Fluorescent Probes

2,4-Dinitrobenzenesulfonyl Fluoresceins as Fluorescent Alternatives to Ellman's Reagent in Thiol-Quantification Enzyme Assays**

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Since its first description in 1959,[1] 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB or Ellman's reagent) has been used widely for the quantification of thiols, especially for assays of various enzymes including acetyl- and butyrylcholinesterase (AChE and BChE, respectively)[2] in which substrates release thiols through enzymatic reactions.[3] 4,4′-Bipyridyl disulfide[4] and 5-(2-aminoethyl)dithio-2-nitrobenzoic acid[3] were also shown to be useful for colorimetric analyses of thiols. However, fluorescent or chemiluminescent thiol probes would be more practical than these chromogenic probes for enzyme assays, especially for high-throughput programs to screen enzyme inhibitors as new drugs. Recently, the use of a dioxetane-disulfide derivative as a chemiluminescent probe in place of DTNB led to an improved sensitivity of the AChE assay with acetylthiocholine (ASCh) by a factor of ten.[5] However, the assay required a rather tedious procedure in

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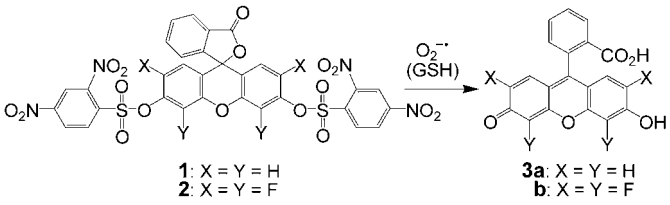
which the enzyme reaction and detection steps were performed independently owing to the limited solubility of the chemiluminescent probe in aqueous media.

At present, a wide variety of fluorescent probes for thiols are available.[6] Among them, maleimide-type probes CPM[6] and ThioGlo_3,[7] and a haloalkyl-type probe monobromobimane[6] may be utilized for thiol-determination enzyme assays. However, these probes have disadvantages that attenuate their utility in enzyme assays. CPM and ThioGlo_3 suffer from limited solubility in aqueous media, similar to the dioxetane-disulfide chemiluminescent derivative mentioned earlier, as well as inevitable side reactions of their maleimide groups to form fluorescent byproducts. Also, under mild conditions, reactions of monobromobimane with thiols are relatively slow.[8] Thus, these fluorescent probes have found applications in the labeling of biopolymers and derivatization of thiols for separation analysis rather than in enzyme assays.

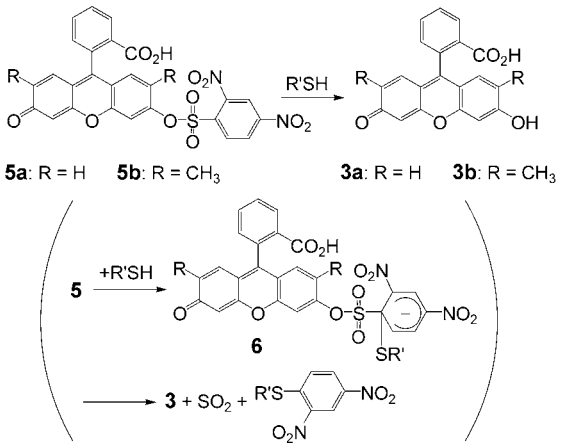
Recently it was found that **2** (Scheme 1) works as a highly specific probe for superoxide ($O_2^{-\bullet}$) based on deprotection of 2,4-dinitrobenzenesulfonyl (DBS) groups by $O_2^{-\bullet}$, whereas **1**



Scheme 1. A previously reported fluorescent probe for superoxide.

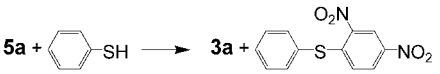
is transformed to **3a** much more effectively upon reaction with glutathione (GSH) through nucleophilic aromatic substitution[9,10] than with $O_2^{-\bullet}$.[11] The latter observation provided us with a clue for the design of novel fluorescent probes for thiols. So far, nucleophilic aromatic substitution has been capitalized only as a derivatizing reaction for the separation analysis of thiols with halogenated benzofurazans.[12] This fact also encouraged us to develop thiol probes based on nucleophilic aromatic substitution. Compound **1** is unlikely to work as a thiol probe as the observed fluorescence response of **1** toward GSH was relatively small. However, changing the protection mode with DBS groups from bis to mono was expected to enhance not only the reactivity toward thiols but also its solubility in aqueous media. Furthermore, it is well known that nucleophilic aromatic substitution proceeds more effectively as the acidity of leaving groups decreases.[10] These considerations led us to design **5b** as well as **5a** (Scheme 2) as fluorescent alternatives to DTNB for thiol-quantification enzyme assays. Herein we describe the characteristics of **5** as thiol probes and their utility in screening assays for ChE inhibitors.

As expected, monoprotection with a DBS group imparted to **5** a high degree of solubility in aqueous media. Working solutions of these probes were prepared by 200-fold dilution of ethanolic solutions of **5** with aqueous HEPES buffer (pH 7.4, HEPES = *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid). Relative quantum efficiencies (Φ_{fl}) of **5a** and **5b** in HEPES buffer (pH 7.4) were 0.0007 and 0.0003,



Scheme 2. Probes **5** for thiols, and the reactions that give rise to their fluorescent products **3**, as used in this study.

respectively, relative to **3a** in 0.1M NaOH ($\Phi_{fl} = 0.85$) as standard.[13] Similar estimates of Φ_{fl} for **3a** and **3b**[14,15] in the same buffer were 0.75 and 0.58, respectively. Compound **5a** was treated at room temperature with PhSH (1.1 equiv) in HEPES buffer (pH 7.4) containing 5% EtOH. After 10 minutes, **5a** was almost completely consumed and (2,4-dinitrophenyl)phenylsulfide was isolated in 96% yield [Eq. (1)]. No byproducts other than PhSSPh (4%) were



(1)

observed. Additionally, only 0.7 and 1.1% of **5a** and **5b**, respectively, decomposed to the corresponding compound **3** after 1 hour of incubation in buffer solution at 37°C. These results demonstrate that conversion of **5** into **3** is a useful reaction for the determination of thiols in aqueous media, through the switching "on" of fluorescence. Also, its hydrolysis at a negligible level is the only expected side reaction in aqueous solution.

Excitation and emission spectra were recorded at several intervals during the reaction of **5** with GSH at 37°C in HEPES buffer (pH 7.4, see Figure 1). As soon as GSH was added, excitation and emission peaks for **3** appeared in the spectra as a result of its generation from **5**, with rapidly increasing intensity that stabilized 10 minutes after the initial incubation. The rate constants k_{obsd}[16] for reaction of **5a** or **5b** with GSH at pH 7.4 and 37°C were 1.7×10^2 and $1.4 \times 10^2\,\text{M}^{-1}\,\text{s}^{-1}$, respectively. Compounds **5** reacted with cysteine (Cys) and $H_2NCH_2CH_2SH$ (AET) with rate constants that were similar to those for GSH, while deprotection of **5** by $HSCH_2CH_2CO_2H$ (MPA) was about ten times slower than that by GSH (Table 1). The rate constant k for the reaction of DTNB with GSH at 25°C is $3.5 \times 10^4\,\text{M}^{-1}\text{s}^{-1}$.[17] For comparison the k values of the present probes are included in Table 1 and indicate that compounds **5** react with GSH approximately ten times slower than DTNB. However, this fact was not a disadvantage for determining thiols with **5**. When an assay

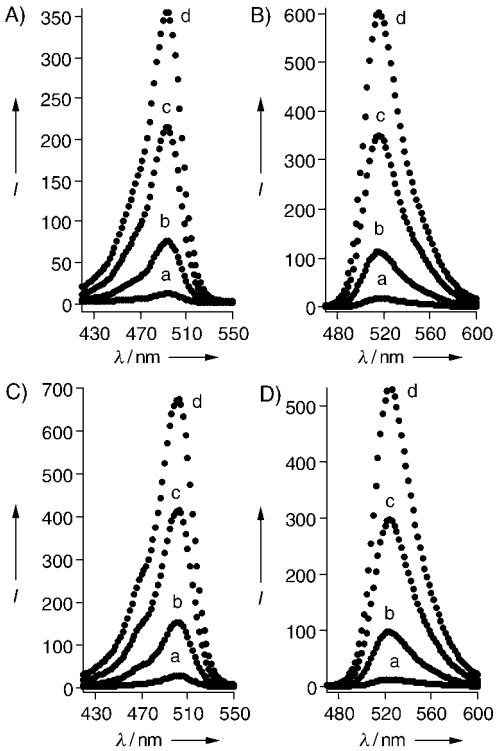


Figure 1. Excitation and emission spectra of **5a** (A, B) and **5b** (C, D) measured at 37 °C at a) $t = 0$, b) $t = 1$, c) $t = 4$, and d) $t = 10$ minutes after addition of GSH (final concentration: 50 μM; $\lambda_{em} = 560$ nm, $\lambda_{ex} = 460$ nm). Final concentrations of solutions of **5** were 25 μM in HEPES buffer (10 mM, pH 7.4, 3 mL) containing 0.5% EtOH.

with **5** was performed for the thiols described in Table 1 in a 96-well microtitre plate, detection limits of less than 2.0 pmol/well were noted for GSH, Cys, and AET, and a linear calibration curve was obtained for each of these thiols up to 1000 pmol/well. Sensitivity, as expressed by the slope of the calibration curves, suggested that **5b** was slightly more sensitive than **5a**.

Dependency of the present fluorometric assay on pH was also examined for the reaction of **5b** with GSH, and **5b** was found to act as a probe down to pH 7.0, although the slope of the calibration curve obtained at pH 7.0 was about one-quarter of that determined at pH 7.4. The fluorescence intensities of **3b** at pH 7.0 and 6.6 (HEPES buffer) were 88 and 74%, respectively, of that measured at pH 7.4. Thus, the lower increase in fluorescence on reaction of **5b** with GSH at pH values lower than 7.4 is ascribed to a decrease in the reactivity of GSH at lower pH values. The observed pH dependency would not militate against application of the present fluorometric assay to thiol-quantification enzyme assays, as assays of various enzymes with DTNB are generally carried out at pH 7.5–8.0.

The results of fluorometric assays using **5** of human erythrocyte AChE and serum BChE with ASCh and butyrylthiocholine (BSCh) as substrates, respectively, are shown in Figure 2. Each of the enzyme reactions was performed in HEPES buffer (pH 7.4) at 37 °C for 10 minutes. Fluorometry with **5b** enabled the determination of AChE and BChE, even at 0.05 and 0.001 mU/well (1 mU of AChE (or BChE) is defined to hydrolyze 1 nmol of ASCh (or BSCh) per minute at pH 7.4 and 37 °C), respectively. The detection limits for these analytes with **5a** were 0.05 and 0.01 mU/well, respectively. Compound **5b** also performed better than **5a** for the ChE assay. Thus, the fluorometric assay with **5b** was applied to determine inhibitory constants (IC_{50}) of some ChE inhibitors. After a mixture of **5b**, ChE, its substrate, and a ChE inhibitor was incubated at 37 °C for 10 minutes, an increase in fluorescence intensity was observed. Inhibition curves were analyzed by a four-parameter logistic regression to afford IC_{50} values. The results are summarized in Table 2, which also includes the constants determined with DTNB. A good correlation was obtained

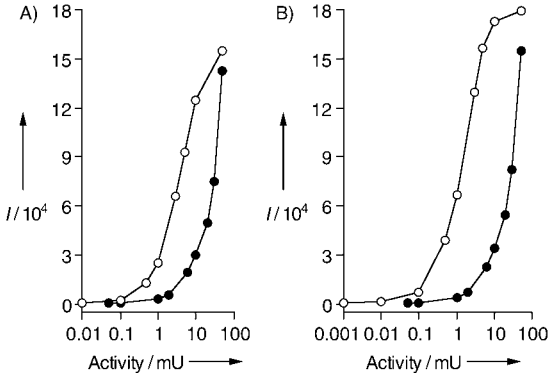


Figure 2. Assays of human erythrocyte AChE (●) and serum BChE (○) in the presence of **5**. Assays were performed after incubating a mixture of the analyte ChE (10 μL), its substrate (ASCh for AChE and BSCh for BChE, respectively; 1.0 mM, 10 μL in H_2O), and either A) **5a** or B) **5b** (25 μM, 170 μL in HEPES (10 mM, pH 7.4) containing 0.5% EtOH) in a 96-well microtitre plate at 37 °C for 10 minutes. The final volume of the reaction mixture was adjusted to 200 μL by addition of H_2O (10 μL). See main text for definition of mU (activity).

Table 1: Characteristics of **5a** and **5b** as fluorescent probes for GSH, Cys, MPA, and AET.[a]

	Thiol	k_{obsd} [$M^{-1}s^{-1}$] for reaction with thiols[b]	Assay	
			Detection limit [pmol]	Sensitivity [au $pmol^{-1}$][c]
5a	GSH	1.7×10^2 (3.9×10^3)	2.0	22.0
	Cys	2.7×10^2 (4.0×10^3)	2.0	27.5
	MPA	1.8×10 (5.0×10^4)	10	2.6
	AET	6.4×10^2 (5.0×10^3)	2.0	27.3
5b	GSH	1.4×10^2 (3.3×10^3)	0.7	31.9
	Cys	2.5×10^2 (3.6×10^3)	2.0	36.3
	MPA	1.5×10 (4.0×10^4)	5.0	3.5
	AET	5.3×10^2 (4.1×10^3)	1.0	31.6

[a] All data were obtained with **5** (final concentration: 25 μM) in HEPES buffer (10 mM, pH 7.4) containing 0.5% EtOH at 37 °C. GSH = glutathione, Cys = cysteine, MPA = 3-mercaptopropanoic acid, AET = 2-aminoethanethiol. [b] The numbers in parentheses are the rate constants k for the reaction of **5** with the corresponding thiolate anion estimated with $k = k_{obsd}(1+10^{pK_a-pH})$, with 8.75, 8.54, 10.84, and 8.23 used as the microscopic pK_a values of sulfhydryl groups in GSH, Cys, MPA, and AET, respectively.[18] [c] au = arbitrary units.

Table 2: Comparison of IC_{50} of ChE inhibitors on human erythrocyte AChE and serum BChE obtained with **5b** and DTNB

Inhibitor	Probe	IC_{50} [nM]		Selectivity for AChE[a]
		AChE	BChE	
donepazil	**5b**	11.8 ± 1.5	5260 ± 290	446
	DTNB[b]	19 ± 7	4100 ± 1800	216
BW284C51	**5b**	58.0 ± 5.4	19800 ± 750	341
	DTNB[c]	18.8	48000	2550
physostigmine	**5b**	142.0 ± 3.9	2090 ± 42	14.7
	DTNB[d]	27.9 ± 2.4	16.0 ± 2.9	0.57
tacrine	**5b**	111.0 ± 5.3	11.5 ± 1.2	0.10
	DTNB[b]	108 ± 7	29 ± 2	0.27

[a] Expressed as IC_{50} for BChE divided by IC_{50} for AChE. [b] See Ref. [2b]. [c] See Ref. [2c]. [d] See Ref. [2d].

between the IC_{50} values afforded by the present method and the DTNB method, except for physostigmine. This is likely a result of not preincubating ChE and each inhibitor before commencing the enzymatic reaction in the present method (all values cited using Ellman's method were estimated after a preincubation step). In fact, physostigmine is known to exhibit strong inhibitory activity against ChE only after preincubation for rather long periods of time.[19]

In conclusion, almost-nonfluorescent compounds **5** are transformed effectively to highly fluorescent **3** through reaction with thiols in aqueous buffer, with significantly shorter reaction times under mild conditions and without any side reactions, except for a low level of their hydrolysis. Thus, compounds **5** may be used as fluorescent probes for thiols and allow a simple and reliable fluorometric system for measuring ChE inhibitory activities to be developed. It is believed that fluorometry with **5** facilitates thiol-quantification assays of various types of enzymes as a high-throughput technique for screening enzyme inhibitors as new drugs. Further studies along these lines are currently underway in our laboratory.

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