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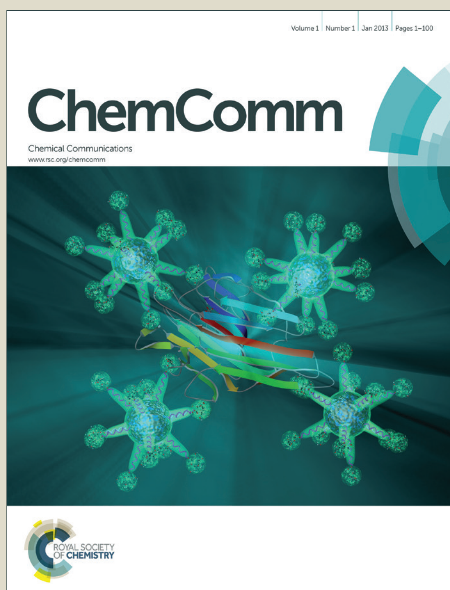

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ARTICLE TYPE

Synthesis and Evaluation of Turn-on Fluorescent Probes for Imaging Steroid Sulfatase Activities in Cells§

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We have synthesized and evaluated two self-immobilizing, turn-on fluorescent probes carrying a coumarin molecular framework for imaging intracellular human steroid sulfatase (STS) activity. The 8-fluoromethyl coumarin derivative, which gives a stronger fluorescent response in the incubation study with STS preparations, was successfully applied to visualize STS activity in cells.

Sulfation and phosphorylation are two major covalent modification strategies that Nature employs to modulate the functions of biomolecules.[1-4] Both processes reversibly introduce negative charges to the biomolecules and their significance in the biological world has been well acknowledged.[5,6] However, our understanding on the sulfated events lags far behind that of phosphorylation, making sulfation/desulfation events more deserving of further studies. Sulfatases are a family of enzymes responsible for removing sulfate groups from sulfated substrates that are involved in various biological events, including hormone regulation, developmental cell signalling, and bacterial pathogenesis.[6-9] It is especially worth noting that recent efforts have focused mainly on one particular member of this family, human steroid sulfatase (STS, EC 3.1.6.2), due to its role in estrogen-dependent tumors.[10,11] Nevertheless, the regulation of expression and activity of STS has not been fully characterized. Hence, a sensitive and selective cell imaging platform of this biomedical marker would be of great value in developing diagnostic tools or cell-based screening approaches for inhibitor evaluation.

We have previously developed the first activity-based probe for STS that could be applied to monitor STS activities with a dot blot assay.[12] The probe, upon incubation with STS, releases an intermediate that could spontaneously generate a reactive quinone methide and react with nearby nucleophiles on STS to afford a probe-STS conjugated adduct. Some variants based on similar working concepts have thereafter been developed.[13-15] Although probes with a fluorescent reporter group offer great detection sensitivity, the interference of background fluorescence of unbound probe would limit the application in microscopic imaging or flow cytometric detection. Nevertheless, the target enzyme-triggered, covalent bond forming feature was well exploited recently in a pioneering work to develop self-immobilizing fluorogenic imaging agents for glycosidases.[16] A variant that acted as phosphotyrosine mimetics to target phosphatases and found applications in bio-imaging and fluorescence-activated cell sorting (FACS) was also reported.[17] The molecular framework of these fluorogenic reagents features coumarin derivatives with an 8-fluoromethyl or 8-difluoromethyl substituent. In this paper, we synthesized two turn-on probes **1a** and **1b** (Figure 1), measured their fluorescent response toward STS preparations, and applied the probes for imaging STS activities in cells.

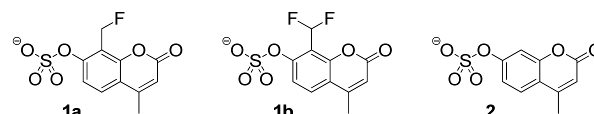
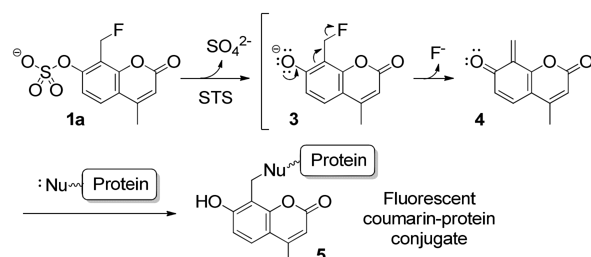


Figure 1. The structure of fluorescent turn-on probes **1a** and **1b**, and a fluorogenic substrate **2** (4-MUS) for STS.

Most arylsulfatases, including STS, could catalyze the hydrolysis of aryl sulfate artificial substrates,[6,18] and this feature has been adopted for the probe design.[12] In particular, 4-methylumbelliferyl sulfate (**2**, 4-MUS) is a widely used fluorogenic substrate in sulfatase assays.[19,20] We envisioned that probes **1a** and **1b**, which resemble compound **2** except with a fluoromethyl or difluoromethyl substituent at position C-8, could also be cleaved by STS to meet one of the most important criteria in the function of activity-based probes. Probes **1a** and **1b** are not innately fluorogenic. The proposed fluorogenic imaging mechanism for probe **1a** is depicted in Scheme 1. When the sulfate group of probe **1a** is cleaved by STS, the released product **3** is expected to rapidly undergo elimination of a fluoride to generate a quinone methide-like reactive intermediate **4**, which will react with nucleophiles on STS or nearby proteins to afford fluorescent coumarin-protein conjugates. The fluorescent coumarin-protein conjugates will not be washed off in the washing steps after staining incubation, therefore constituting the



Scheme 1. Proposed working mechanism for the STS-triggered fluorescent turn-on imaging of cells with probe **1a**.

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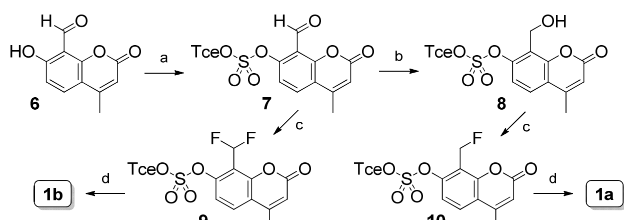
basis for imaging. Probe **1b** is expected to go through similar cleavage, elimination, and labeling/immobilization events, except both fluorine atoms would be consecutively eliminated in the process.[21]

Starting from 8-formyl-7-hydroxyl-4-methyl-coumarin **6**,[22] the probes **1a** and **1b** could be conveniently prepared in three and four steps, respectively, using slightly modified procedures from the literatures (Scheme 2).[12,16,21,23] Compound **6** was first subjected to sulfation reaction using trichloroethylsulfuryl chloride and 4-dimethylaminopyridine (DMAP) in CH_2Cl_2 to give compound **7** in 72% yield. The aldehyde functionality of compound **7** was converted to a difluoromethyl group by treatment with DAST in CH_2Cl_2 to afford compound **9** in 85% yield. Final deprotection of the trichloroethyl (Tce) group was achieved by treatment with Zn dust and $HCOONH_4$ in MeOH to give **1b** as its ammonium salt in 84% yield. For the preparation of probe **1a**, compound **7** was reduced with $NaBH_3CN$ in MeOH to give alcohol **8**. The hydroxymethyl group of compound **8** was then subjected to the same fluorination and deprotection reactions as those for probe **1b** to give probe **1a** in high yield.



Scheme 2. Synthesis of probes **1a** and **1b**. *Reagents and conditions:* (a) trichloroethylsulfuryl chloride, DMAP, TEA, CH_2Cl_2, -18 °C, 3 h, 72%; (b) $NaBH_3CN$, CH_3OH, 3h, 96%; (c) DAST, CH_2Cl_2, -18 °C, 30 min, 85 % for **9**, 74% for **10**; (d) Zn dust, $HCOONH_4$, CH_3OH, for **1a**: 30 min, 95%; for **1b**: 10 min, 84%.

Prior to the evaluation of probes, CHO-K1 cells stably expressing human STS (CHO/STS) were established according to previous reports.[12,24] Both probes **1a** and **1b** showed good stability when stored in buffers at low temperatures in the absence of sulfatases. Whether probes **1a** and **1b** could be hydrolyzed by STS was first examined by incubating the probes with STS preparations from CHO/STS cell lysates respectively. Since both probes **1a** and **1b** contain fluorine atoms, the reactions would generate inorganic fluoride. Thus the STS-catalyzed hydrolytic reactions were carried out in NMR tubes containing probe **1a** or **1b** (2.5 mM) and CHO/STS lysate (1.47 mg) in Tris-HCl D_2O buffer (100 mM, pH 7.5). The progress of reactions was continually monitored with ^{19}F NMR at 37 °C. It is interesting to note that in the reaction with probe **1a**, only two signals were observed in the reaction mixture (Figure 2a); a triplet (enlarged in inset) at -209 ppm is the signal for the starting material **1a** and a singlet at -121 ppm represents the inorganic fluoride. The same phenomenon was also observed for probe **1b**, a doublet at -115 ppm for **1b** and a singlet at -121 ppm for fluoride (Figure 2b). Since no other fluorine-containing intermediates were present in significant amounts during the reactions, the integration ratios of these two species could be used to calculate the conversion of reactions (spectra with proper integration are shown in Figure S1). The data not only confirmed both probes **1a** and **1b** could be hydrolyzed by STS, but also indicated that the hydrolysis occurs

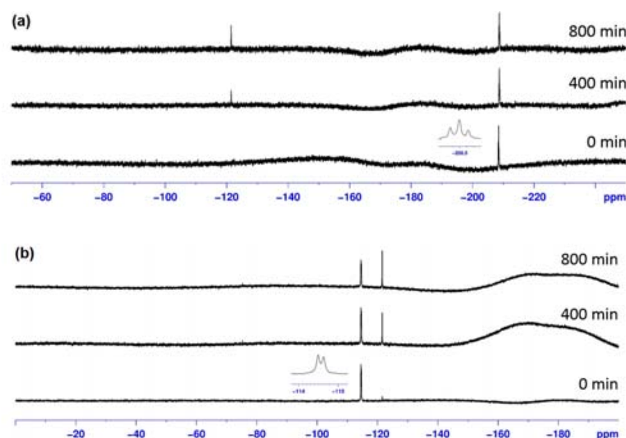


Figure 2. Selective ^{19}F NMR spectra for the hydrolysis of (a) probe **1a** and (b) probe **1b** by STS at 0, 400, and 800 min. The triplet at -209 ppm and the doublet at -115 ppm (enlarged in inset) represent the signals for the probes **1a** and **1b**, respectively. The singlet at -121 ppm is the inorganic fluoride.

at relatively the same rate (Figure 3). These results indicated that the 8-fluoromethyl and 8-difluoromethyl substituents have a negligible effect on STS substrate preference. More significantly, with the current probe concentration (2.5 mM) we observed a nearly complete inaction of STS in both hydrolytic reactions after 25-30% of the probes were consumed. This type of inhibitory behavior is in support of covalent modifications of the enzyme by the probes.

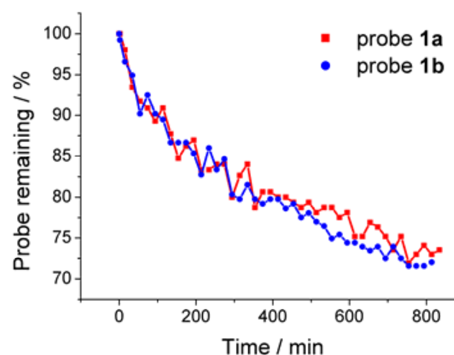


Figure 3. Time course study for the hydrolysis of probes **1a** and **1b** by STS as continually monitored with ^{19}F NMR.

We next examined the fluorogenic properties of probes **1a** and **1b** toward STS. The incubation experiments were carried out in a 96-well microplate reader. Each well contains the corresponding probe (5 μM) and CHO/STS cell lysates (50 μg) in Tris buffer (pH 7.5). The fluorescence intensities were continuously recorded (λ_{ex} = 360 nm, λ_{em} = 465 nm) for 2 h at 37 °C. As shown in Figure 4, the fluorescence intensity (F/F_0) of the mixture containing probe **1a** increased with time and the signals increased by more than seven fold over a two-hour period of time, where F_0 and F stand for the fluorescence intensity in the absence and the presence of STS, respectively. On the contrary, probe **1b** did not produce any significant change in its fluorescence intensity under the same circumstance. The dramatic difference in their fluorogenic properties strongly favors probe **1a** for further cell imaging experiments. The surprisingly poor fluorogenic response of probe **1b** could be due to the following reason. At low probe concentrations (5 μM) the competitive quenching pathway of the

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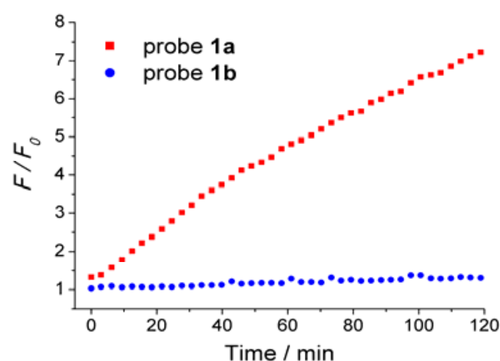

Figure 4. Time-course of fluorescence intensity (F/F_0) for the STS-catalyzed hydrolysis of probes **1a** and **1b** (5 μM) in Tris-HCl buffer (0.1 M Tris-HCl, pH 7.5) at 37 °C, where F_0 and F represent the fluorescence intensity (λ_{ex} = 360 nm, λ_{em} = 465 nm) in the absence and the presence of STS, respectively.

quinine methide by water might become dominant, leading to the formation of aldehyde **6** as the major hydrolytic product. A similar observation for the formation of aldehyde product from difluoromethyl derivatives was previously reported.[21] The weak fluorescent property of aldehyde **6** matches well with this observation (Figure S2).

Finally, to evaluate the self-immobilizing fluorogenic potential of the probes for the imaging of STS activities in cells, we tested probes **1a**, **1b** and compound **2** on CHO/STS cells. CHO/STS cells (10^5 cells/well) were first loaded with the corresponding probe (10 μM) after cell fixation, incubated for 16 h at 37 °C, washed with PBS (pH 7.4) thoroughly to remove unbound fluorophore/probes, and monitered directly using confocal fluorescence microscopy. The imaging results are shown in Figure 5. As expected, probe **1a** resulted in a strong fluorescent image (Figure 5b) and probe **1b** produced a negligible fluorescent image (Figure 5c). This trend is consistent with their fluorogenic behavior toward STS preparations discussed above. As a control, no fluorescent images were generated in the absence of the probes (Figure 5a). It is important to note that compound **2** also gave a negative result (Figure 5e), despite it is a fluorogenic reagent. The fluorescence generated by compound **2** was completely removed by the washing steps, making the self-immobilizing feature of the probes extremely critical to the success of cell imagining purpose.

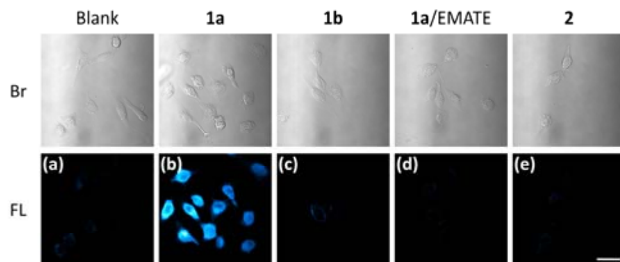

Figure 5. Comparative confocal fluorescence imaging study of CHO/STS cells in the absence of probes (a), or in the presence of probes **1a** (b,d), **1b** (c), and compound **2** (e). In the inhibitor blockade study (d), EMATE was added and incubated for 1 h prior to the addition of probe **1a**. Scale bar represents 30 μm.

To further confirm that the fluorescent image produced by probe **1a** was triggered by intracellular STS activity, we performed inhibitor blockade study by pre-incubating the cells with 10 μM of estrone 3-*O*-sulfamate (EMATE), which is a potent inhibitor for human STS.[25] In the presence of EMATE, probe **1a** failed to generate fluorescent image (Figure 5d). The data also imply that the current cell imaging study could be further developed into a cell-based platform for screening inhibitors against human STS. The significance of developing probes toward disease-related sulfatases was further substantiated by a recent report, in which a highly fluorogenic probe was used to profile sulfatases in mycobacterial lysates with native protein gels.[26] Although the probes for microbes could allow rapid pathogen discrimination for infectious diseases, they did not work on human sulfatases, making our current research an valuable tool for human STS-related diseases.

Conclusions

In conclusion, we have synthesized and evaluated two self-immobilizing, turn-on fluorescent probes carrying a coumarin molecular framework. Probe **1a**, which carries an 8-fluoromethyl substituent and displayed a much stronger fluorescent response than its 8-difluoromethyl counterpart did, was successfully applied to give fluorescent images on CHO/STS cells. Probe **1a** represents the first fluorescent turn-on probe triggered by STS for cell imaging and could have versatile application potential as valuable tools for STS-related diseases, including cell-based inhibitor screening and diagnostic application.

Notes and references

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† Electronic Supplementary Information (ESI) available: synthetic procedures as well as ^{1}H, ^{13}C, and ^{19}F NMR spectra for probes **1a** and **1b** are included. See DOI: 10.1039/b000000x/

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