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A Highly Selective Fluorescent Probe for Thiol Bioimaging

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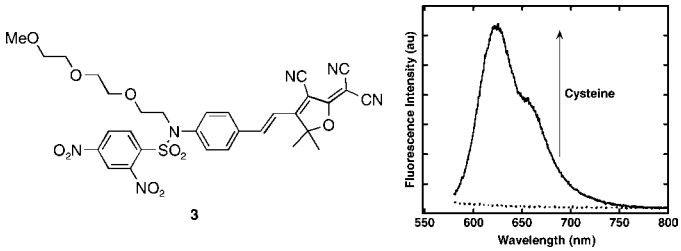
ABSTRACT



A new fluorescent turn-on probe (3) for the selective sensing and bioimaging of thiols is reported. In aqueous buffer solutions at physiological pH, thiols cleave the 2,4-dinitrobenzenesulfonyl group to release the red-emissive donor–acceptor fluorophore (4). The probe displays excellent immunity to interference from nitrogen and oxygen nucleophiles and the imaging of thiols in living cells is demonstrated.

The rapid, sensitive, and selective sensing of thiols is of significant interest in areas ranging from the petrochemical industry,[1] to food quality control[2] and medicine. The optical sensing of thiols is particularly relevant in biological systems. Intracellular thiols such as glutathione (GSH), cysteine (Cys), and homocysteine (HCys) play a crucial role in maintaining biological redox homeostasis through the equilibrium established at a given electrical potential between reduced free thiols (RSH) and oxidized disulfides (RSSR). Thiols are also active in the catalytic sites of enzymes, and play important roles in metabolic pathways.[3] The levels of certain thiols, such as homocysteine, have been linked to a number of diseases, including cancer, Alzheimer's, and cardiovascular disease.[4] Thiol levels may also be affected in response to the oxidative stress that has been associated with some of these conditions.[5] Furthermore, thiol probes are desirable for assaying enzymes that release a thiol upon reaction with a substrate (e.g., thioesterases) and for finding and evaluating new inhibitors of these enzymes.[6]

Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)) is the classical probe for the determination of sulfhydryl groups by UV−vis absorption spectrophotometry.[7] A large number

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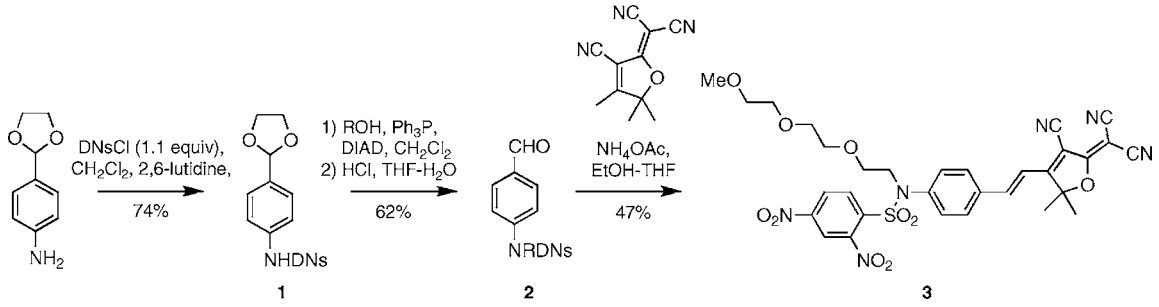
Scheme 1. Synthesis of Fluorescent Thiol Probe **3**



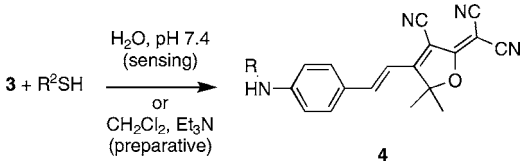
of chromophores and fluorophores bearing electrophilic groups (e.g., iodoacetamides, maleimides, and benzyl halides) to which thiols may be covalently attached have been developed for thiol bioconjugation and are commercially available. However, these often suffer from low on/off signal ratios, necessitating washing and isolation steps, thus precluding rapid quantification.[8]

The area of thiol-sensitive sensors and imaging agents is undergoing a renaissance that is largely driven by the development of selective fluorescent turn-on probes. In one of these approaches, the reaction between a thiol and a maleimide interrupts an intramolecular PET (photoinduced electron transfer) process, converting the quenched probe into an efficient fluorophore.[9] In an alternative strategy, the use of a thiol nucleophile as a reagent for the deprotection of an electron-poor arenesulfonate ester[10] results in the release of a fluorophore.[6b,c,11] Many of these probes still suffer from poor solubility in aqueous media, requiring the use of organic cosolvents.[12] Arenesulfonate protecting groups are also susceptible to attack by oxygen or nitrogen nucleophiles. These parasitic side reactions (e.g., unselective hydrolysis) lower both sensitivity (higher blank signals) and selectivity for thiol analytes. Finally, many of these probes are excited and/or emit in the near-UV to green region of the spectrum. However, long-wavelength probes with emission in the red or near-infrared are optimal for biological imaging applications due to decreased light scattering, increased optical transparency, reduced autofluorescence, and greater photostability of tissues at these wavelengths.

In this report, the design and synthesis of a new fluorescent probe for thiols (**3**) that addresses some of these drawbacks is detailed. Probe **3**'s design is based on a classical donor−π-acceptor architecture. Thiol-mediated cleavage of the electron-withdrawing sulfonyl group releases an aniline donor, increasing the push−pull character of the dye and resulting in a higher quantum yield and in large bathochromic shifts in the absorption and emission spectra. The choice of the arenesulfonamide[13] protecting group improves the probe's resistance toward oxygen and nitrogen nucleophiles, and increase selectivity toward thiols in comparison to arenesulfonate-based probes.[6b,c] Substitution of the sulfonamide N−H with a side chain extends the pH range in which probe **3** can be used compared to the corresponding acidic secondary 2,4-dinitrobenzenesulfonamides. The latter decompose to the corresponding 2,4-dinitroanilines after extrusion of sulfur dioxide under basic conditions.[10,13] Furthermore, the side chain influences the properties of the probe. The triethyleneglycol methyl ether chain confers higher water solubility to probe **3**. The synthesis of probe **3** is presented in Scheme 1.

Probe **3** is prepared in moderate yield in three steps starting from an acetal-protected 4-aminobenzaldehyde. The modular design allows for the independent variation of each component (arenesulfonyl group, side chain, π-conjugated bridge, electron acceptor) and facilitates the tailoring of probes for specific applications. For instance, side chains bearing molecular recognition groups or surface anchors may be introduced without the need to redesign the probe's synthesis. The validity of the proposed sensing mechanism was confirmed by the preparative-scale synthesis of the free dye **4** from **3** and thioglycolic acid (Scheme 2).

Scheme 2. Cleavage of Sulfonamide **3** with Thiols



The sensory response of probe **3** is exemplified by its reaction with cysteine (Figure 1). Upon addition of cysteine

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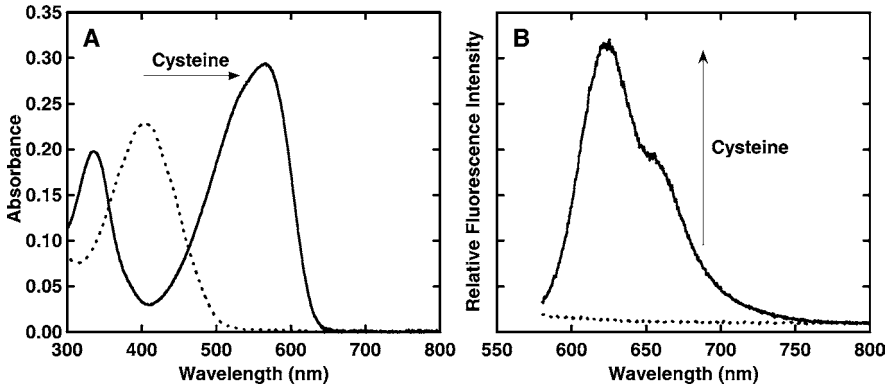

Figure 1. (a) Absorption and (b) emission spectra (λ_{ex} = 560 nm) of probe **3** before (dotted line) and after (solid line) addition of cysteine in HEPES buffer solution (10 mM, pH 7.4). The spectra were obtained immediately after addition of cysteine. The final concentrations of probe and cysteine are 10 μM and 1 mM, respectively.

to a solution of **3** in HEPES buffer (10 mM, pH 7.4), the solution instantly turns from yellow to pink, and the absorption spectrum shows the development of the characteristic absorption band of **4** that is red-shifted by 158 nm compared to that of **3**. A remarkable increase in the fluorescence intensity of up to 120-fold, depending on the concentrations of the reagents, is also observed.

The selectivity of probe **3** for thiols was confirmed by screening its response to biologically relevant analytes under physiological conditions (37 °C, 10 mM HEPES buffer, pH 7.4) (Figure 2). Probe **3** shows a positive fluorescence

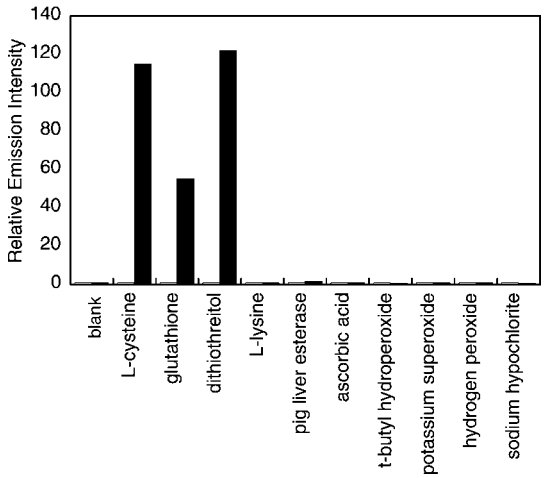

Figure 2. Response of probe **3** to different analytes. Relative fluorescence intensity of 10 μM probe **3** (HEPES buffer, pH 7.4) at 627 nm (λ_{ex} = 565 nm) before (white bars, left) and after (black bars, right) incubation at 37 °C for 15 min in the presence of 100 μM (final concentrations) analytes. Porcine liver esterase was added to a concentration of 1.4 units/mL.

response only in the presence of thiols (cysteine, reduced glutathione, dithiothreitol), for which fluorescence signals were increased 60- to 120-fold. No significant signal increases above blank levels were observed in the presence of amines (lysine), reactive oxygen species (*tert*-butylhydroperoxide, hydrogen peroxide, sodium hypochlorite), or reducing agents (ascorbic acid). Probe **3** also remains essentially unaffected by a hydrolytic enzyme (porcine liver esterase). It is worth noting that probe **3** is responsive to thiols at a pH lower than their pK_a (∼10−11), indicating that complete deprotonation of the thiols is not required for their detection.

In aqueous media (10 mM HEPES buffer, pH 7.4), probe **3** has an absorption maximum at 405 nm (ϵ = 21 200 mol L^{-1} cm^{-1}) and is essentially nonfluorescent with a trace emission centered at λ_{ex} = 560 nm (Φ_F = 0.0008). The quenched fluorescence is attributed to the absence of charge-transfer character in the protected probe and to an efficient donor-excited photoinduced electron transfer (PET) to the dinitrobenzenesulfonyl group. Upon deprotection, free dye **4** exhibits a significant bathochromic shift in its absorption spectrum, with an absorption maximum at 563 nm (ϵ = 35 100 mol L^{-1} cm^{-1}), indicative of the more efficient delocalization in the push−pull chromophore.

The absorption and emission spectra of **3** and **4** remain essentially unchanged over a wide pH range (5.6−9.5) that is compatible with most biological applications. Furthermore, in aqueous buffer solution at pH 7.4, no hydrolysis of probe **3** is detected over 12 h at 37 °C.

In aqueous solution, the fluorescence quantum yield of **4** remains modest with Φ_F = 0.01 (λ_{em} = 623 nm). However, like other donor−acceptor fluorophores bearing dicyanomethylene-based acceptors,[14] the fluorescence quantum yield increases in a more viscous or rigid environment. In 90:10 glycerol:methanol (v/v), the quantum yield reaches a value of 0.18. This dependence on media can be advantageous for

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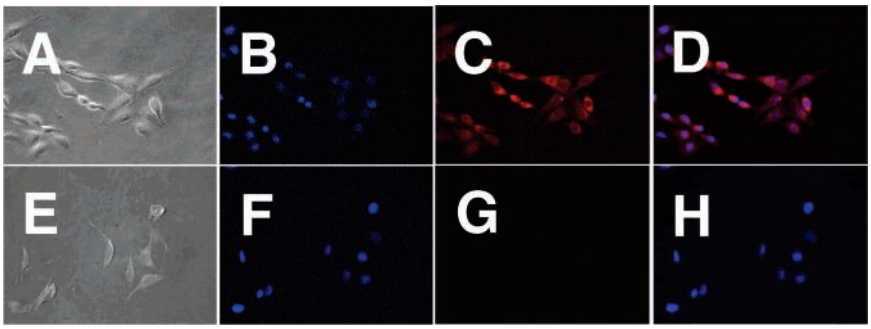

Figure 3. Fluorescence images of 3T3 cells: (A) brightfield image of cells incubated with probe **3** (25 μM) for 10 min at 37 °C, and stained with a nucleus-staining dye, Hoechst 33342; (B) fluorescence image collected with a Hoechst dye filter set; (C) fluorescence image collected with a Cy3 dye filter set; (D) overlay image of B and C; and (E−H) corresponding control images of cells pretreated with *N*-methylmaleimide (1 mM) for 60 min at 37 °C and then incubated with **3** as above.

biological imaging. Upon interaction or adsorption with macromolecules and surfaces in cellular environments, the Φ_F of **4** increases, while probe **3** remains essentially nonemissive. The photophysical properties of the free dye **4** in the presence of human serum albumin (HSA) illustrate this effect. The addition of 1% HSA to a solution of **4** in HEPES buffer increased its Φ_F to 0.24. Small bathochromic and hyperchromic shifts are also observed upon addition of HSA. The beneficial influence on the fluorescence quantum yield of interactions between dye **4** and biological surfaces and macromolecules suggests that turn-on fluorescence signal improvements greater than the 120-fold on/off ratios observed in ordinary aqueous solutions may be achieved in a number of biosensing schemes. Furthermore, the photophysical properties of fluorophores closely related to **4** suggest that this thiol probe could be adequate for single-molecule or 2-photon sensing schemes.[14b]

The monitoring of thiols in living cells by probe **3** was undertaken. Albino Swiss mouse embryo fibroblast cells (3T3 cell line) were incubated with a solution of probe **3** (25 μM in 1:100 DMSO−PBS v/v, pH 7.4) for 10 min at 37 °C. Probe **3** was found to be cell-permeable and to react with intracellular thiols, resulting in strong fluorescence emission as observed by fluorescence microscopy (Figure 3C). In a control experiment, cells that were pretreated with an excess of the thiol-reactive *N*-methylmaleimide, which consumes all of the free thiols within the cell, and then incubated with probe **3** do not show a significant fluorescence signal (Figure 3G). This confirms the specificity of probe **3** for thiols over other analytes in living cells.

In conclusion, probe **3** has been shown to be a thiol-selective turn-on fluorescence probe with high on/off ratios (up to 120-fold) with emission in the red region of the spectrum. Probe **3** shows good resistance toward unselective hydrolysis. The emissive free dye **4** released after reaction with a thiol exhibits large batho- and hyperchromic shifts with respect to **3**, and features a fluorescence quantum efficiency that is greatly enhanced in the presence of biological macromolecules. The application of probe **3** for the bioimaging of thiols in live cells has been demonstrated, and shows potential for in vivo small animal imaging.[15] The modular nature of the probe's design opens the possibility of tailored probes for specific applications.

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Supporting Information Available: Experimental procedures and full spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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