Supporting Information

Design, Synthesis, and Characterization of Bis(7-(*N*-(2-morpholinoethyl)sulfamoyl)benzo[c][1,2,5]oxadiazol-5-yl)sulfane for Nonprotein Thiol Imaging in Lysosomes in Live Cells

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1. Materials and methods

Unless otherwise stated, all chemical reagents and solvent were obtained from commercial sources and used without further purification. 4-(2-Aminoethyl)morpholine and sodium hydrosulfide hydrate were purchased from Sigma-Aldrich (St. louis, MO, USA). 4-Chloro-7-chlorosulfonyl-2,1,3-benzoxadiazole was purchased from TCI (Portland, OR, USA).

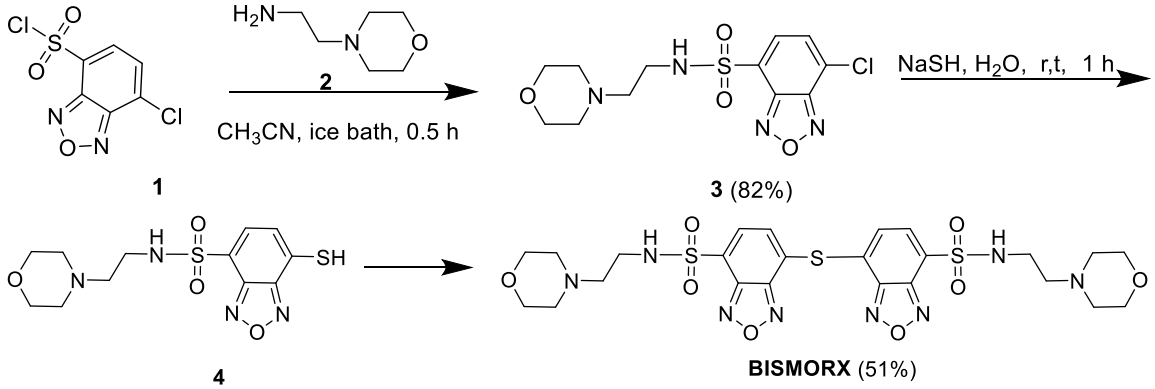
Flash column chromatography was carried out on a W-Prep 2XY Yamazen Dual Channel Flash Chromatography System (San Bruno, California). ^{1}HNMR spectra were recorded on a Bruker Varian 600 MHZ spectrometer in deuterated solvents as indicated. All NMR peaks were given as chemical shift in part per million relative to TMS (Tetramethylsilane) as the internal standard. Multiplicities are indicated by s (singlet), d (doublets), 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).

HPLC analysis was achieved on an Agilent HPLC system connected with an 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% 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 fluorescence detection.

2. Synthesis and characterization of BISMORX

The synthetic scheme for BISMORX is outlined in Scheme S1. The synthesis of BISMORX started with addition of a commercially available 4-(2-aminoethyl)morpholine to 4-chloro-7-chlorosulfonyl-2,1,3-benzoxadiazole (**1**) to produce the intermediate **3** in 82% yield. BISMORX was obtained in 51% yield after the treatment of **3** with sodium hydrosulfide hydrate to yield a thiophenol (**4**) followed by air-oxidation in a one-pot reaction (Scheme S1). BISMORX was characterized by ^{1}HNMR and HRMS. The purity of BISMORX was confirmed by HPLC to be > 97%.



Scheme S1. Synthesis of BISMORX

*7-Chloro-N-(2-morpholinoethyl)benzo[c][1,2,5]oxadiazole-4-sulfonamide (**3**).* To a stirred solution of 4-chloro-7-chlorosulfonyl-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 stirred in an ice bath for 0.5 h before the solvents were removed by a rotary evaporator under a reduced pressure. The resulting residue was purified through a silica gel column (CH_2Cl_2/MeOH) to obtain the desired compound (**3**) as a yellow solid product (0.566 g, 82%). The product was characterized by ^{1}H NMR and LRMS. ^{1}H NMR (600 MHz, acetone-d6) δ 7.97 (d, *J* = 7 Hz, 1H), 7.74 (d, *J* = 7 Hz, 1H), 3.28 [t, *J* = 6 Hz, 4H], 3.09 (t, *J* = 6 Hz, 2H), 2.28 [t, *J* = 6 Hz, 2H), 2.09 (brs, 4H). $C_{12}H_{15}ClN_4O_4S$ (M+H, 347.10) was confirmed by LRMS.

*Bis(7-(N-(2-morpholinoethyl)sulfamoyl)benzo[c][1,2,5]oxadiazol-5-yl)sulfane (**BISMORX**).* 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 a reduced pressure by a rotary evaporator to produce a yellow residue. The residue was purified through a silica gel column (CH_2Cl_2/MeOH) to obtain the desired product (BISMORX) as a yellow solid 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, acetone-d6) δ 7.97 (d, *J* = 7 Hz, 2H), 7.74 (d, *J* = 7 Hz, 2H), 3.33-3.19 (m, 8 H), 3.09 [dt, *J* = 18, 6 Hz, 4H), 2.3-2.23 (m, 4H), 2.13-2.04 (m, 8H). Exact mass calculated for $C_{24}H_{31}N_8O_8S_3$ (M+1): 655.1417, found: 655.1417.

3. Chemical stability of BISMORX

The chemical stability of BISMORX in a DBPS buffer (pH 7, 1x) was investigated by HPLC. As presented in Figure S1, no change in BISMORX was observed over the 28 h period indicating that BISMORX was stable over the 28 h period at the experimental condition.

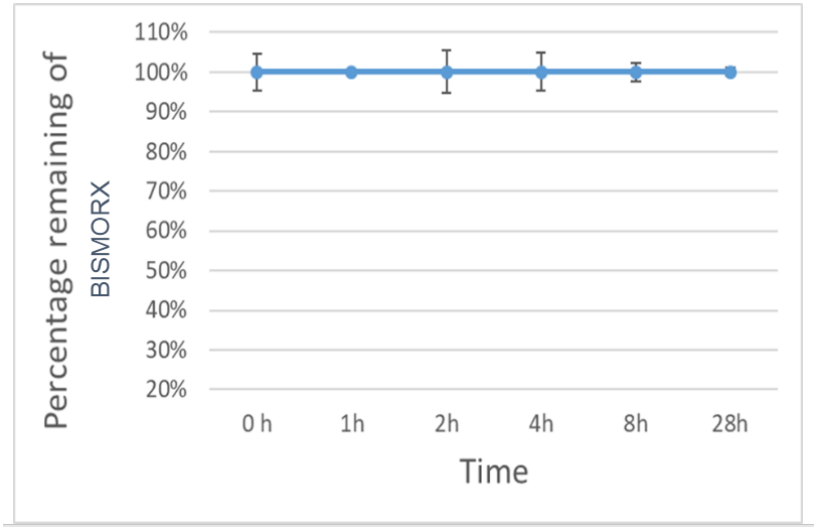


Figure S1. Stability of BISMORX. BISMORX (100 μM) was dissolved in a DBPS buffer (pH 7, 1x) at 37 °C for 28 h. An aliquot (10 μL) was withdrawn for HPLC analysis. The results were expressed as a percentage of the remaining BISMORX and representing an average of three injection of the same sample. The experiment was repeated, and the same results were obtained

4. Chemical reactivity of BISMORX with NAC methyl ester

The chemical reactivities of BISMORX with -NH_2, -OH, or -COOH groups were determined by using NAC methyl ester as a model molecule. No reaction was observed when BISMORX was mixed with serine even at a ratio of 1:50 for 6 h. Figure S2 shows that BISMORX peak remained unchanged at 0 min and 60 min of the reaction. The data suggest that BISMORX does not react with the nucleophilic functional groups -NH_2, -OH, or –COOH.

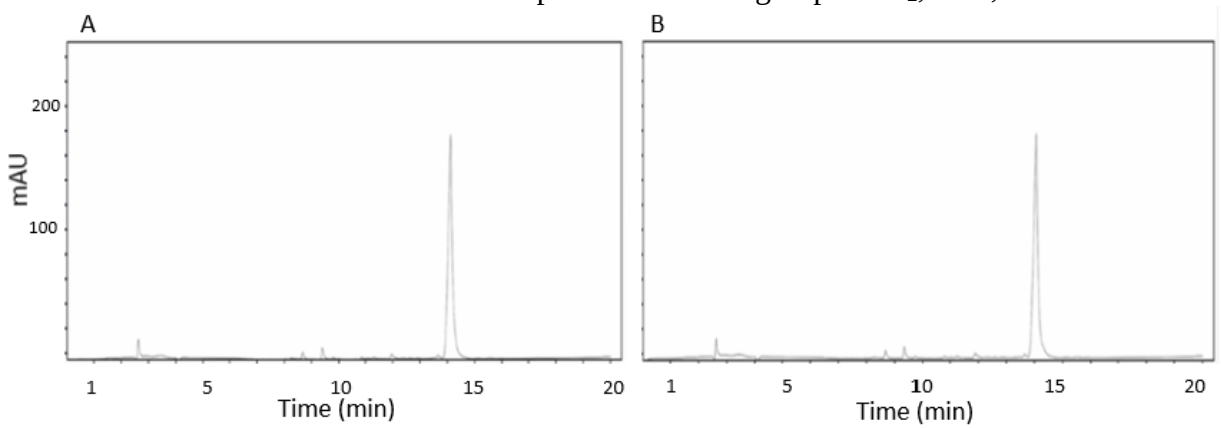


Figure S2. Representative HPLC chromatograms from a reaction of BISMORX with serine. BISMORX (100 μM) was mixed with serine (5 mM) in a DPBS buffer (pH 7, 1x) at 37 °C for 0 min (**A**) and 6 h (**B**). The chromatograms were obtained by using a diode array detector at 254 nm.

5. Cell viability determination

Cell viability in the presence of BISMORX was determined by using the trypan blue assay and NCI-H226 cells (human lung cancer from the National Cancer Institute). Briefly, NCI-H226 cells were seeded in a 12-well plate at a concentration of 75000 cells/well and placed in a 37 °C and 5% CO_2 incubator. After reaching to about 80% confluence, the cells were treated with different concentration (100 μM, 200 μM, 400 μM) of BISMORX for 6 h. After the 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 conducted according to the manufacture protocol. it was found that the cells remained alive (>95%) when treated with 100 μM of BISMORX. However, 50% and 80% of the cells died when the cells were treated with 200 μM and 400 μM of BISMORX respectively. Therefore, BISMORX at 100 μM was chosen as the concentration for live cell thiol imaging.

6. Effect of pH on the reactivity of BISMORX

Since BISMORX was designed to detect thiols in lysosomes which are acidic (pH 4.5-6), BISMORX's reactivity toward NPSH was checked at pH 5 to ensure that BISMORX would be able to react with NPSH in lysosomes. BISMORX (100 μM) was mixed with NAC methyl ester (500 μM) in DPBS buffer (pH 5, 1x) at 37 °C for 4 h. The reaction was monitored for its change in fluorescence intensity. An experiment at pH 7 was also conducted in parallel for comparison. Figure S3 shows that BISMORX's reaction with NAC methyl ester was completed in 1 h at pH 5. Interestingly, BISMORX completed the reaction also in 1 h at pH 7 though the fluorescence intensity was stronger at pH 5.

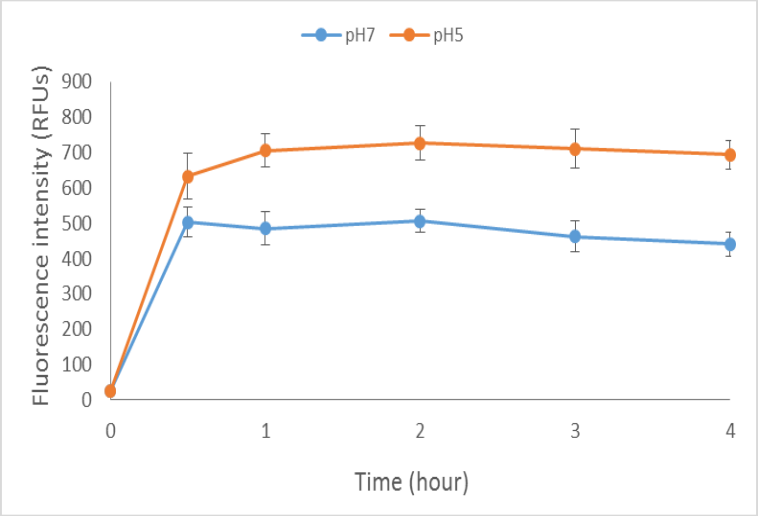


Figure S3. Effects of pH on the reaction rate of BISMORX with NAC methyl ester. BISMORX was mixed with NAC methyl ester at a ratio of 1 : 5 in a DPBS buffer (pH 7 or pH 5, 1x) at 37 °C. An aliquot (150 μL) was withdrawn and transferred to a 96-well plate for fluorescence detection on a SpectraMax M2 microplate reader. The results were expressed as the observed fluorescence intensity.

7. Reactivity of BISMORX with protein thiols (PSH)

In order to determine the BISMORX's reactivity toward PSH, PSH from bovine serum albumin (BSA) and cell homogenates were employed. When BISMORX (100 μM) was mixed with BSA (500 μM) in a DPBS buffer (pH 5, 1x) at 37 °C, no increase in fluorescence intensity was observed (Figure S4) suggesting that BISMORX was unable to react with PSH in BSA.

Similar results were obtained for the reaction of BISMORX with PSH from cell homogenates. When proteins from cell homogenates were mixed with BISMORX in a DPBS buffer (pH 5, 1x), no new peak was observed (Figure S5A), and no peak area change of BISMORX was observed either (Figure S5B) when the reaction was monitored by HPLC. These results confirm that no reaction occurred between BISMORX and PSH.

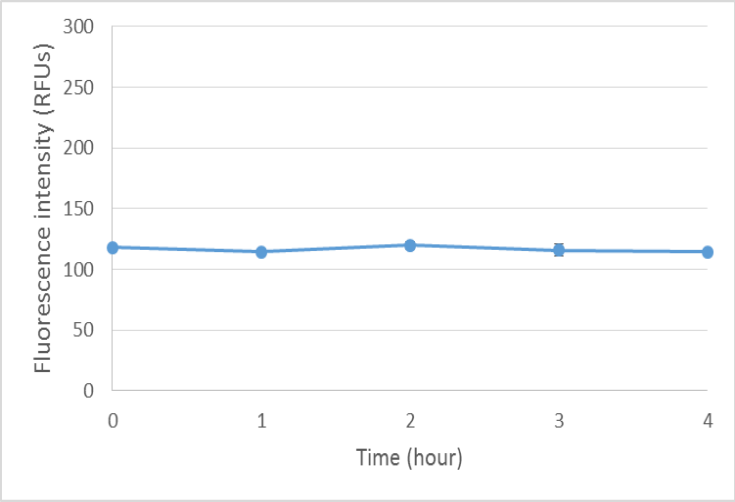


Figure S4. The reactivity of BISMORX toward PSH in BSA. BISMORX (100 μM) was mixed with BSA (500 μM) in a DPBS buffer (pH 5, 1x) for 4 h at 37 °C. An aliquot (100 μL) was withdrawn at different time points and transferred to a 96 well plate to monitor fluorescence on a

SpectraMax M2 microplate reader. The results were expressed as the observed fluorescence intensity.

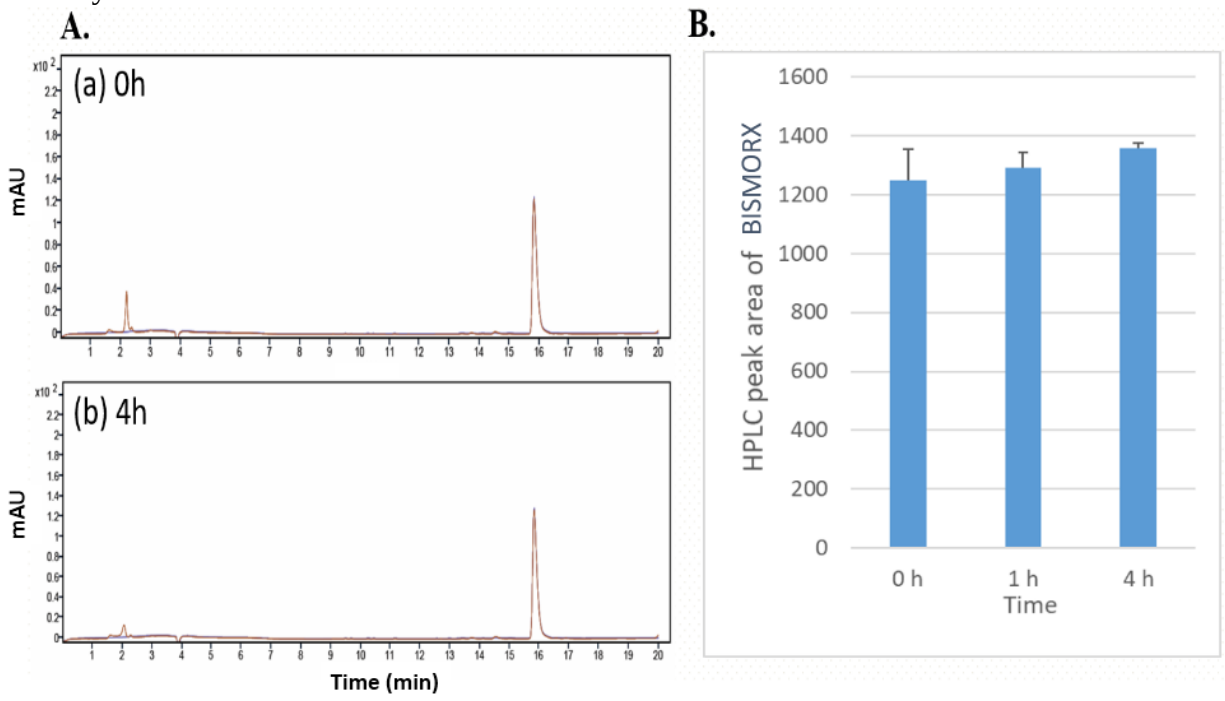


Figure S5. Reactivity of BISMORX towards PSH from cell homogenates. NCI-H226 cells (6 $\times 10^6$) were washed once with DPBS, homogenized by ultrasonication with 1 mL of 3% SSA for 10 min, followed by centrifugation (14000 rpm, 5 min) at 4 °C to remove NPSH. Protein precipitates were washed five times with 1 mL of 3% of SSA before re-suspended in a DPBS buffer (pH 5, 280 μL), and treated with BISMORX (0.5 mM, 20 μL) at 37 °C. An aliquot (50 μL) at different time points (0 h, 1 h, 4 h) was withdrawn, added with acetonitrile (150 μl), and followed by centrifugation (14000 rpm, 5 min) to remove proteins before 20 μL being used for HPLC analysis. **A:** Representative HPLC chromatograms derived from a reaction of BISMORX with PSH from cell homogenates at t = 0 h (a), 4 h (b). **B:** BISMORX HPLC peak areas obtained from a reaction of BISMORX with PSH from cell homogenates at pH 5 and 37 °C.