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Sulfatase activity assay using an activity-based probe by generation of *N*-methyl isoindole under reducing conditions

Hey Young Yoon, Jong-In Hong*

Department of Chemistry, College of Natural Sciences, Seoul National University, Seoul 151-747, Republic of Korea

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ABSTRACT

Sulfatases catalyze the hydrolysis of sulfate esters that are present in a range of biomolecules. This is an important step in several biological processes such as cellular degradation, hormone regulation, and cell signaling. We have developed a new activity-based sulfatase probe (probe 1) that generates a fluorescent *N*-methylisoindole upon hydrolysis by sulfatase. Because of the autoxidation of *N*-methylisoindole, the sulfatase activity was also tested under reducing conditions, containing either glutathione (GSH) or tris(2-carboxyethyl)phosphine (TCEP), exhibiting little change in kinetic parameters compared to non-reducing conditions. Probe **1** displayed reasonable kinetic parameters under both non-reducing and reducing conditions, among which the use of Tris buffer and Tris buffer containing GSH appeared to be appropriate conditions for inhibitor screening. Probe **1** showed stronger intensity upon treatment with sulfatase under neutral conditions than under acidic conditions, but it still has limitations in the selectivity for a specific sulfatase. Nevertheless, the fluorescent signal generated as a result of the release of *N*-methylisoindole after treatment of probe **1** with sulfatase provides a new assay for measuring sulfatase activity that could be adapted for high throughput screening.

Introduction

In many biological systems, the sulfation state of biomolecules is important in determining their function. Sulfation state is regulated by sulfatases (EC 3.1.5.6) which catalyze the cleavage of sulfate esters present in biomolecules such as carbohydrates, steroids, and proteins [1–3]. Human sulfatases are broadly classified based on their subcellular location. The three classes are, lysosomal sulfatases, non-lysosomal sulfatases, and extracellular sulfatases. Sulfatases in each subcellular location play important roles in many biological processes including cellular degradation, hormone regulation, and cell signaling. In breast carcinoma cells, for example, it has been reported that the mRNA level is increased but the activity of steroid sulfatases is decreased [4,5]. In order to deepen our understanding of sulfatase biology, the development of probes capable of measuring sulfatase activity is required. Optical probes have proven to be powerful tools for measuring sulfatase activity and these assays have contributed to the diagnosis and treatment of sulfatase-related diseases.

In general, sulfatase activity assays have been performed using *p*-nitrophenyl sulfate (*p*-NPS) or 4-methylumbelliferyl sulfate (4-MUS). However, these sulfatase substrates have slow reaction rates and lack sensitivity [6–8]. Several other activity-based sulfatase probes have been reported. These are comprised of luminophores and a sulfate group, and they exhibit an optical change after cleavage of the sulfate ester by sulfatases [9–13]. These probes have better kinetics and increased sensitivity compared to *p*-NPS and 4-MUS [9–14], and they were able to discriminate mycobacterium strains through individual sulfatase activity patterns [13].

Here, we introduce a new activity-based probe for measuring sulfatase activity assay using the formation of fluorescent *N*-methylisoindole [15]. Based on the ability of sulfatase to cleave sulfate groups, we designed a pro-fluorescent probe (probe **1**) which consists of a phenyl sulfate moiety as a substrate and 2-formyl benzylcarbamate moiety as a signaling unit. When the sulfate group of probe **1** is cleaved by sulfatase, self-immolation and intramolecular cyclization occur, resulting in the generation of a fluorescent *N*-methylisoindole (Scheme 1). Isoindole derivatives has been reported to be unstable and decompose easily, resulting in reduced fluorescence intensity [16–21]. To avoid this reduction in fluorescence intensity, we also examined sulfate ester hydrolysis under reducing conditions, namely in the presence of glutathione

* Corresponding author.
E-mail address: jihong@snu.ac.kr (J.-I. Hong).

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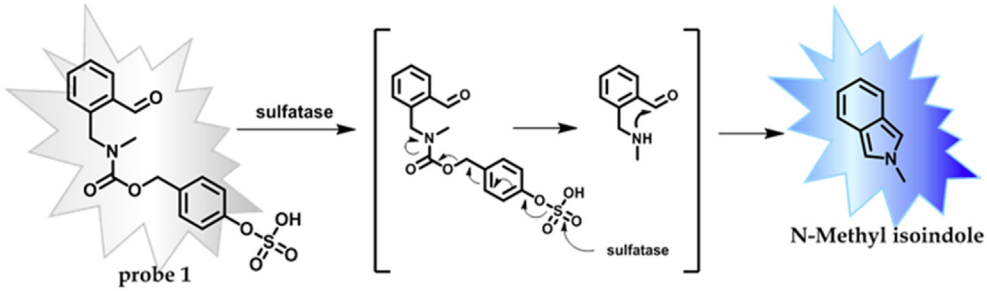


Scheme 1. Probe **1** for sulfatase activity assay.

(GSH) or tris(2-carboxyethyl)phosphine (TCEP).

Materials and methods

Synthesis of probe 1

*Synthesis of neopentyl(4-((((4-nitrophenoxy)carbonyl)oxy)methyl) phenyl) sulfate (**8**)*

Compound **7** was prepared according to a published procedure [22] and its synthesis is described in more detail in the Data in Brief [23]. To a solution of 4-nitrophenyl chloroformate (640 mg, 1.1 equiv.) in anhydrous THF was added pyridine (257 μL, 1 equiv.) at 0 °C. After stirring for 20 min, a solution of compound **7** (970 mg, 3.54 mmol) in anhydrous THF was added dropwise over a 10-min period, and the mixture was allowed to warm up to room temperature [24]. Stirring was continued at room temperature for 16 h and then the solvent was removed *in vacuo*. The residue was dissolved in ethyl acetate and washed with saturated aqueous NH_4Cl solution several times and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (chloroform:acetone = 100:1) to yield compound **8** (784 mg, 1.78 mmol, 50% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.03 (9H, s), 4.13 (2H, s), 5.32 (2H, s), 7.38 (2H, d, J = 8.5 Hz), 7.41 (2H, d, J = 9.0 Hz), 7.52 (2H, d, J = 8.6 Hz), 8.30 (2H, d, J = 8.5 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.86, 31.90, 69.75, 83.69, 115.331, 121.43, 121.81, 125.28, 125.46, 130.30, 133.64, 145.39, 150.48, 155.45; HRMS (FAB): m/z calcd. for [$C_{19}H_{21}NO_9S + Na^+$] 462.0835, found 462.0838.

*Synthesis of methyl 2-(dimethoxymethyl)benzoate (**3**)*

To a solution of methyl 2-formylbenzoate (**2**) (1.62 g, 9.87 mmol) in methanol (20 mL) at 0°Cunder N_2 was added 1 M $TiCl_4$ (1 mL, 0.1 equiv.) solution in CH_2Cl_2 in a single step. After stirring for 30 min, the resulting mixture was treated with triethylamine (3 mL, 10 equiv.), and the mixture was stirred for an additional 3 h at room temperature. After removal of all volatile compounds under reduced pressure, the residue was dissolved in ethyl acetate and washed with brine 3 times, dried over Na_2SO_4 and concentrated. The crude residue was purified by silica gel column chromatography (hexane:ethyl acetate = 5:1) to yield compound **3** (1.8 g, 8.56 mmol, 87% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.39 (6H, s), 3.93 (3H, s), 6.08 (1H, m), 7.40 (1H, t, J = 7.5 Hz), 7.53 (1H, t, J = 7.6 Hz), 7.76 (1H, d, J = 7.7 Hz), 7.82 (1H, d, J = 7.6 Hz).

*Synthesis of 2-(dimethoxymethyl)benzaldehyde (**4**)*

To a suspension of lithium aluminum hydride (250 mg, 2 equiv.) in anhydrous THF compound **3** (900 mg, 4.3 mmol) dissolved in anhydrous THF was slowly added at 0 °C under N_2. The reaction mixture was stirred at room temperature for 5 h, and was then cooled to 0 °C and quenched with a 1 M aqueous NaOH solution. The mixture was dried over Na_2SO_4 and filtered through a Celite pad. The filtrate was concentrated *in vacuo*. The resulting product was used for the next synthetic step without further purification.

To a solution of the reduction product (653 mg, 3.59 mmol) dissolved in CH_2Cl_2 was added MnO_2 (3.7 g, 10 equiv.). After being stirred at room temperature overnight, the mixture was filtered with Celite and a silica pad. The filtrate was concentrated *in vacuo* and the residue was purified by silica column chromatography (hexane:ethyl acetate = 3:1) to yield compound **4** (540 mg, 2.98 mmol, 69% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.42 (6H, s), 5.90 (1H, s), 7.52 (1H, t, J = 7.3 Hz), 7.62 (1H, t, J = 7.4 Hz), 7.70 (1H, d, J = 7.5 Hz), 7.95 (1H, d, J = 7.5 Hz), 10.46 (1H, s).

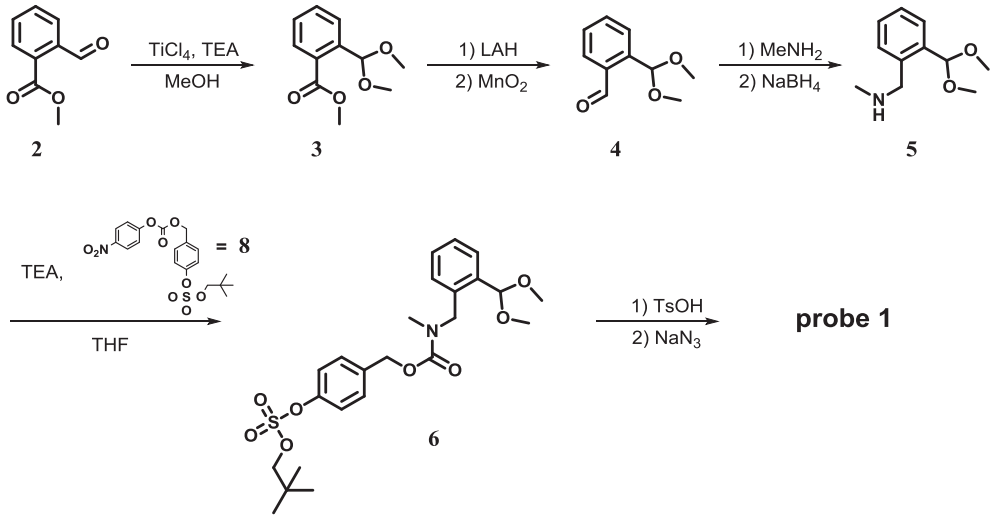
*Synthesis of 4-((((2-(dimethoxymethyl)benzyl)(methyl)carbamoyl) oxy)methyl)phenyl neopentyl sulfate (**6**)*

To a solution of compound **4** (540 mg, 2.98 mmol) in methanol was added a 2 M solution of methylamine in THF (4 mL, 2 equiv.). The resulting mixture was stirred at room temperature overnight, cooled to 0 °C and treated with sodium borohydride (570 mg, 5 equiv). The mixture was stirred for 1 h and then quenched with water. After removal of all volatile compounds under reduced pressure, the residue was dissolved in ethyl acetate and washed with saturated aqueous $NaHCO_3$ solution and brine, dried over Na_2SO_4 and concentrated *in vacuo*. Product **5** was used for the next synthetic step without further purification (219 mg).

To a solution of compound **5** (89 mg, 0.46 mmol) in THF were added triethylamine (192 μL, 3 equiv.) and compound **8** (200 mg, 1 equiv.). The mixture was stirred at room temperature overnight and concentrated *in vacuo*, and the residue was dissolved in ethyl acetate and washed with aqueous $NaHCO_3$ solution and saturated aqueous NH_4Cl solution, dried over Na_2SO_4 and concentrated. The residue was purified by silica column chromatography (chloroform:acetone = 50:1) to yield compound **6** (107 mg, 0.22 mmol, 47% yield). ^{1}H NMR (300 MHz, CDCl3) δ 1.02 (9H, s), 2.92 (3H, d, J = 24 Hz), 3.31(6H, d, J = 10 Hz), 4.10 (2H, s), 4.69 (2H, s), 5.19 (2H, d, J = 15.6 Hz) 5.41 (1H, d, J = 23 Hz), 7.16–7.23 (2H, br), 7.31 (3H, m), 7.46 (1H, m), 7.55 (1H, s).

*Synthesis of 4-((((2-formylbenzyl)(methyl)carbamoyl)oxy)methyl) phenyl hydrogen sulfate (probe **1**)*

To a solution of compound **6** (57 mg, 0.12 mmol) in acetone was added *p*-toluenesulfonic acid monohydrate (6.5 mg, 0.3 equiv.) and the resulting solution was stirred at room temperature for 2 h. Acetone was removed *in vacuo* and the residue was purified by silica column chromatography (chloroform:acetone = 30:1). The resulting product (50 mg, 0.11 mmol) and sodium azide (10 mg, 1.2 equiv.) were then dissolved in DMF. The resulting solution was heated with stirring at 70 °C overnight and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (CH_2Cl_2:methanol = 10:1) and yielded probe **1** as the sodium salt (37 mg, 0.098 mmol, 88% yield). ^{1}H NMR (300 MHz, CD_3OD) δ 2.95 (3H, s), 4.96 (2H, s), 5.12 (2H, d, J = 21 Hz) 7.21–7.39 (5H, m), 7.52 (1H, t, J = 7.3 Hz), 7.60 (1H, s), 7.92 (1H, d, J = 7.1 Hz), 10.15 (1H,



Scheme 2. Synthesis of probe **1**.

d, $J = 19$ Hz); ^{13}C NMR (75 MHz, CD_3OD) δ 29.38, 49.67, 66.68, 121.11, 125.85, 127.45, 128.74, 133.58, 133.58, 133.91, 152.35, 193.72; HRMS (FAB): m/z calcd. for [$C_{17}H_{16}NNaO_7S$ + H^+] 402.0623, found 402.0621 (See Scheme 2).

Fluorescent changes of probe 1 upon treatment with sulfatases

Fluorescence changes in probe **1** were measured by treating 20 μM probe **1** with 0.25 mg/mL sulfatase at 37 °C in 50 mM Tris-buffer (100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4) in the presence and absence of 1 mM GSH or 1 mM TCEP over a period of 0–70 min as follows; A stock solution of 10 mM probe **1** was prepared in DMSO. A second stock solution of 500 μM probe **1** was prepared by dissolving 50 μL of the 10 mM probe stock solution in 1 mL of 50 mM Tris buffer. Thereafter, 10 μL of the second probe stock solution (500 μM) was diluted with 200 μL of 50 mM Tris-buffer to give a 20 μM solution of probe **1**. A stock solution of 0.625 mg/mL *Helix pomatia* arylsulfatase (S9626, Sigma-Aldrich) as prepared in 50 mM Tris buffer, and 100 μL of this 0.625 mg/mL sulfatase stock solution and 150 μL of 50 mM Tris-buffer were mixed in a 96-well plate to give a 0.25 mg/mL sulfatase solution.

A stock solution of 0.625 mg/mL sulfatase was prepared in 50 mM Tris buffer containing 1.25 mM GSH or in 50 mM Tris buffer containing 1.25 mM TCEP, and 100 μL of each sulfatase stock solution and 100 μL of 50 mM Tris-buffer containing 1.25 mM GSH or TCEP were mixed with 40 μL of 50 mM Tris buffer without GSH or TCEP and 10 μL of a 500 μM probe stock solution in a 96-well plate to give final concentrations of 0.25 mg/mL sulfatase, 20 μM probe **1**, and 1 mM reducing agents, respectively.

Diels-Alder reactions

A mixture of 40 μL of 500 μM probe stock solution and 400 μL of 0.625 mg/mL sulfatase stock solution in 560 μL of 50 mM Tris buffer was incubated for 45 min to generate *N*-methylisoindole. The final concentrations of probe **1** and sulfatase were 20 μM and 0.25 mg/mL, respectively. To generate *N*-methylisoindole under reducing conditions, 40 μL of 500 μM probe stock solution and 160 μL of 50 mM Tris buffer without reducing agents were added to a microtube containing 400 μL of 0.625 mg/mL sulfatase stock solution dissolved in 1.25 mM GSH- or TCEP-containing buffer and 400 μL of 50 mM Tris buffer with 1.25 mM GSH or TCEP, and the resulting solution was incubated for 45 min. The final concentrations of probe **1**, sulfatase, and reducing agents were 20 μM, 0.25 mg/mL, and 1 mM, respectively. The resulting solution was then poured into a *N*-phenylmaleimide solution in THF and stirred at 70 °C overnight. The reaction mixture was extracted with CH_2Cl_2, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The residue was dissolved in 1 mL of methanol and subjected to liquid chromatography-mass spectrometry (LC/MS) analysis (Agilent technologies 1260 infinity) (Fig.2 in Ref. [23]) to monitor for the formation of Diels-Alder adducts.

Enzyme kinetics

Kinetic experiments were carried out using 0.25 mg/mL sulfatase and various concentrations of probe **1** (50, 20, 10, 5, 2.4, 1.2 μM) at 37 °C in 50 mM Tris buffers with and without 1 mM reducing agents (pH 7.4). Fluorescence measurements were performed using a SpectraMax M2 multi-detection reader (Molecular Device). The rate of increase in fluorescence intensity at 415 nm when excited at 325 nm was measured to determine the kinetic parameters of enzyme hydrolysis. The values of the kinetic parameters (K_m and V_{max}) were obtained by fitting the data to the Michaelis-Menten equation. K_m and V_{max} values were calculated by nonlinear fitting of the Michaelis–Menten equation using SigmaPlot 8.0 (Systat Software Inc.) (Fig.3 in Ref. [23]).

Inhibitory potency measurements

Measurements of inhibitory potency were carried out with 0.25 mg/mL sulfatase, 20 μM probe **1**, and various concentrations of estrone 3-O-sulfamate (EMATE, 0, 12, 25, 50, 100, 200 and 500 nM) at 37 °C in 50 mM Tris buffers with and without 1 mM reducing agents (pH 7.4) as follows:

A mixture of 100 μL of sulfatase stock solution (0.625 mg/mL) and 40 μL of various concentrations of EMATE in 100 μL of 50 mM Tris buffer was incubated in a 96-well plate for 1 h at 37 °C. Following this, 10 μL of the second probe stock solution (0.5 mM) was added to make the final volume 250 μL and fluorescence intensities were measured. The final concentrations of EMATE were 0, 12, 25, 50, 100, 200 and 500 nM. For the measurements of inhibitory potency under reducing conditions, sulfatase stock solutions dissolved in 50 mM Tris buffer containing 1.25 mM GSH or TCEP were used. The rate of decrease in fluorescence intensity at 415 nm was measured to determine the IC_{50} of EMATE. The IC_{50} values were

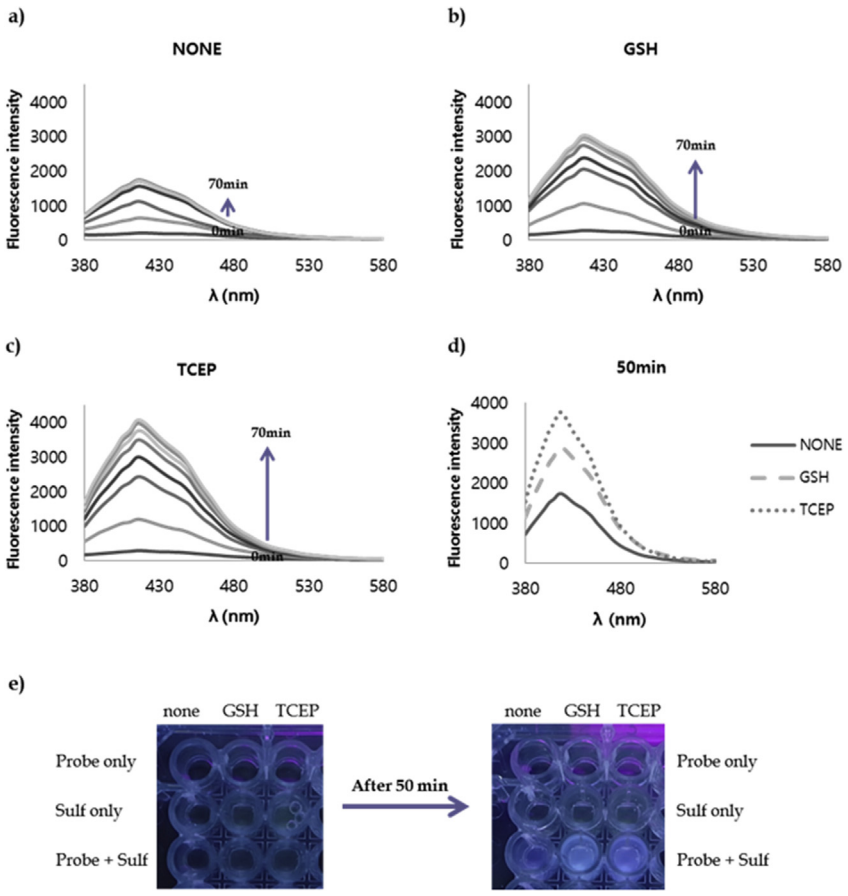


Fig. 1. Time-dependent fluorescence changes of probe **1** (20 μM) in the presence of sulfatase (0.25 mg/mL) at 37 °C a) in 50 mM Tris buffer at pH 7.4, b) in 50 mM Tris buffer containing 1 mM GSH, c) in 50 mM Tris buffer containing 1 mM TCEP, d) fluorescence intensities of probe **1** under three different buffer conditions after 50 min incubation with sulfatase at 37 °C and e) fluorescence changes of probe **1** under UV light (360 nm).

Table 1
Kinetic parameters.

	Probe 1			*p-nitrophenyl sulfate (p-NPS)*[a]	*3-O-methylfluorescein-sulfate (MFS)*[b]
	none	GSH	TCEP		
K_m (μM)	203 ± 57	204 ± 59	206 ± 73	1850 ± 320	24.8 ± 2.2
V_{max} (μM/min)	0.27 ± 0.06	0.21 ± 0.04	0.17 ± 0.04	33.8 ± 4.8	0.00041 ± 0.00001

[a] Assay was carried out with sulfatase from *Helix pomatia* in 100 mM Tris buffer (pH 7.43) at 30 °C [11].
[b] Assay was carried out with sulfatase from *Helix pomatia* in 100 mM potassium acetate buffer (pH 5.0) at 37 °C [12].

calculated by fitting with the Hill equation using Sigmaplot 8.0 (Fig.4 in Ref. [23]).

Results and discussion

Fluorescence changes in probe 1 upon sulfatase treatment under three different buffer conditions

To examine the fluorescence changes upon treatment with sulfatases, we measured the fluorescence intensity of probe **1** in the absence and presence of sulfatases. When excited at 325 nm, probe **1** was non-fluorescent in the absence of sulfatase, but in the presence of sulfatase, the fluorescence of probe **1** was enhanced in a time-dependent manner (Fig. 1a). After 60 min incubation of the probe with sulfatase, the fluorescence intensity at 415 nm increased up to 8-fold and became saturated (Fig. 1a). The fluorescent signal arose from the *N*-methylisoindole generated as a result of sulfatase activity. To confirm the generation of *N*-methylisoindole, a Diels-Alder reaction using *N*-phenylmaleimide was carried out to trap isoindoles [18], and mass spectrometric analysis was also performed. The peak of the Diels-Alder adduct was observed at m/z 304 which corresponds to the calculated molecular weight of the adduct (Figure 2a in Ref. [23]). These results confirmed that enzymatic cleavage of the sulfate group in the probe triggered the generation of a fluorescent *N*-methylisoindole.

However, *N*-methylisoindoles are known to be unstable and readily autoxidize in air and water [17,19,21]. Such autoxidation will attenuate the fluorescence intensity of *N*-methylisoindole and reduce the utility of this molecular probe. In order to reduce the autoxidation rate of *N*-methylisoindole, we used two different buffers that contained 1 mM of GSH or 1 mM of TCEP as reducing agents. GSH was used as a representative physiological reducing agent and TCEP was used since it is a common reducing agent in many enzyme activity assays and inhibitor screens. As shown in

Fig. 1b and c, the fluorescence intensity at saturation was increased 10-fold in GSH buffer and 14-fold in TCEP buffer compared to the intensity in absence of sulfatase. Moreover, after 50 min of incubation, the fluorescence intensities of *N*-methylisoindole in buffers containing 1 mM GSH or 1 mM TCEP were 1.7 times and 2 times stronger compared to those under 50 mM Tris buffer without reducing agents, respectively (Fig. 1d). In addition, the increase in fluorescence intensity was dependent on the concentrations of GSH and TCEP (Fig. 1 in Ref. [23]), which confirmed that the stronger fluorescence emission was due to the reducing agents. To identify the products of the enzymatic reaction in GSH- or TCEP-containing buffer, a Diels-Alder reaction using *N*-phenylmaleimide was also carried out. Mass spectrometry showed the presence of a peak at m/z 304, indicating formation of the same Diels-Alder adduct as was obtained without reducing agents (Figure 2b and 2c in Ref. [23]). Therefore, the stronger fluorescence intensity in GSH- or TCEP-containing buffer solutions could be attributed to inhibition of *N*-methylisoindole autoxidation by the reducing agents.

The optimal pH of most human sulfatases including arylsulfatase A, B, and galactosamine-6-sulfatase was reported to be around 5, while the optimal pH of arylsulfatase C (steroid sulfatase) and endo sulfatases is 7 [3]. To confirm the utility of probe **1** under neutral and acidic conditions, the fluorescence changes of probe **1** upon the treatment of sulfatase were monitored in acidic buffer (pH 5.0) and 1 mM GSH- or 1 mM TCEP-containing acidic buffer (Figure 5 in Ref. [23]). Compared to the fluorescence intensity of probe **1** in neutral buffer solutions, the fluorescence intensity of probe **1** upon sulfatase treatment in acidic buffer was considerably weak. Given that sulfatase from *Helix pomatia* also showed proper activity at acidic pH [6,11], these phenomena could be caused by more rapid decomposition of *N*-methylisoindole in acidic conditions [17–21]. This result indicates that probe **1** would be more feasible to detect sulfatase activity at neutral pH.

Enzymatic kinetics

To analyze the kinetic parameters of sulfatases, we measured the reaction rates over a range of probe concentration. Concentrations of probe **1** from 0.5 μM to 100 μM, and 0.25 mg/mL of sulfatase in 50 mM Tris buffer at pH 7.4 were used and the same experiments were performed in two different buffers containing 1 mM GSH and 1 mM TCEP, respectively. From the enzyme experiments, the K_m and V_{max} values were obtained by plotting enzyme reaction rates as a function of probe concentration (Table 1 and Figure 3 in Ref. [23]). Compared to previous reported kinetic values obtained using *p*-NPS (K_m = 1.85 ± 0.32 mM, V_{max} = 33.8 ± 4.8 μM/min) [12], the calculated K_m and V_{max} values of probe **1** indicated a slower reaction rate with sulfatase but a higher affinity for sulfatase. In addition, despite using different reducing agents in enzyme reactions, we observed similar K_m and V_{max} values, which indicated that the interaction between probe **1** and sulfatase was not affected by the reducing agents.

Inhibition potency

High-throughput screening for the identification of drugs has become widely used and many probes have been developed for such screening. To investigate whether the probe was appropriate for inhibitor screening, we measured the IC_{50} values of estrone 3-O-sulfamate (EMATE) which is a known inhibitor of human steroid sulfatase [18,19] using a range of EMATE concentrations and 0.25 mg/mL sulfatase and 20 μM probe **1**. The IC_{50} values determined by the Hill plots are shown in Table 2 and Figure 4 in Ref. [23]. Although these values were larger than the reported IC_{50} value of EMATE (80 nM) measured using placental microsomes [25,26], considering the large amount of sulfatase used, the IC_{50} values are acceptable. The inhibition potency in TCEP-containing buffer was greater than the other IC_{50} values in Tris buffer with and without GSH. Despite the fact that reducing agents are commonly used in enzyme assays and inhibitor screens, they can generate false positive hits [27]. These data implied that TCEP could interrupt the interaction between EMATE and sulfatases, resulting in a greater IC_{50} value. Therefore, it is recommended probe **1** be used in the presence of Tris buffer, or Tris-buffer containing GSH, for any potential high-throughput sulfatase inhibitor screen.

Table 2
Inhibitory potency.

	Probe 1			*[H^3]estrone sulfate*	
	none	GSH	TCEP		
IC_{50} (nM)	324.3 ± 182.3	317.6 ± 86.4	1076.5 ± 1338.5	6.5×10^{-7}[a]	80[b]

[a] The measurement was performed with [H^3] estrone sulfate and MCF-7 cell [25].
[b] The measurement was performed with [H^3] estrone sulfate and placental microsome [26].

Conclusion

We have developed an activity-based sulfatase probe that generates fluorescent *N*-methylisoindole after cleavage by sulfatases. The reduced fluorescence intensity resulting from the autoxidation of *N*-methylisoindoles was improved by using reducing agents, such as GSH and TCEP, causing a 2-fold increase in the fluorescence intensity compared to the fluorescence intensity in the buffer with no additives. We also demonstrated that this probe had reasonable kinetic parameters under three different assay conditions, among which Tris buffer and Tris buffer containing GSH appeared to be feasible for potent inhibitor screening. Probe **1** would not be selective for a specific sulfatase, but it showed more significant fluorescence changes with the treatment of sulfatase at neutral pH. Therefore, probe **1** provides a new assay for measuring sulfatase activity at neutral pH, which could be applied to high throughput screening.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2017.03.012.

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