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

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Materials and Methods

Scheme S1. Synthesis of the probes 1a and 1b

Figure S1. Selective $^{19}$F NMR spectra for the hydrolysis of (a) probe 1a and (b) probe 1b by STS at 0, 200, 400, 600 and 800 min.

Figure S2. Emission spectra ($\lambda_{ex} = 360$ nm) of compounds 6 (red line) and 11 (blue line) at 10 $\mu$M.

References

$^1$H, $^{13}$C, and $^{19}$F NMR spectra of probe 1a

$^1$H, $^{13}$C, and $^{19}$F NMR spectra of probe 1b
Materials and Methods. All of the chemical reagents (except where indicated otherwise) were purchased from Acros, Aldrich, and Merck and were used without further purification. Analytical thin-layer chromatography (TLC) plates (silica gel, 60F-54, Merck) were visualized under UV light and/or phosphomolybdic acid–ethanol stain. Column chromatography was performed with Kieselgel 60 (70–230 mesh) silica gel (Merck). IR spectra were recorded on a Varian 640 FT-IR spectrometer. $^1$H and $^{13}$C NMR spectra were recorded using a Bruker Avance 400 spectrometer. The proton and carbon chemical shifts were reported in ppm downfield from tetramethylsilane ($\delta$) as the internal standard and coupling constants are in hertz (Hz). The proton and carbon chemical shifts are given in ppm using CDCl$_3$ ($\delta$H 7.24 and 77.0), CD$_3$OD ($\delta$H 3.31 and 49.00), DMSO–d$_6$ ($\delta$H 2.50 and 39.52), acetone–d$_6$ ($\delta$H 2.05 and 29.84), and D$_2$O ($\delta$H 4.70) as internal standards. IR spectra were recorded using Varian 640 FT–IR spectrometer. The high–resolution mass spectra (HRMS) were recorded on a LCT Premier XE mass spectrometer. Melting points are uncorrected. The images were obtained using a Zeiss LSM710 NLO confocal spectral microscope.

Scheme S1. Synthesis of the products 1a and 1b. Reagents and conditions: (a) trichloroethylsulfonyl chloride, DMAP, TEA, CH$_2$Cl$_2$, -18 °C, 3 h, 72%; (b) NaBH$_3$CN, CH$_3$OH, 3h, 96%; (c) DAST, CH$_2$Cl$_2$, -18 °C, 30 min, 85% for 9, 74% for 10; (d) Zn dust, HCOONH$_4$, CH$_3$OH, for 1a: 30 min, 95%; for 1b: 10 min, 84%

8-Formyl-4-methylumbelliferyl (2,2,2-trichloroethyl) sulfate (7). To a stirred solution of 6 (149.3 mg, 0.724 mmol) in freshly distilled CH$_2$Cl$_2$ (6.0 mL) was added TEA (402.6 µL, 2.896 mmol) and DMAP (88.5 mg, 0.724 mmol). The solution was cooled by ice-salt bath for 15 min and then chlorosulfuric acid 2,2,2-trichloroethyl ester (233.4 mg, 0.941 mmol) in 1.2 mL CH$_2$Cl$_2$ was added. The mixture was stirred for 3 h, diluted with CH$_2$Cl$_2$ (50 mL), and washed successively with 5% NaHCO$_3$ (10
mL x 3), 5% citric acid (10 mL x 3), water (10 mL x 3), and brine (10 mL x 1). The organic layer was dried over Na₂SO₄, filtered, and evaporated. The desired product was purified with silica gel column chromatography eluted with hexane/EtOAc (1/1). Compound 7 was obtained as a colorless oil (218.0 mg, 72%). Rₖ = 0.50 (hexane/EtOAc = 1/1, UV). ^1H NMR (400 MHz, CDCl₃): δ 10.7 (s, 1 H, –CHO), 7.87 (d, J = 8.8 Hz, 1 H, aromatic), 7.51 (d, J = 8.8 Hz, 1 H, aromatic), 6.39 (s, 1 H, aromatic), 5.10 (s, 2 H, –CH₂CCl₃), 2.48 (s, 3 H, –CH₃); ^13C NMR (100 MHz, CDCl₃): δ 185.6 (CHO), 157.9 (C), 155.8 (C), 151.2 (C), 149.2 (C), 130.6 (CH), 120.0 (C), 118.4 (CH), 116.5 (C), 115.9 (CH), 92.3 (C), 81.1 (CH₂), 19.0 (CH₃). IR (neat): 1745, 1704, 1633, 1419, 1199, 1056, 997, 866, 727 cm⁻¹. HRMS calcd for C₁₃H₈Cl₃O₇S (M – H)⁺ 412.9062, found 412.9060.

8-Hydroxymethyl-4-methylumbelliferyl (2,2,2-trichloroethyl) sulfate (8). To a stirred solution of 7 (50.7 mg, 0.122 mmol) in 1.2 mL MeOH was added NaBH₃CN (11.5 mg, 0.183 mmol) and stirred for 3 h. Silica gel (1 g) was added into the resulting mixture and the solvent was evaporated. The desired product was purified with silica gel column chromatography eluted with hexane/EtOAc (1/1). Compound 8 was obtained as a colorless oil (49.1 mg, 96%). Rₖ = 0.43 (hexane/EtOAc = 1/1, UV). ^1H NMR (400 MHz, CDCl₃): δ 7.63 (d, J = 8.9 Hz, 1 H, aromatic), 7.44 (d, J = 8.9 Hz, 1 H, aromatic), 6.31 (s, 1 H, aromatic), 4.96-4.95 (m, 4 H), 2.43 (s, 3 H, –CH₃); ^13C NMR (100 MHz, CDCl₃): δ 159.6 (C), 152.8 (C), 152.0 (C), 150.1 (C), 125.5 (CH), 121.8 (C), 119.5 (C), 117.0 (CH), 115.3 (CH), 92.3 (C), 80.8 (CH₂), 53.5(CH₂), 18.9 (CH₃). IR (neat): 3445, 1731, 1601, 1415, 1196, 1056, 997, 870, 787, 724 cm⁻¹. HRMS calcd for C₁₃H₁₁Cl₃NaO₇S (M + Na)⁺ 438.9183, found 438.9171.

8-Fluoromethyl-4-methylumbelliferyl (2,2,2-trichloroethyl) sulfate (10). To an ice-salt bath solution of 8 (29.7 mg, 0.0711 mmol) in 0.5 mL of anhydrous CH₂Cl₂ was slowly added DAST (17.4 μL, 0.142 mmol) in 0.2 mL of anhydrous CH₂Cl₂
through a syringe. The reaction was stirred for 30 min. When no more starting material was observed, it was quenched by adding one drop of MeOH. The mixture was concentrated and the desired product 10 (22.1 mg, 74%) was purified by silica gel column chromatography (66-75% gradient CH₂Cl₂ in hexane) as colorless foam. Rf = 0.63 (hexane/EtOAc = 1/1, UV). ¹H NMR (400 MHz, CD₃OD): δ 7.99 (d, J = 8.9 Hz, 1 H, aromatic), 7.60 (d, J = 8.9 Hz, 1 H, aromatic), 6.43 (s, 1 H, aromatic), 5.73 (d, J = 47.5 Hz, 2 H, -CH₂F), 5.20 (s, 2 H, -CH₂CCl₃), 2.51 (s, 3 H, -CH₃); ¹³C NMR (100 MHz, CD₃OD): δ 161.1 (C), 154.4 (C), 154.2 (C), 152.2 (C), 129.2 (d, J = 3.2 Hz, CH), 121.0 (d, J = 2.3 Hz, C), 117.9 (d, J = 1.6 Hz, CH), 117.9 (d, J = 15.5 Hz, C), 116.0 (CH), 93.9 (C), 82.2 (CH₂), 73.6 (d, J = 165.5 Hz, -CH₂F), 19.0 (CH₃); ¹⁹F NMR (376 MHz, CD₃OD): δ -212.8 (t, J = 47.5 Hz, 1 F). IR (neat): 1740, 1602, 1417, 1201, 1064, 979, 867 cm⁻¹. HRMS calcd for C₁₃H₁₀Cl₃FNaO₆S (M + Na)⁺ 440.9140, found 440.9128.

Ammonium 8-fluoromethyl-4-methylumbelliferyl sulfate (1a). To a stirred solution of 10 (10.6 mg, 0.0253 mmol) in MeOH (0.5 mL) was added HCOONH₄ (12.8 mg, 0.202 mmol) and Zn dust (13.2 mg, 0.202 mmol). The resulting mixture was stirred for 30 min. The Zn dust was filtered off through Celite, and the filtrate was concentrated. The desired product 1a (7.3 mg, 95%) was purified by C18 column chromatography (0-20% gradient MeOH in H₂O) as colorless foam. Rf = 0.60 (BuOH/AcOH/H₂O = 4/1/1, UV). ¹H NMR (400 MHz, CD₃OD): δ 7.85 (dd, J = 8.9, 2.2 Hz, 1 H, aromatic), 7.62 (dd, J = 8.9, 0.5 Hz, 1 H, aromatic), 6.33 (d, J = 0.8 Hz, 1 H, aromatic), 5.73 (d, J = 47.9 Hz, 2 H, -CH₂F), 2.50 (d, J = 1.0 Hz, 3 H, -CH₃); ¹³C NMR (100 MHz, CD₃OD): δ 161.0 (C), 154.4 (d, J = 3.6 Hz, C), 154.0 (C), 153.0 (d, J = 2.5 Hz, C), 126.5 (d, J = 3.6 Hz, CH), 117.7 (d, J = 2.3 Hz, CH), 116.7 (d, J = 2.4 Hz, C), 116.3 (d, J = 15.5 Hz, C), 112.7 (CH), 72.6 (d, J = 163.1 Hz, -CH₂F), 17.4 (CH₃); ¹⁹F NMR (376 MHz, CD₃OD): δ -211.7 (t, J = 47.8 Hz, 1 F). IR (neat): 3441,
1731, 1628, 1601, 1253, 1054, 946, 856, 576 cm$^{-1}$. HRMS calcd for C$_{11}$H$_{13}$FNO$_6$S (M + NH$_4$)$^+$ 306.0448, found 306.0445.

8-Difluoromethyl-4-methylumbelliferyl (2,2,2-trichloroethyl) sulfate (9). To a stirred solution of 7 (46.6 mg, 0.112 mmol) in 0.5 mL of anhydrous CH$_2$Cl$_2$ was added EtOH (3.3 µL, 0.055 mmol) and DAST (54.9 µL, 0.442 mmol) in 0.1 mL of anhydrous CH$_2$Cl$_2$ through a syringe. The reaction was stirred for 18 h. When no more starting material was observed, it was quenched by adding 5% NaHCO$_3$ (1 mL). The mixture was diluted with EtOAc (50 mL), and washed successively with 5% aqueous NaHCO$_3$ (10 mL x 3), water (10 mL x 3), brine (10 mL x 1). The organic layer was dried over Na$_2$SO$_4$, filtered, and evaporated. The desired product was purified with silica gel column chromatography eluted with hexane/EtOAc (8/2). Compound 9 was obtained as a colorless foam (41.9 mg, 85%). $R_f$ = 0.41 (hexane/EtOAc = 6/4, UV). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.79 (d, $J$ = 8.9 Hz, 1 H, aromatic), 7.58 (d, $J$ = 8.9 Hz, 1 H, aromatic), 7.32 (t, $J$ = 52.8 Hz, 1 H, –CHF$_2$), 6.35 (d, $J$ = 1.0 Hz, 1 H, aromatic), 4.93 (s, 2 H, –CH$_2$CCl$_3$), 2.45 (d, $J$ = 1.1 Hz, 3 H, –CH$_3$); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 158.1 (C), 152.2 (C), 151.3 (C), 150.0 (C), 128.5 (CH), 119.3 (C), 116.9 (CH), 115.7 (CH), 114.2 (t, $J$ = 23.3 Hz, C), 109.1 (t, $J$ = 238.5 Hz, -CHF$_2$), 92.1 (C), 81.0 (CH$_2$), 18.9 (CH$_3$); $^{19}$F NMR (376 MHz, CDCl$_3$): $\delta$ -114.4 (d, $J$ = 52.9 Hz, 2 F). IR (neat): 1744, 1605, 1418, 1187, 1053, 996, 864 cm$^{-1}$. HRMS calcd for C$_{13}$H$_9$Cl$_3$F$_2$NaO$_6$S (M + Na)$^+$ 458.9046, found 458.9045.

Ammonium 8-difluoromethyl-4-methylumbelliferyl sulfate (1b). To a stirred solution of 9 (39.1 mg, 0.0894 mmol) in 1.8 mL MeOH was added HCOONH$_4$ (45.1 mg, 0.715 mmol) and Zn dust (46.7 mg, 0.715 mmol). The resulting mixture was stirred for 10 min. The Zn dust was filtered off through Celite, and the filtrate was concentrated. The desired product 1b (24.3 mg, 84%) was purified by C18 column chromatography (0–20% gradient MeOH in H$_2$O) as colorless foam. $R_f$ = 0.65
(BuOH/AcOH/H2O = 4/1/1, UV). $^1$H NMR (400 MHz, CD$_3$OD): $\delta$ 7.90 (d, $J$ = 8.8 Hz, 1 H, aromatic), 7.59 (d, $J$ = 8.8 Hz, 1 H, aromatic), 7.25 (t, $J$ = 53.2 Hz, 1 H, $-\text{CHF}_2$), 6.34 (s, 1 H, aromatic), 2.49 (s, 3 H, $-\text{CH}_3$); $^{13}$C NMR (100 MHz, CD$_3$OD): $\delta$ 161.8 (C), 155.1 (C), 155.0 (C), 153.4 (C), 129.2 (CH), 119.5 (CH), 118.5 (C), 115.6 (t, $J$ = 22.9 Hz, C), 114.5 (CH), 112.3 (t, $J$ = 235.6 Hz, $-\text{CHF}_2$), 18.9 (CH$_3$); $^{19}$F NMR (376 MHz, CD$_3$OD): $\delta$ -116.7 (d, $J$ = 53.2 Hz, 2 F). IR (neat): 3417, 1711, 1603, 1362, 1316, 1181, 1047, 1011, 846, 600 cm$^{-1}$. HRMS calcd for C$_{11}$H$_7$O$_6$F$_2$S (M $-$ H)$^-$ 304.9931, found 304.9929.

7-Hydroxy-8-(hydroxymethyl)-4-methylumbelliferyl (11). To a stirred solution of 6 (218.9 mg, 1.072 mmol) in 10 mL EtOH/CH$_2$Cl$_2$ (1/1) was added NaBH$_4$ (121.6 mg, 3.216 mmol). The reaction mixture was stirred for 3.5 h, then quenched by adding 1 N HCl (1 mL). The mixture was diluted with CH$_2$Cl$_2$ (30 mL), and washed successively with H$_2$O (5 mL x 1) and brine (5 mL x 1). The organic layer was dried over Na$_2$SO$_4$, filtered, and evaporated. The desired product was recrystallized from CH$_2$Cl$_2$ and CH$_3$OH. Compound 11 was obtained as a white solid (154.7 mg, 70%), m.p. = 160–161 °C. $R_f$ = 0.38 (hexane/EtOAc = 3/7, UV). $^1$H NMR (400 MHz, CD$_3$OD): $\delta$ 7.57 (d, $J = 8.8$ Hz, 1 H, aromatic), 6.87 (d, $J = 8.7$ Hz, 1 H, aromatic), 6.12 (d, $J = 1.0$ Hz, 1 H, aromatic), 4.89 (s, 2 H, $-\text{CH}_2\text{OH}$), 2.42 (d, $J = 1.0$ Hz, 3 H, $-\text{CH}_3$); $^{13}$C NMR (100 MHz, CD$_3$OD): $\delta$ 163.6 (C), 161.4 (C), 156.2 (C), 154.3 (C), 126.5 (CH), 115.1 (C), 113.8 (CH), 113.8 (C), 111.1 (CH), 54.0 (CH$_2$), 18.8 (CH$_3$). IR (neat): 3326, 2949, 1687, 1609, 1581, 1393, 1370, 1323, 1294, 1288, 1077, 1003, 848 cm$^{-1}$. HRMS calcd for C$_{11}$H$_{10}$NaO$_4$ (M + Na)$^+$ 229.0471, found 229.0479.

**STS protein expression and fractionation:** CHO/K1 cells were cultured in Ham’s F-12 medium supplemented with fetal bovine serum (10 %), penicillin (100 units/mL),
streptomycin (100 μg/mL) and of amphotericin B (0.25 μg/mL) at 37°C with 5% CO₂.

STS was expressed using a stable CHO/STS cell line. The transfection procedures and
cell lysate collection were described in our previously report.¹ ²

**Time course study for the hydrolysis of probes 1a and 1b by STS:** The fluorine
signals were monitored with ¹⁹F NMR. Two NMR tubes containing probe 1a or 1b
(2.5 mM) were incubated with CHO/STS lysate (1.47 mg) in Tris-HCl D₂O buffer
(100 mM, pH 7.5) at 37 °C, respectively. The mixtures were continually monitored
with ¹⁹F NMR at intervals of 20 min. The concentrations of the remaining probes in
the reaction mixture were calculated according to the integration of ¹⁹F NMR.
Figure S1. Selective $^{19}$F NMR spectra for the hydrolysis of (a) probe 1a and (b) probe 1b by STS at 0, 200, 400, 600 and 800 min.

**Time-course of fluorescence intensity** ($F/F_0$): Fluorescent intensities were assayed on a 96-well microplate. Each reaction mixture contains CHO/STS cell lysate (50 µg) and probe 1a or probe 1b (5 µM) in a final volume of 100 µL of Tris-HCl buffer (100 mM, pH 7.5). The fluorescence intensities were continuously recorded for 2 h ($\lambda_{ex} = 360$ nm, $\lambda_{em} = 465$ nm) at 37°C using a Paradigm Multi-Mode Plate Reader (Beckman Biomek 3000).

**Comparison of emission spectra of compounds 6 and 11:** Fluorescence spectra were recorded on a spectrofluorometer (Jobin Yvon Fluorolog-3) using 10 mm quartz cuvettes with a path length of 1 cm ($\lambda_{ex} = 360$ nm). Solutions of compounds 6 and 11 (10 µM), containing 0.05% and 0.1% of DMSO respectively, were prepared in Tris-HCl buffer (100 mM, pH 7.5) for fluorescence measurements.
**Figure S2.** Emission spectra ($\lambda_{ex} = 360$ nm) of compounds 6 (red line) and 11 (blue line) at 10 $\mu$M.

**Comparative fluorescent imaging study of CHO/STS cells:** CHO/STS cells were seeded on a 6-well culture plate with coverslips overnight for attachment. After washing coverslips with PBS, cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, and washed with PBS three times, followed by staining with 10 $\mu$M of the corresponding probe (1a, 1b and compound 2) in 100 mM Tris-HCl, pH 7.5 for 16 h at 37°C. For inhibitor blockade study, pre-incubation with 10 $\mu$M of EMATE for 1 h was performed prior to the addition of probe 1a in the same buffer condition. After washing with PBS (x3) and distilled water (x2), cells were mounted for image captured. Images were captured with a Zeiss LSM710 NLO confocal spectral microscope and operated by Zen software. Excitation channel: 405 nm Diode laser. All images were acquired the same way.

**References:**
$^1$H, $^{13}$C and $^{19}$F NMR spectra of probes 1a and 1b.