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

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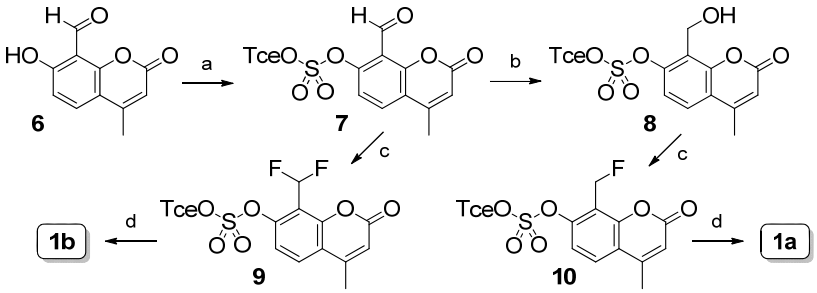
Materials and Methods. All of the chemical reagents (except where indicated otherwise) were purchased from Acros, Aldrich, and Merck and were used without further purification. Analytical thin-layer chromatography (TLC) plates (silica gel, 60F-54, Merck) were visualized under UV light and/or phosphomolybdic acid−ethanol stain. Column chromatography was performed with Kiesegel 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 (δ) as the internal standard and coupling constants are in hertz (Hz). The proton and carbon chemical shifts are given in ppm using $CDCl_3$ (δ_H 7.24 and 77.0), CD_3OD (δ_H 3.31 and 49.00), DMSO−d_6 (δ_H 2.50 and 39.52), acetone−d_6 (δ_H 2.05 and 29.84), and D_2O (δ_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) trichloroethylsulfuryl chloride, DMAP, TEA, CH_2Cl_2, -18 ^{o}C, 3 h, 72%; (b) $NaBH_3CN$, CH_3OH, 3h, 96%; (c) DAST, CH_2Cl_2, -18 ^{o}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%

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_2Cl_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_2Cl_2 was added. The mixture was stirred for 3 h, diluted with CH_2Cl_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 Na_2SO_4, 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%). R_f = 0.50 (hexane/EtOAc = 1/1, UV). ^{1}H NMR (400 MHz, $CDCl_3$): 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, $–CH_2CCl_3$), 2.48 (s, 3 H, $–CH_3$); ^{13}C NMR (100 MHz, $CDCl_3$): 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 (CH_2), 19.0 (CH_3). IR (neat): 1745, 1704, 1633, 1419, 1199, 1056, 997, 866, 727 cm^{-1}. HRMS calcd for $C_{13}H_8Cl_3O_7S$ $(M – H)^–$ 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 $NaBH_3CN$ (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%). R_f= 0.43 (hexane/EtOAc = 1/1, UV). ^{1}H NMR (400 MHz, $CDCl_3$): 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, $–CH_3$); ^{13}C NMR (100 MHz, $CDCl_3$): 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 (CH_2), 53.5(CH_2), 18.9 (CH_3). IR (neat): 3445, 1731, 1601, 1415, 1196, 1055, 997, 870, 787, 724 cm^{-1}. HRMS calcd for $C_{13}H_{11}Cl_3NaO_7S$ $(M + Na)^+$ 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 CH_2Cl_2 was slowly added DAST (17.4 μL, 0.142 mmol) in 0.2 mL of anhydrous CH_2Cl_2

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 CH_2Cl_2 in hexane) as colorless foam. R_f= 0.63 (hexane/EtOAc = 1/1, UV). ^{1}H NMR (400 MHz, CD_3OD): 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, –CH_2F), 5.20 (s, 2 H, –CH_2CCl_3), 2.51 (s, 3 H, –CH_3); ^{13}C NMR (100 MHz, CD_3OD): 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 (CH_2), 73.6 (d, *J* = 165.5 Hz, -CH_2F), 19.0 (CH_3); ^{19}F NMR (376 MHz, CD_3OD): δ -212.8 (t, *J* = 47.5 Hz, 1 F). IR (neat): 1740, 1602, 1417, 1201, 1064, 997, 867 cm^{-1}. HRMS calcd for $C_{13}H_{10}Cl_3FNaO_6S$ $(M + Na)^+$ 440.9140, found 440.9128.

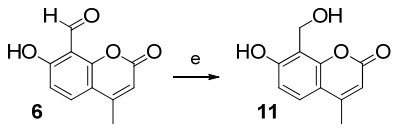
Ammonium 8-fluoromethyl-4-methylumbelliferyl sulfate (1a). To a stirred solution of **10** (10.6 mg, 0.0253 mmol) in MeOH (0.5 mL) was added $HCOONH_4$ (12.8 mg, 0.202 mmol) and Zn dust (13.2 mg, 0.202 mmol). The resulting mixture was stirred for 30 min. The Zn dust was filtered off through Celite, and the filtrate was concentrated. The desired product **1a** (7.3 mg, 95%) was purified by C18 column chromatography (0-20% gradient MeOH in H_2O) as colorless foam. R_f = 0.60 (BuOH/AcOH/H_2O = 4/1/1, UV). ^{1}H NMR (400 MHz, CD_3OD): 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, –CH_2F), 2.50 (d, *J* = 1.0 Hz, 3 H, –CH_3); ^{13}C NMR (100 MHz, CD_3OD): 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, -CH_2F), 17.4 (CH_3); ^{19}F NMR (376 MHz, CD_3OD): 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 $C_{11}H_{13}FNO_6S$ $(M + NH_4)^+$ 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_2Cl_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_2Cl_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_2SO_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, $CDCl_3$): δ 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_2CCl_3), 2.45 (d, *J* = 1.1 Hz, 3 H, –CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 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, $CDCl_3$): δ -114.4 (d, *J* = 52.9 Hz, 2 F). IR (neat): 1744, 1605, 1418, 1187, 1053, 996, 864 cm^{-1}. HRMS calcd for $C_{13}H_9Cl_3F_2NaO_6S$ $(M + Na)^+$ 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_2O) as colorless foam. R_f = 0.65

(BuOH/AcOH/H_2O = 4/1/1, UV). ^{1}H NMR (400 MHz, CD_3OD): δ 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, –CHF_2), 6.34 (s, 1 H, aromatic), 2.49 (s, 3 H, –CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 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, –CHF_2), 18.9 (CH_3); ^{19}F NMR (376 MHz, CD_3OD): δ -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 $C_{11}H_7O_6F_2S$ $(M - H)^-$ 304.9931, found 304.9929.

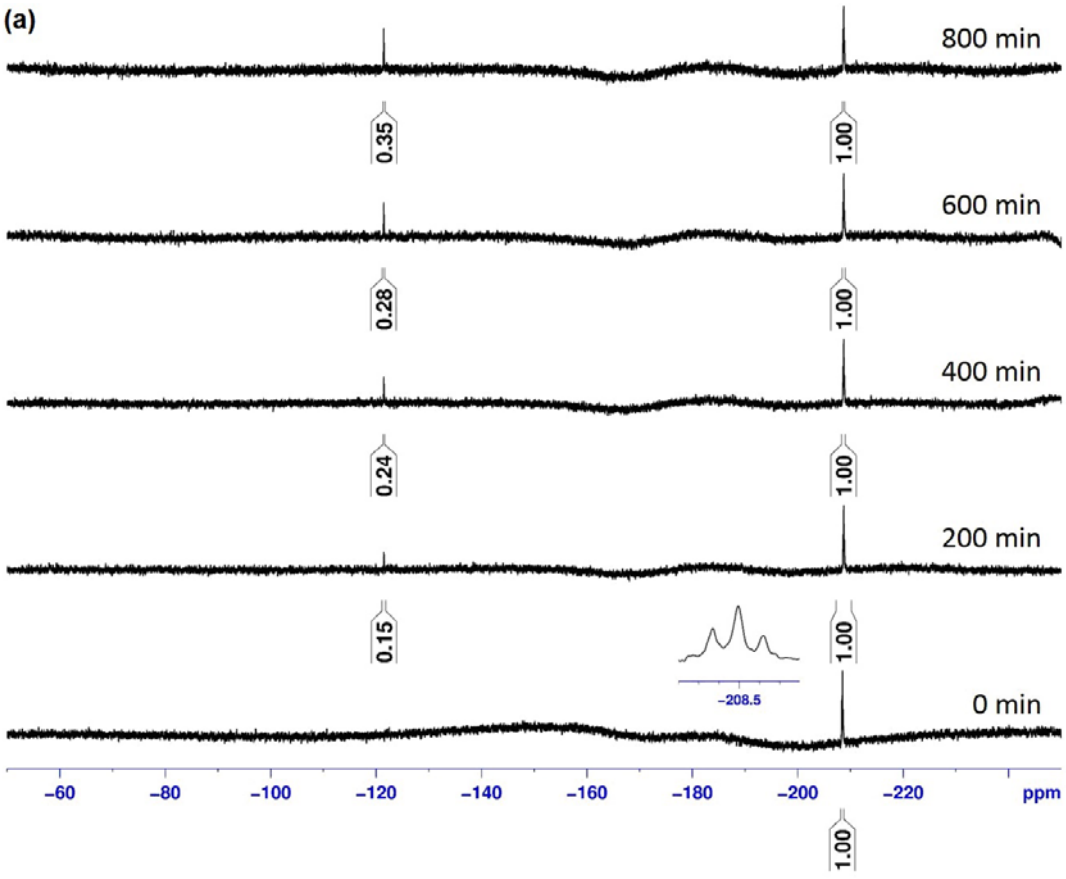


7-Hydroxy-8-(hydroxymethyl)-4-methylumbelliferyl (11). To a stirred solution of **6** (218.9 mg, 1.072 mmol) in 10 mL EtOH/CH_2Cl_2 (1/1) was added $NaBH_4$ (121.6 mg, 3.216 mmol). The reaction mixture was stirred for 3.5 h, then quenched by adding 1 N HCl (1 mL). The mixture was diluted with CH_2Cl_2 (30 mL), and washed successively with H_2O (5 mL x 1) and brine (5 mL x 1). The organic layer was dried over Na_2SO_4, filtered, and evaporated. The desired product was recrystallized from CH_2Cl_2 and CH_3OH. Compound **11** was obtained as a white solid (154.7 mg, 70%), m.p. = 160–161 ^{o}C. R_f = 0.38 (hexane/EtOAc = 3/7, UV). ^{1}H NMR (400 MHz, CD_3OD): δ 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, –CH_2OH), 2.42 (d, *J* = 1.0 Hz, 3 H, –CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 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 % CO_2. 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 ^{19}F NMR. Two NMR tubes containing probe **1a** or **1b** (2.5 mM) were incubated with CHO/STS lysate (1.47 mg) in Tris-HCl D_2O buffer (100 mM, pH 7.5) at 37 ^{o}C, respectively. The mixtures were continually monitored with ^{19}F NMR at intervals of 20 min. The concentrations of the remaining probes in the reaction mixture were calculated according to the integration of ^{19}F NMR.



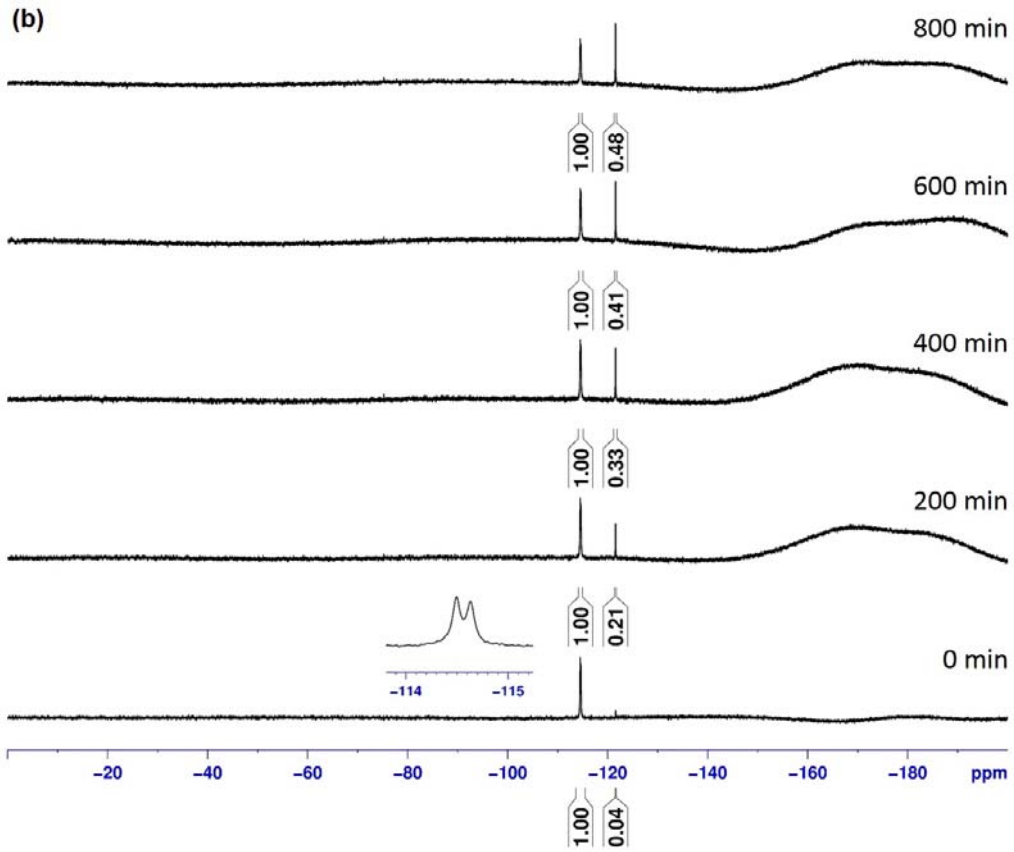


Figure S1. Selective ^{19}F NMR spectra for the hydrolysis of (a) probe **1a** and (b) probe **1b** by STS at 0, 200, 400, 600 and 800 min.

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 (λ_{ex} = 360 nm, λ_{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 spetra were recorded on a spectrofluorometer (Jobin Yvon Fluorolog-3) using 10 mm quartz cuvettes with a path length of 1 cm (λ_{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.

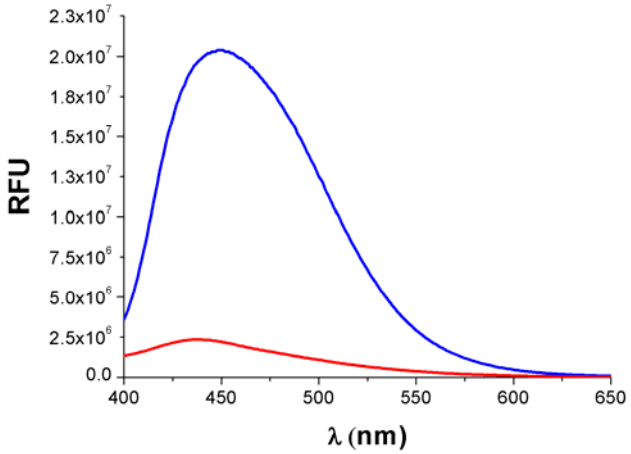


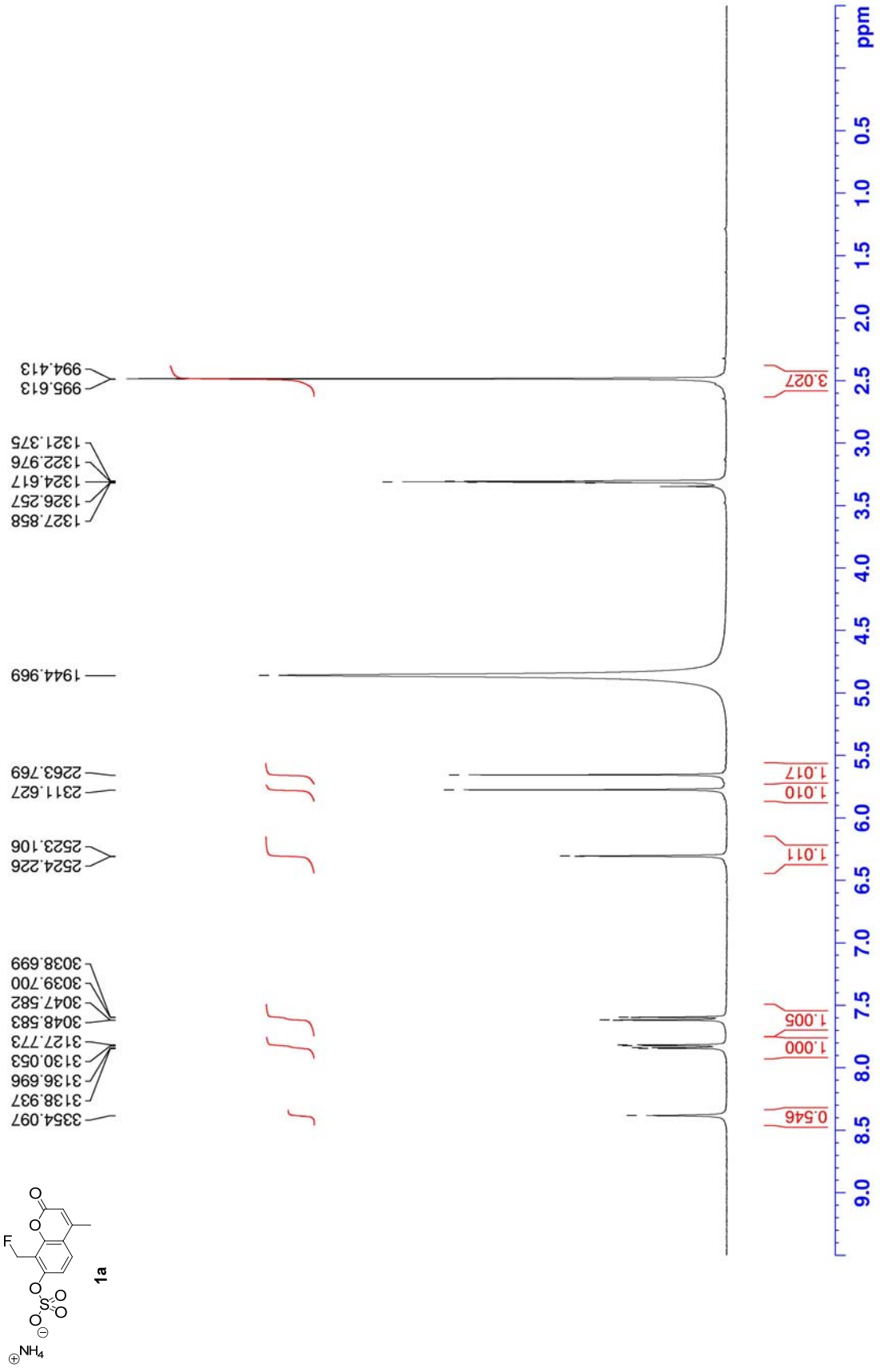
Figure S2. Emission spectra (λ_{ex} = 360 nm) of compounds **6** (red line) and **11** (blue line) at 10 μM.

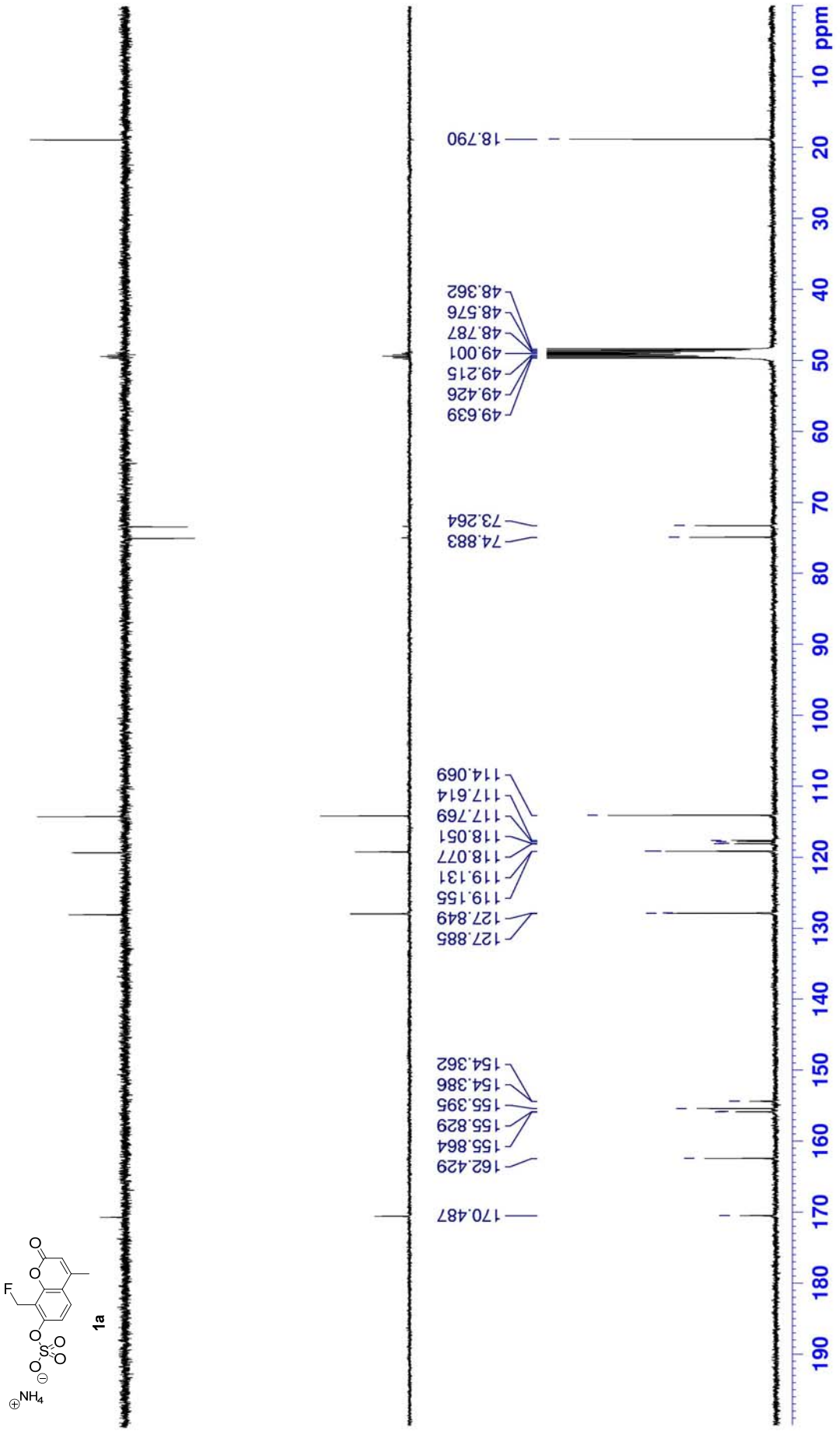
Comparative fluorescent imaging study of CHO/STS cells: CHO/STS cells were seeded on a 6-well culture plate with coverslips overnight for attachment. After washing coverslips with PBS, cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, and washed with PBS three times, followed by staining with 10 μM 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 μM 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. C. P. Lu, C. T. Ren, S. H. Wu, C. Y. Chu and L. C. Lo, *ChemBioChem*, 2007, **8**, 2187
2. P. J. Hughes, L. E. Twist, J. Durham, M. A. Choudhry, M. Drayson, R. Chandraratna, R. H. Michell, C. J. Kirk and G. Brown, *Biochem. J.*, 2001, **355**, 361.

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

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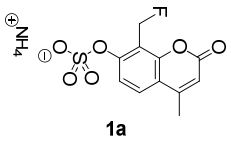

1a

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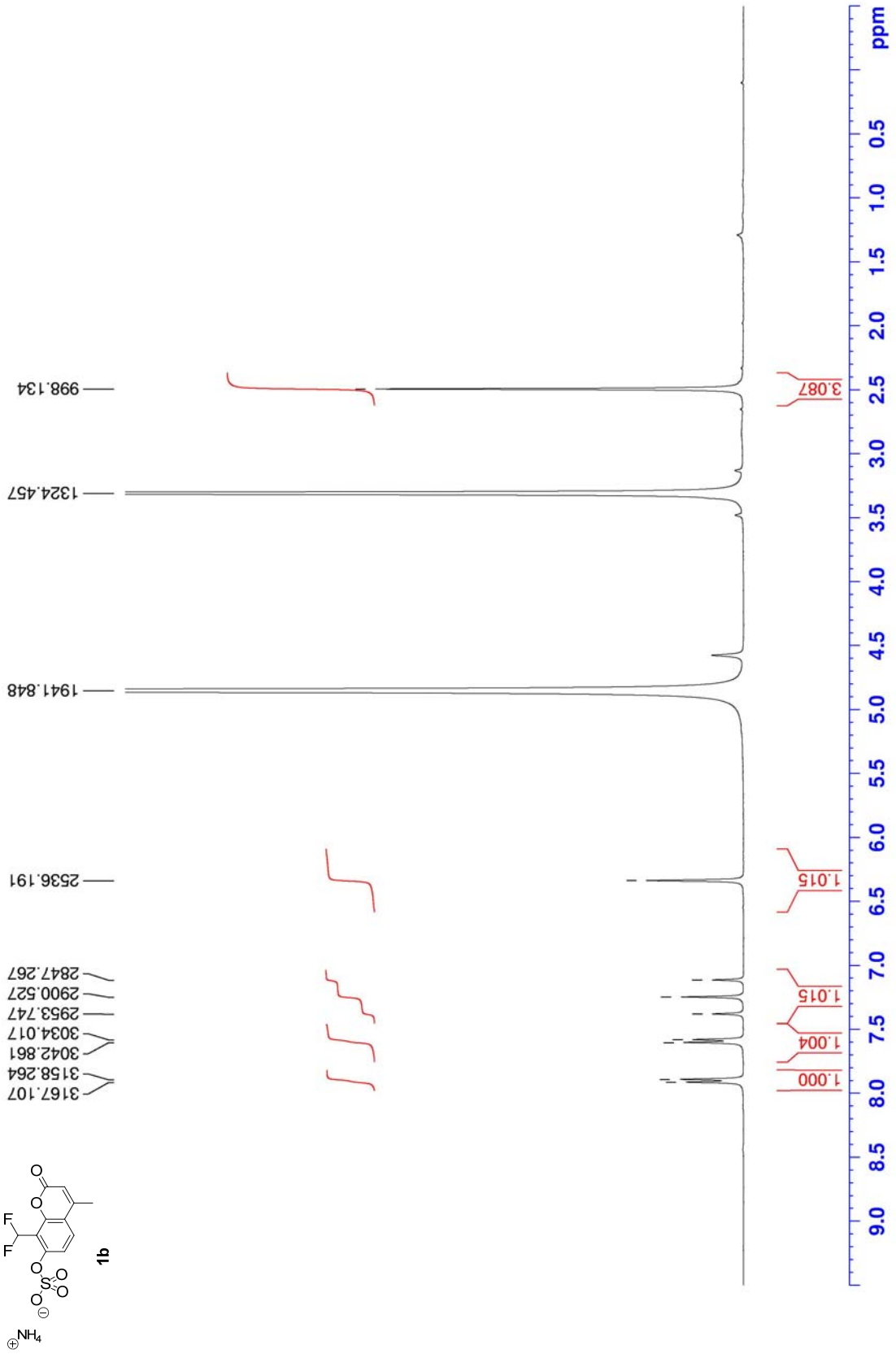
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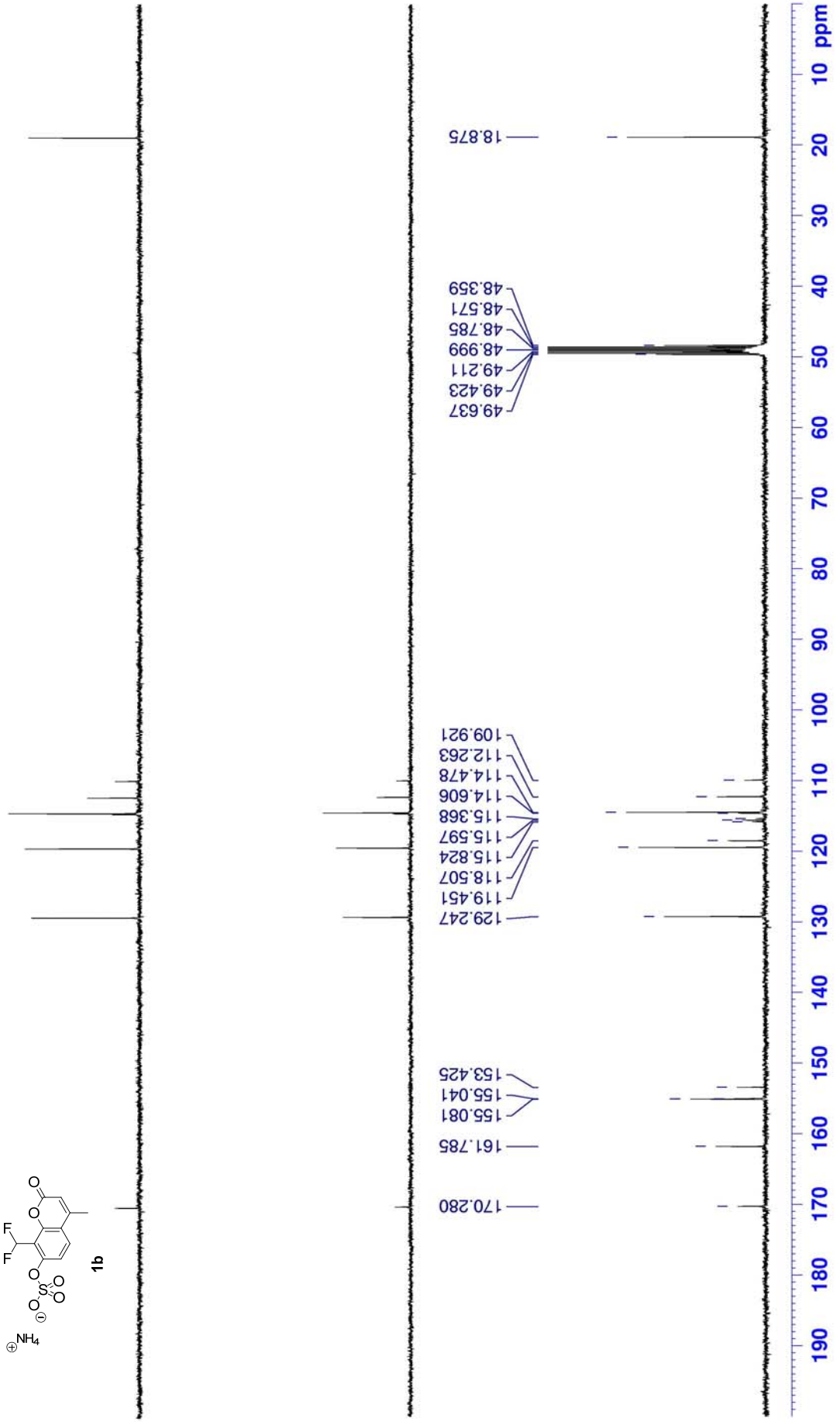
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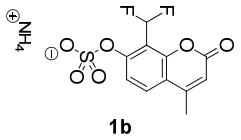

1b