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Sulfation and phosphorylation are two major covalent modification strategies that Nature employs to modulate the functions of biomolecules.\(^1\,\text{4}\) Both processes reversibly introduce negative charges to the biomolecules and their significance in the biological world has been well acknowledged.\(^5\,\text{6}\) 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.\(^6\,\text{9}\) 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.\(^10\,\text{11}\) 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.\(^13\,\text{15}\) 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 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,\(^6\,\text{18}\) 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.\(^19\,\text{20}\) 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 intrinsically 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
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.  

Starting from 8-formyl-7-hydroxyl-4-methyl-coumarin 6, the probes 1a and 1b could be conveniently prepared in three and four steps, respectively, using slightly modified procedures from the literatures (Scheme 2).  

Compound 6 was first subjected to sulfation reaction using trichloroethylsulfuryl chloride and 4-dimethylaminopyridine (DMAP) in CH2Cl2 to give compound 7 in 72% yield. The aldehyde functionality of compound 7 was converted to a difluoromethyl group by treatment with DAST in CH2Cl2 to afford compound 9 in 85% yield. Final deprotection of the trichloroethyl (Tce) group was achieved by treatment with Zn dust and HCOONH4 in MeOH to give compound 1 as its ammonium salt in 84% yield. For the preparation of probe 1a, compound 7 was reduced with NaBH3CN 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.

Prior to the evaluation of probes, CHO-K1 cells stably expressing human STS (CHO/STS) were established according to previous reports.  

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 D2O buffer (100 mM, pH 7.5). The progress of reactions was continually monitored with 19F 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 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.

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 lysate (50 μg) in Tris buffer (pH 7.5). The fluorescence intensities were continuously recorded ($\lambda_{ex} = 360$ nm, $\lambda_{em} = 465$ 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
give a negative result (Figure 5e), despite it is a fluorogenic

... immobilizing feature of the probes extremely critical to the

completely removed by the washing steps, making the self-

reagent. The fluorescence generated by compound

difluoromethyl derivatives was previously reported. 21 The weak

similar observation for the formation of aldehyde product from

behavior toward STS preparations discussed above. As a control,

image (Figure 5c). This trend is consistent with their fluorogenic

fluorescent property of aldehyde

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Notes and references

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