is likely that TBONES and TBOZEN bind to an organic anion transporter, not able to be transported into cells due to the bulkiness, and, therefore, undergo endocytosis to enter lysosomes where they turn NPSH into fluorescent adducts. This hypothesis remains to be confirmed.

In addition to TBONES and TBOZEN, this dissertation also described the rational design, synthesis, and validation of TIMBOS as a thiol specific fluorogenic agent for imaging NPSH in lysosomes in live cells through fluorescence microscopy. The molecule was hoped to be able to image both PSH and NPSH in lysosomes. Unfortunately, the molecule was also found to fail to react with NPSH.

In summary, we have synthesized and characterized the rationally designed three thiol specific fluorogenic reagents (TBONES, TBOZEN and TIMBOS) for NPSH imaging in lysosomes in live cells. These reagents will be useful tools in exploring the roles of lysosomal thiols in cellular functions/dysfunctions

## 5.2 Significance

Thiols play significant roles in various cellular functions/dysfunctions. Most of our knowledge on thiols' roles at the cellular and subcellular levels were obtained from cell homogenates or subcellular organelle homogenates. Significant information can be lost during cell breakage. Reagents that can help visualize thiols in the cell and provide quantitively thiol information in intact cells would be valuable in helping understand thiols' roles at cellular and subcellular levels. Fluorescence microscopy is a technique that can help visualize an analyte in intact cells. The major challenge in using fluorescence microscopy to visualize an analyte is to turn the non-fluorescent analyte into a fluorescent molecule for fluorescence detection.

Various reagents have been reported to turn thiols into fluorescent molecules including monochlorobimane, chloromethyl fluorescein, *O*-phthaldialdehyde, rosamine-based, rhodamine-based, naphthalimide-based, maleimide, and iodoacetamide dyes. The chemical reaction mechanisms of these reagents to turn a thiol into a fluorescent molecule include Michael addition, cyclization with aldehyde, and nucleophilic substitution. Unfortunately, it is known that reactions involve these mechanisms are not thiol specific and can cause overestimation of thiols due to their reactivity with a nucleophile other than a thiol.

We developed three reagents (TBONES, TBOZEN, and TIMBOS) that can react with lysosomal NPSH through a thiol specific thiol-sulfide exchange reaction to produce corresponding fluorescent thiol adducts that can be imaged and quantified in intact live cells through fluorescence microscopy. This mechanism, thiol specific thiol-sulfide exchange reaction, ensure that these reagents will only react with thiols in target

subcellular organelles to provide specificity toward thiol molecules. These fluorescence probes can be valuable tools in exploring thiols' roles in the function and dysfunction in the biological system.

### 5.3 Future direction

TBONES and TBOZEN were initially designed to target cell surface thiols and were not expected to image any intracellular thiols. A mechanistic study that involves the use of an organic anion transporter inhibitor would help determine if an organic anion transporter is involved in the mechanism of lysosomal NPSH imaging by these two molecules. Alternatively, a cell culture experiment can be conducted at 4 ℃. Transport of an acid into cells by an organic anion transporter requires ATP. By conducting the experiment at 4 ℃, ATP would not be produced which would make an organic anion transporter non-functionable. We can also run a cell culture experiment in the presence of an organic anion transporter substrate. A slowdown of NPSH imaging in the presence of another substrate of the transporter will confirms the involvement of the transporter.

Further, benzofurazan sulfides with small substitutes will be designed for imaging both PSH and NPSH on cell surface and lysosomes. We believe benzofurazan with small substitutes can avoid the issue of steric bulkiness that prevents TBONES, TBOZEN, and TIMBOS from reacting with PSH.

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