

**Synthesis and properties of chemiluminescent acridinium ester labels with  
fluorous tags**

Supplementary Section

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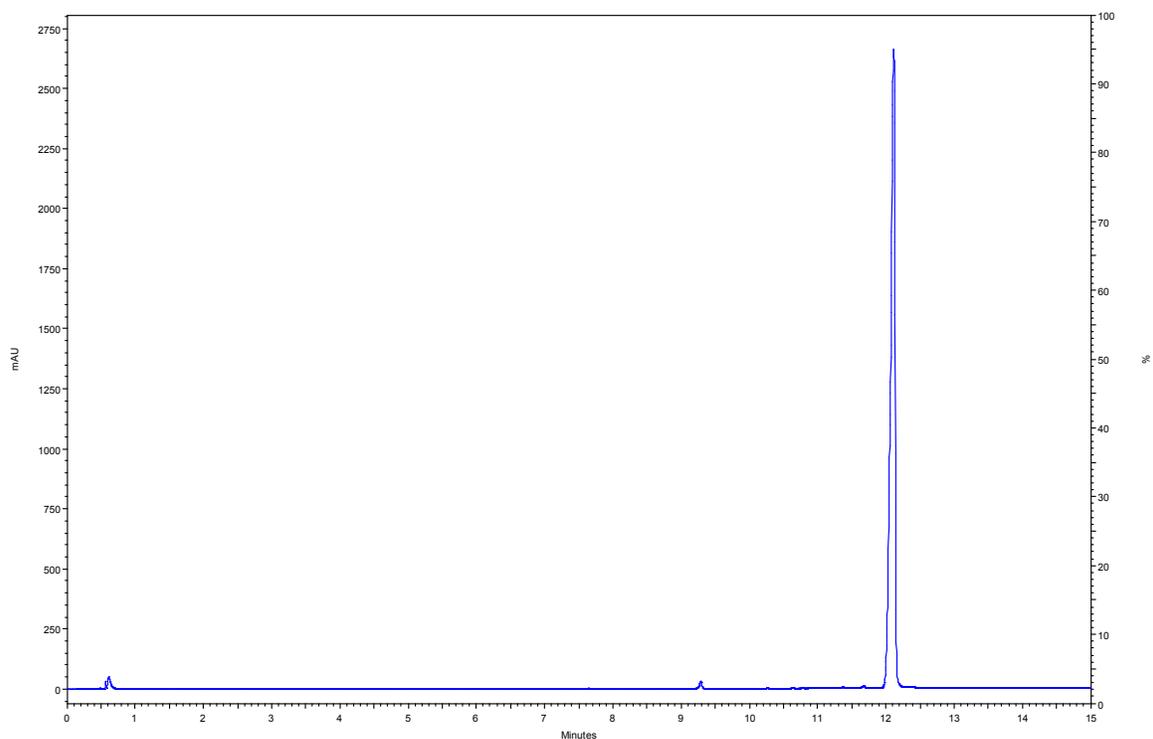
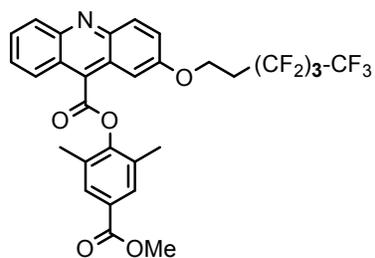
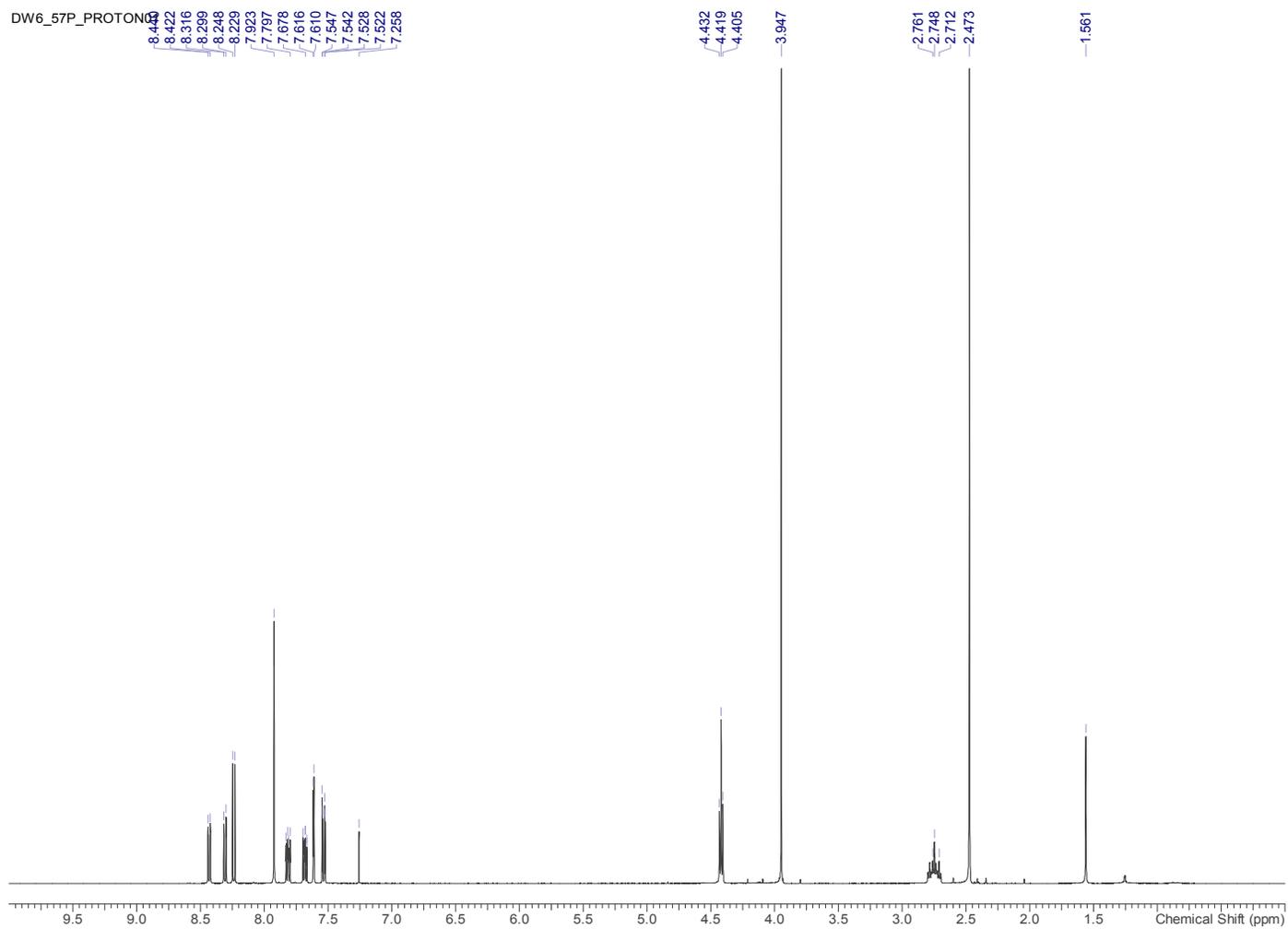


Figure S1. HPLC trace of compound **2a**. HPLC analysis was performed using a Phenomenex, Kinetex XB-C18, 2.6 micron, 4.6 x 50 mm column and a 10 minute gradient of 10 → 90% MeCN/water (each with 0.05% trifluoroacetic acid) followed by a 1.2 minute gradient of 90 → 100% MeCN/water (each with 0.05% trifluoroacetic acid) followed by 100% MeCN (with 0.05% trifluoroacetic acid) for 3.8 minutes.



**2a**



$^1\text{H}$  NMR of **2a** in  $\text{CDCl}_3$

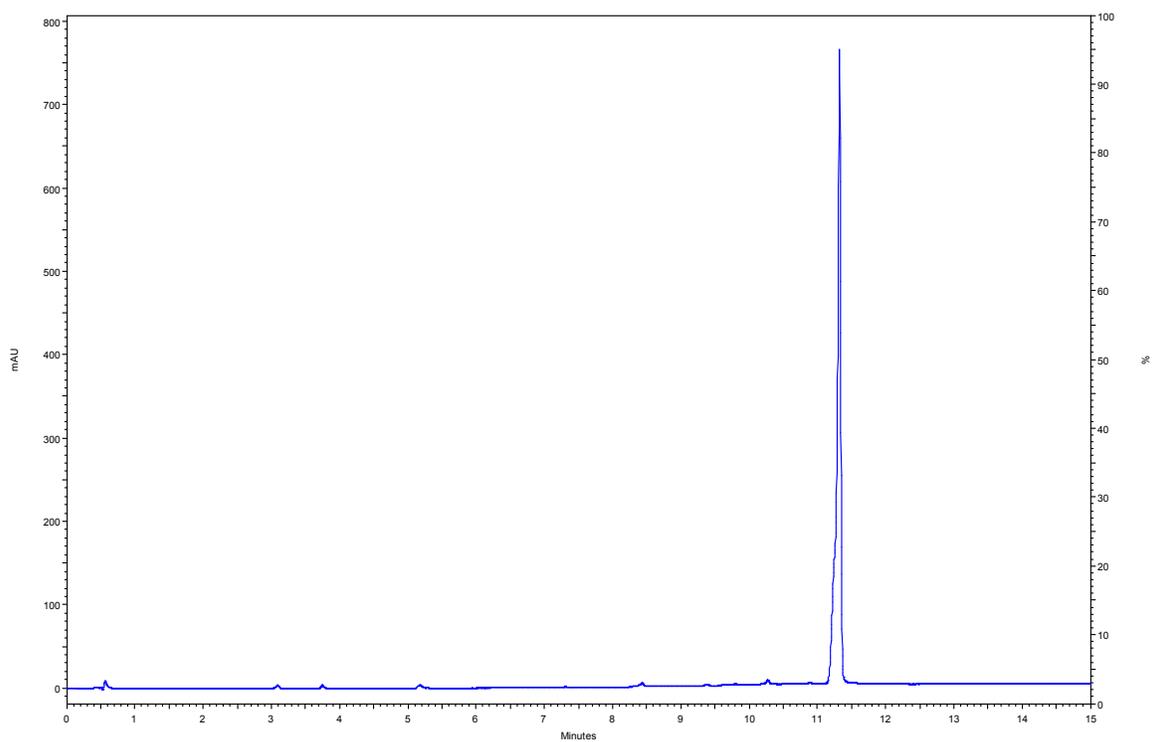
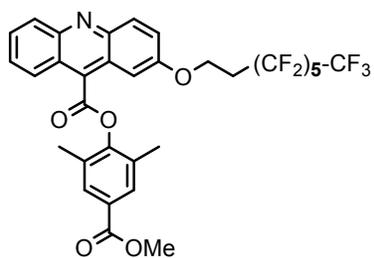
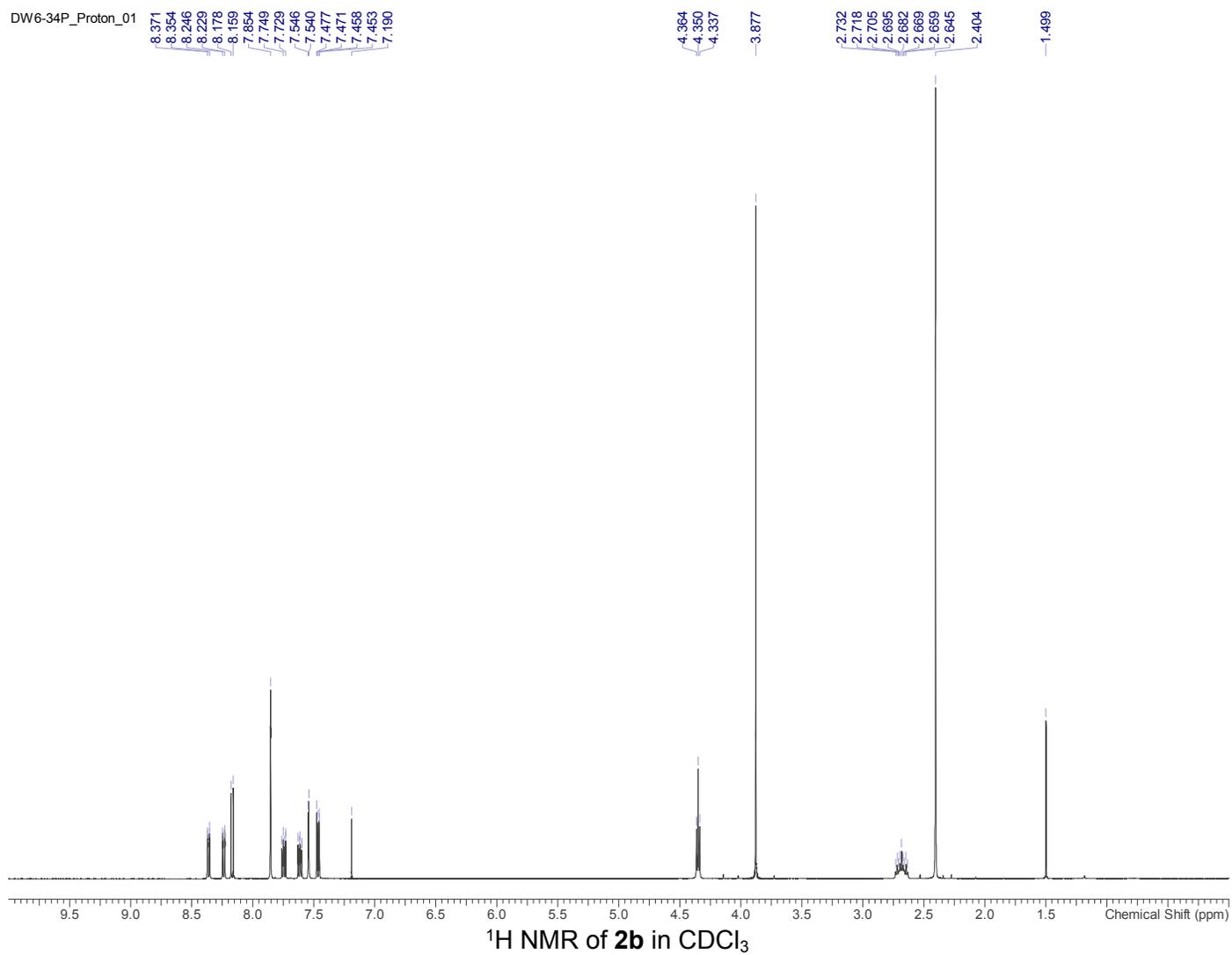


Figure S2. HPLC trace of compound **2b**. HPLC analysis was performed using a Phenomenex, Kinetex XB-C18, 2.6 micron, 4.6 x 50 mm column and a 10 minute gradient of 30 → 100 % MeCN/water (each with 0.05% trifluoroacetic acid) followed by 100% MeCN (with 0.05% trifluoroacetic acid) for 5.0 minutes.



**2b**



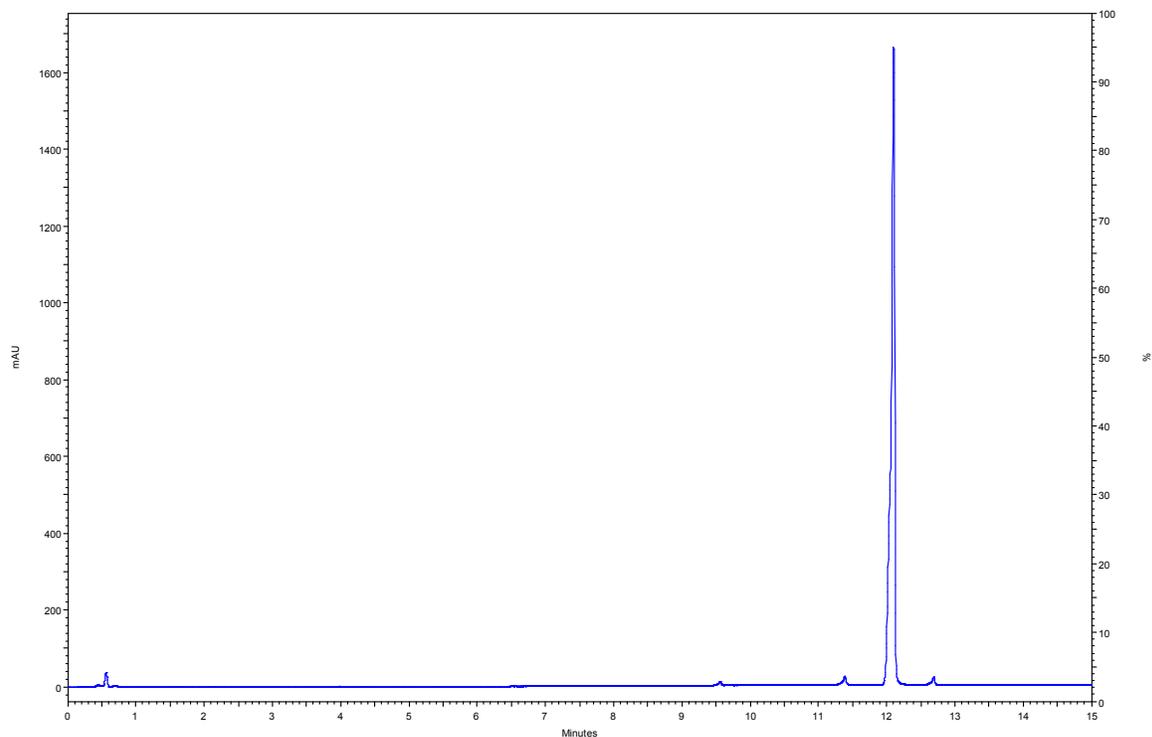
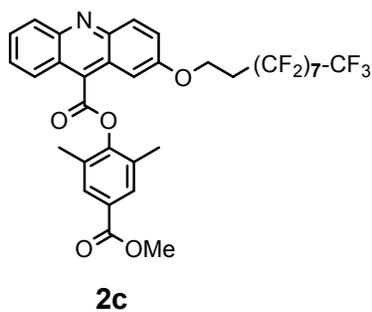
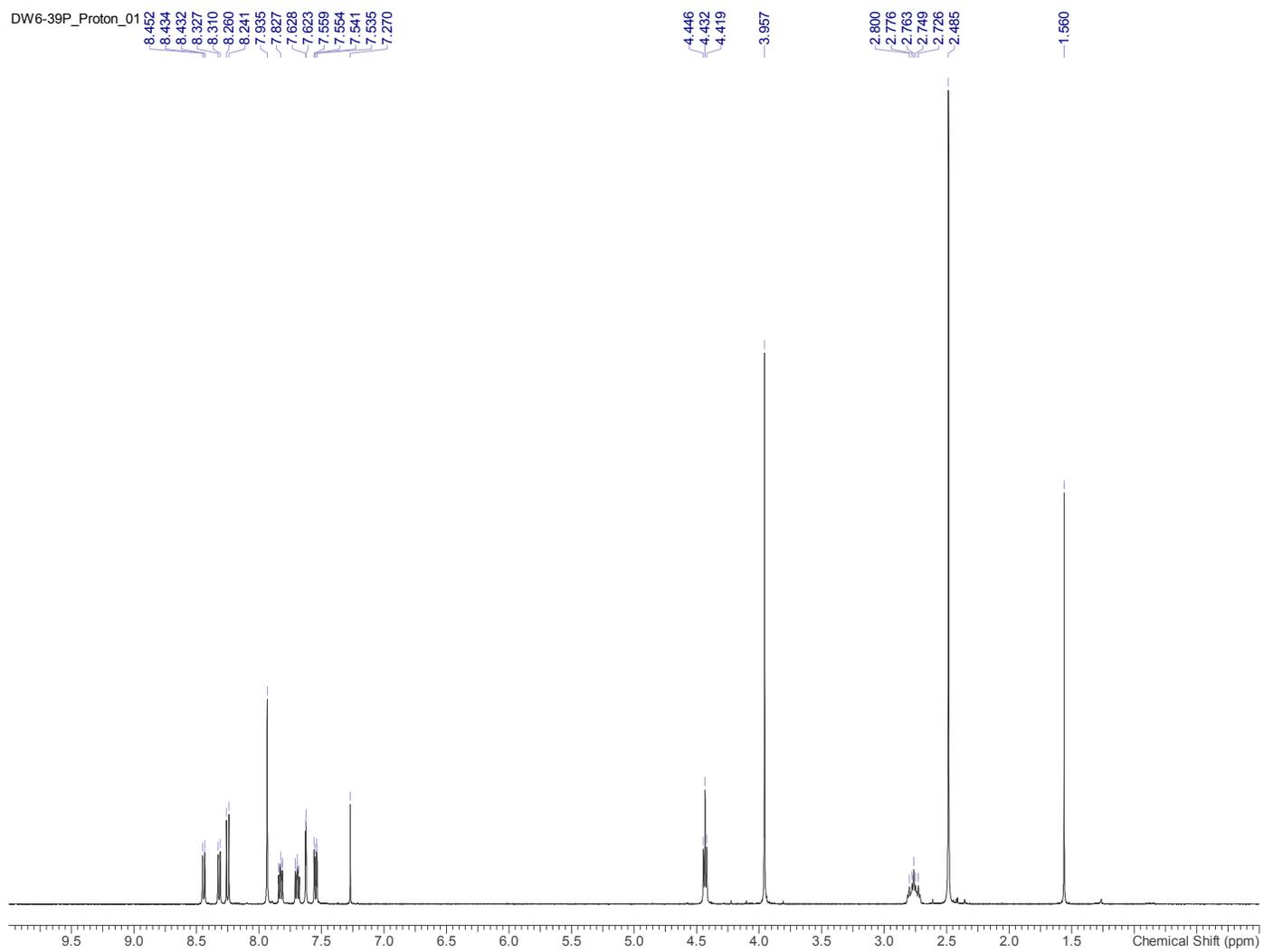


Figure S3. HPLC trace of compound **2c**. HPLC analysis was performed using a Phenomenex, Kinetex XB-C18, 2.6 micron, 4.6 x 50 mm column and a 10 minute gradient of 30 → 100 % MeCN/water (each with 0.05% trifluoroacetic acid) followed by 100% MeCN (with 0.05% trifluoroacetic acid) for 5.0 minutes.





$^1\text{H}$  NMR of **2c** in  $\text{CDCl}_3$

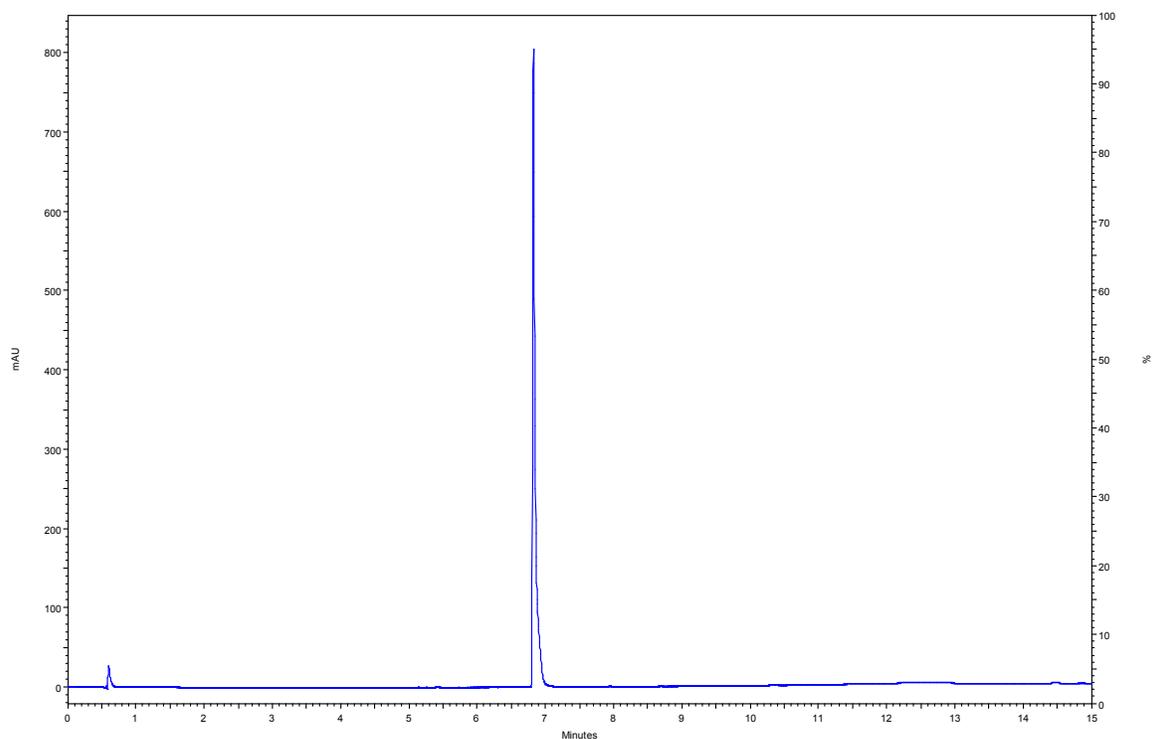
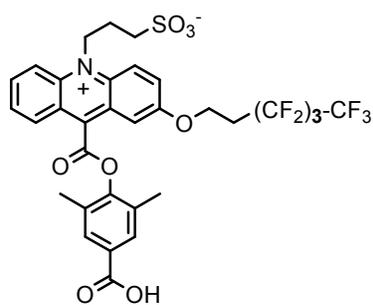
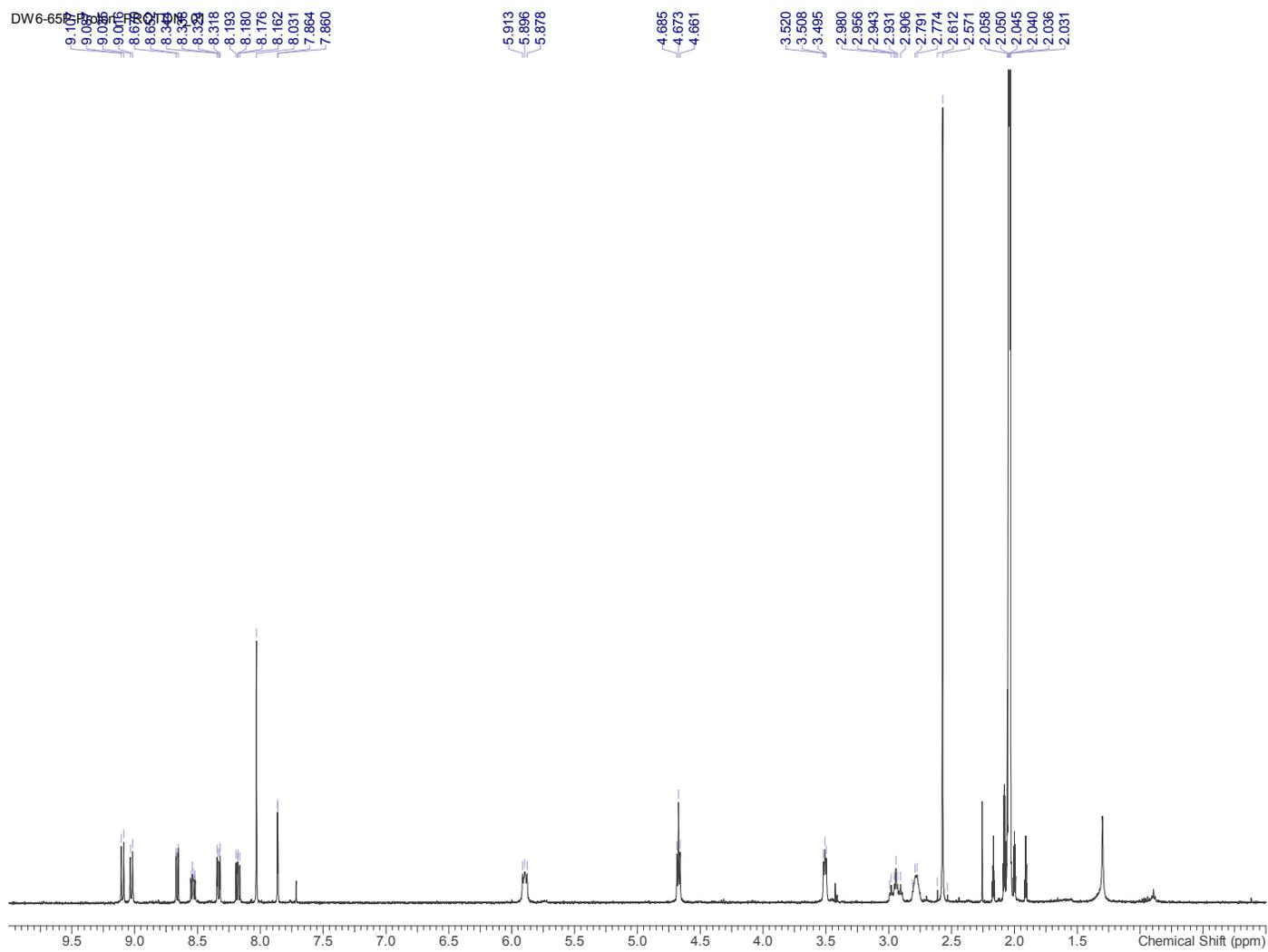


Figure S4. HPLC trace of compound **3a**. HPLC analysis was performed using a Phenomenex, Kinetex XB-C18, 2.6 micron, 4.6 x 50 mm column and a 10 minute gradient of 10 → 90% MeCN/water (each with 0.05% trifluoroacetic acid) followed by a 1.2 minute gradient of 90 → 100% MeCN/water (each with 0.05% trifluoroacetic acid) followed by 100% MeCN (with 0.05% trifluoroacetic acid) for 3.8 minutes.



**3a**



$^1\text{H}$  NMR of **3a** in  $\text{CD}_3\text{COOD}$

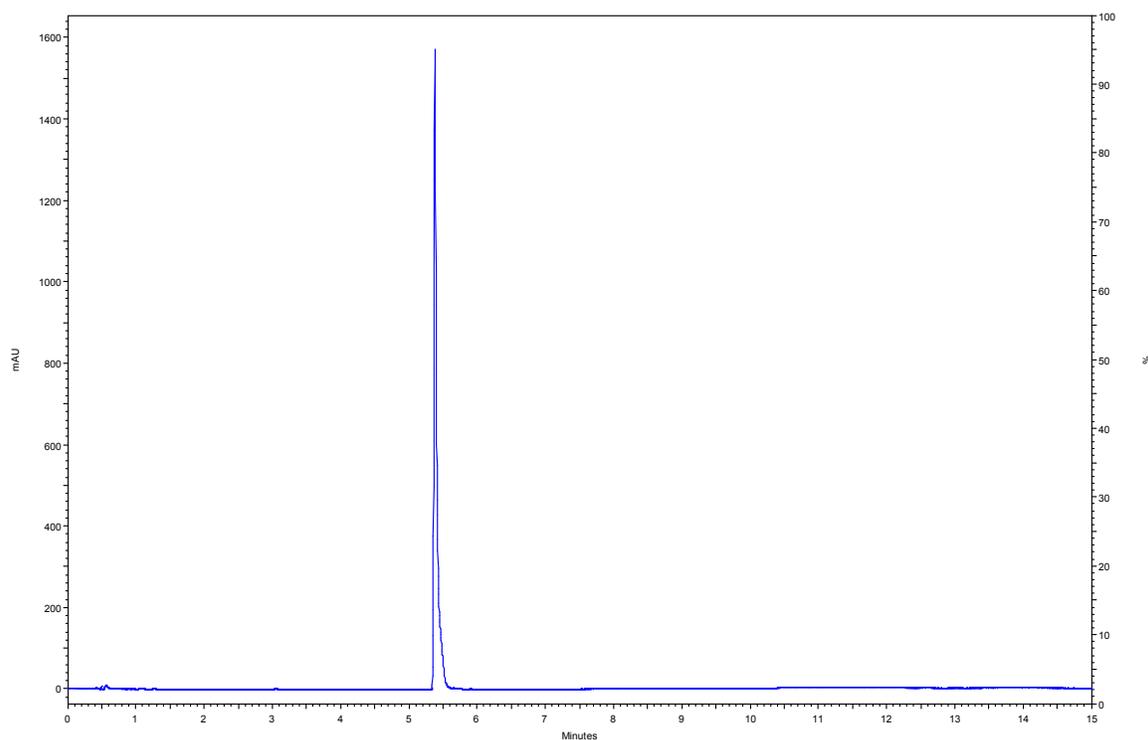
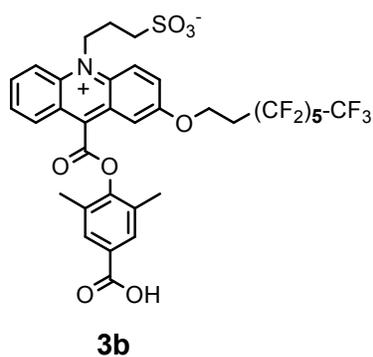
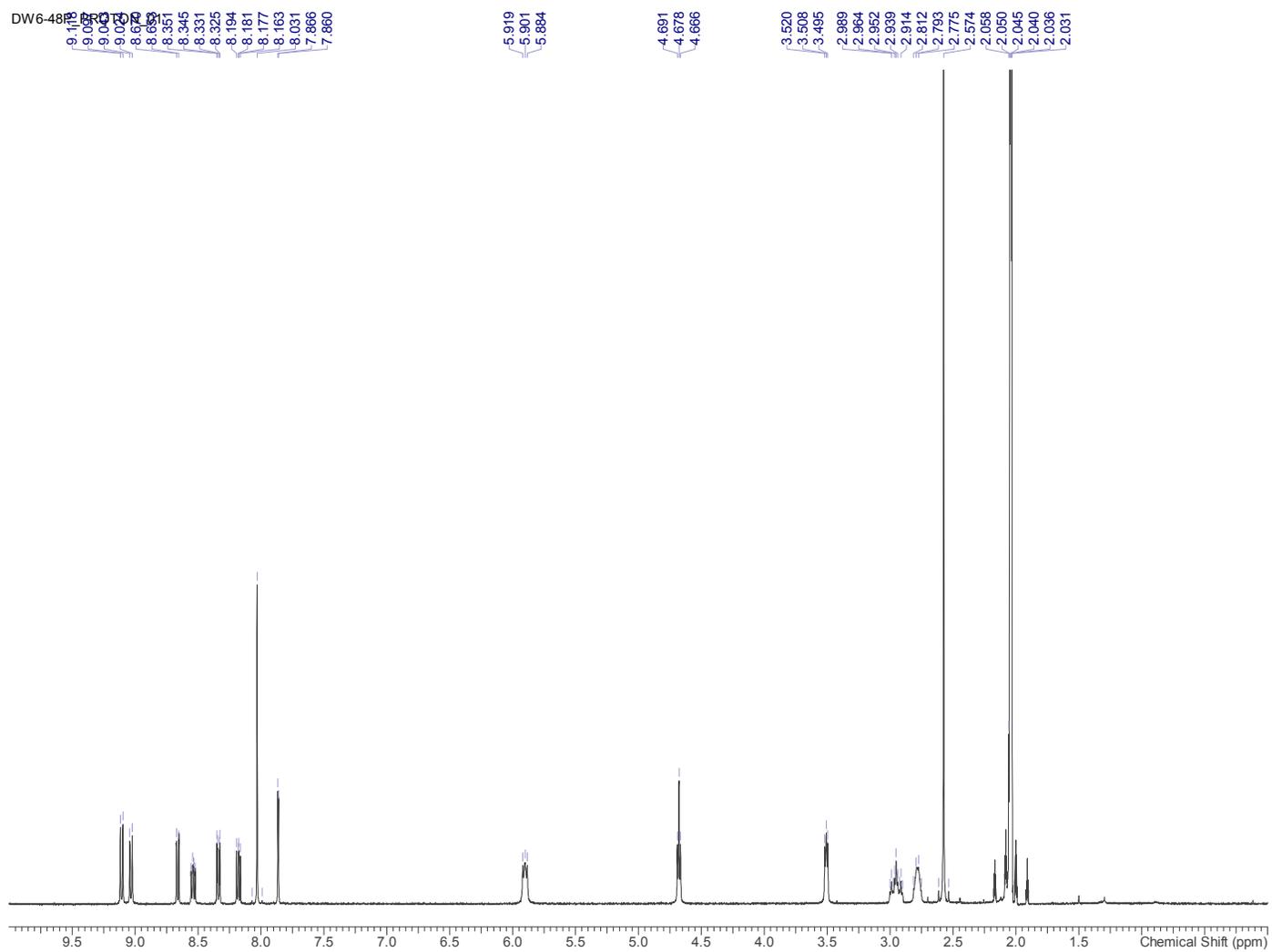


Figure S5. HPLC trace of compound **3b**. HPLC analysis was performed using a Phenomenex, Kinetex XB-C18, 2.6 micron, 4.6 x 50 mm column and a 10 minute gradient of 30 → 100 % MeCN/water (each with 0.05% trifluoroacetic acid) followed by 100% MeCN (with 0.05% trifluoroacetic acid) for 5.0 minutes.





$^1\text{H}$  NMR of **3b** in  $\text{CD}_3\text{COOD}$

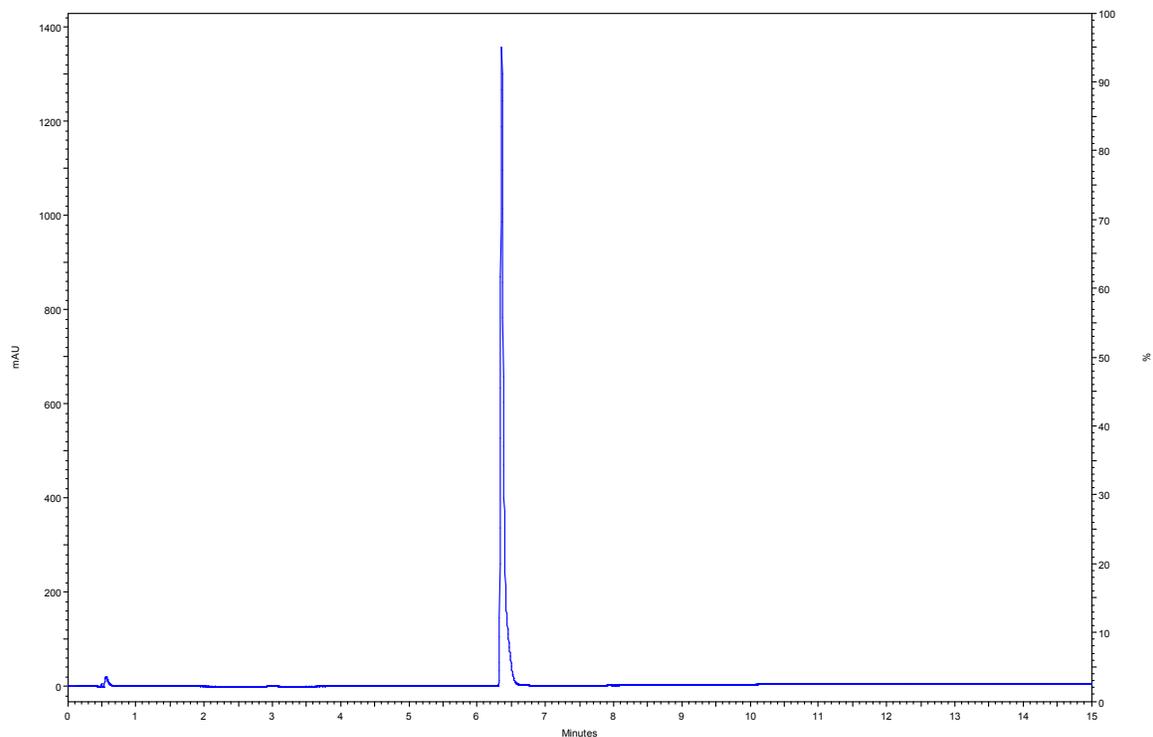
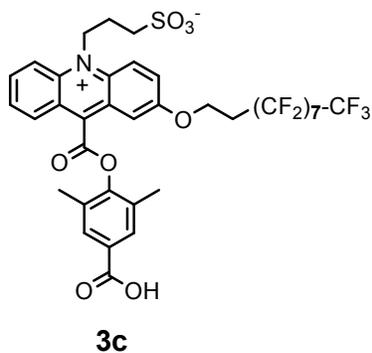
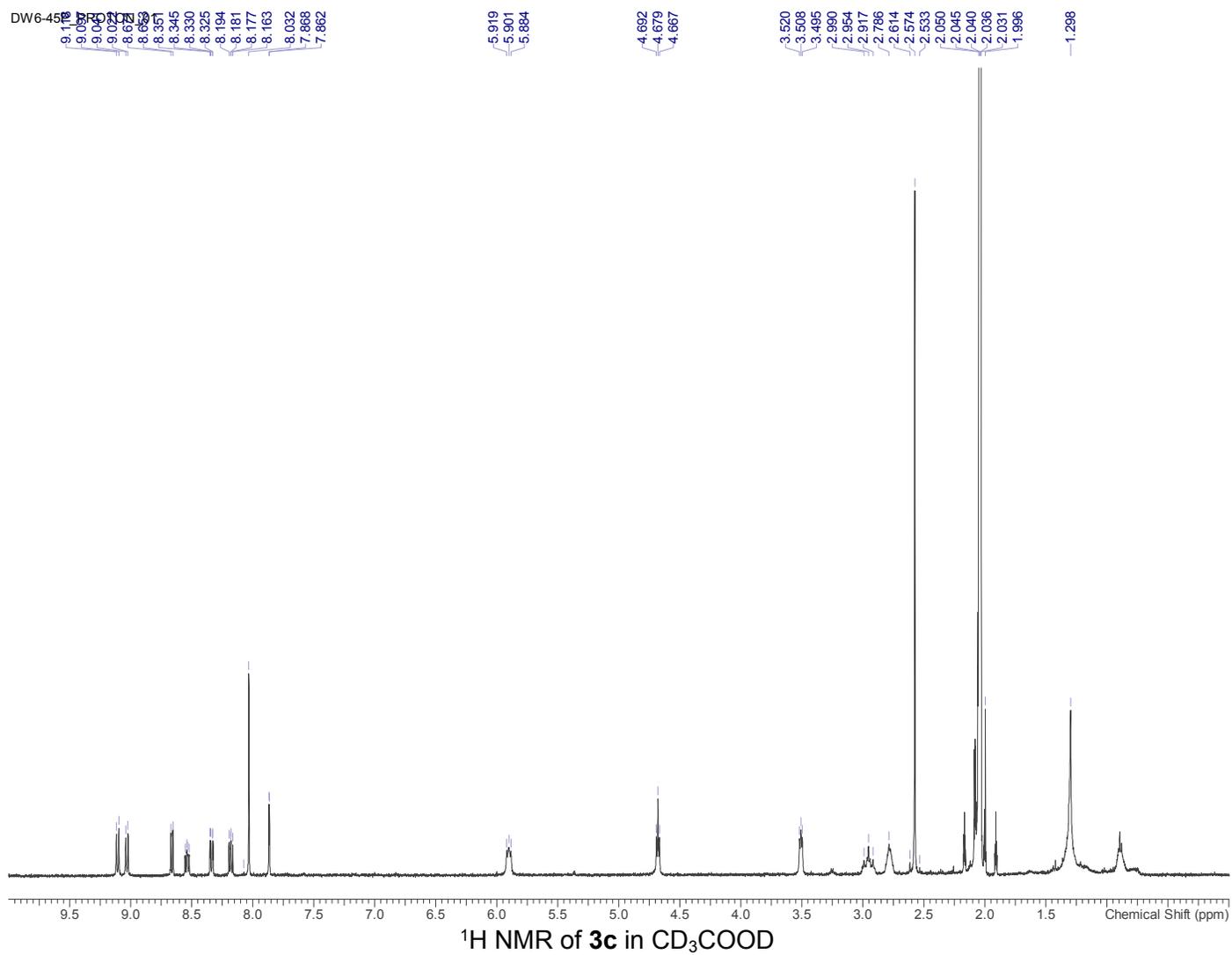


Figure S6. HPLC trace of compound **3c**. HPLC analysis was performed using a Phenomenex, Kinetex XB-C18, 2.6 micron, 4.6 x 50 mm column and a 10 minute gradient of 30 → 100 % MeCN/water (each with 0.05% trifluoroacetic acid) followed by 100% MeCN (with 0.05% trifluoroacetic acid) for 5.0 minutes.





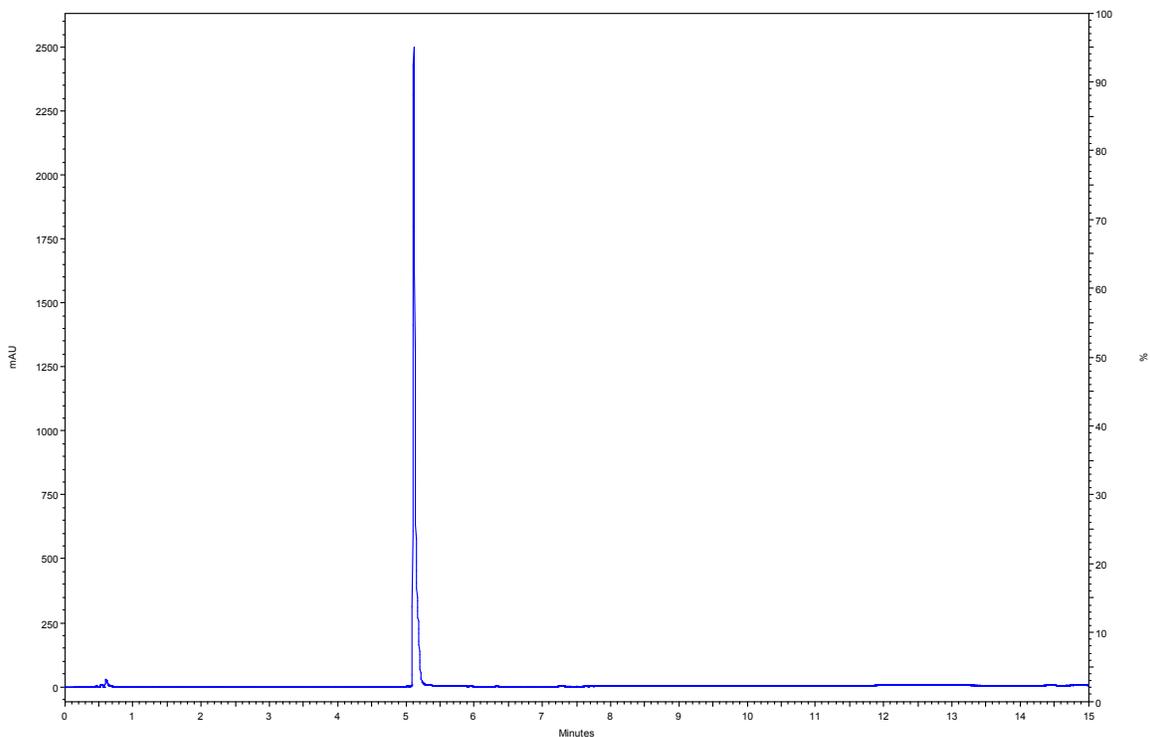
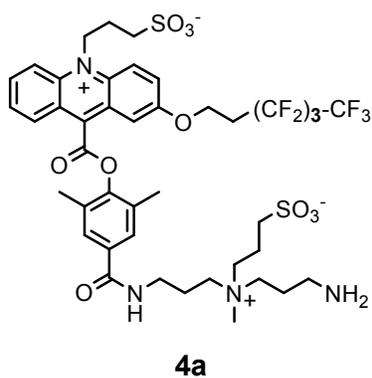
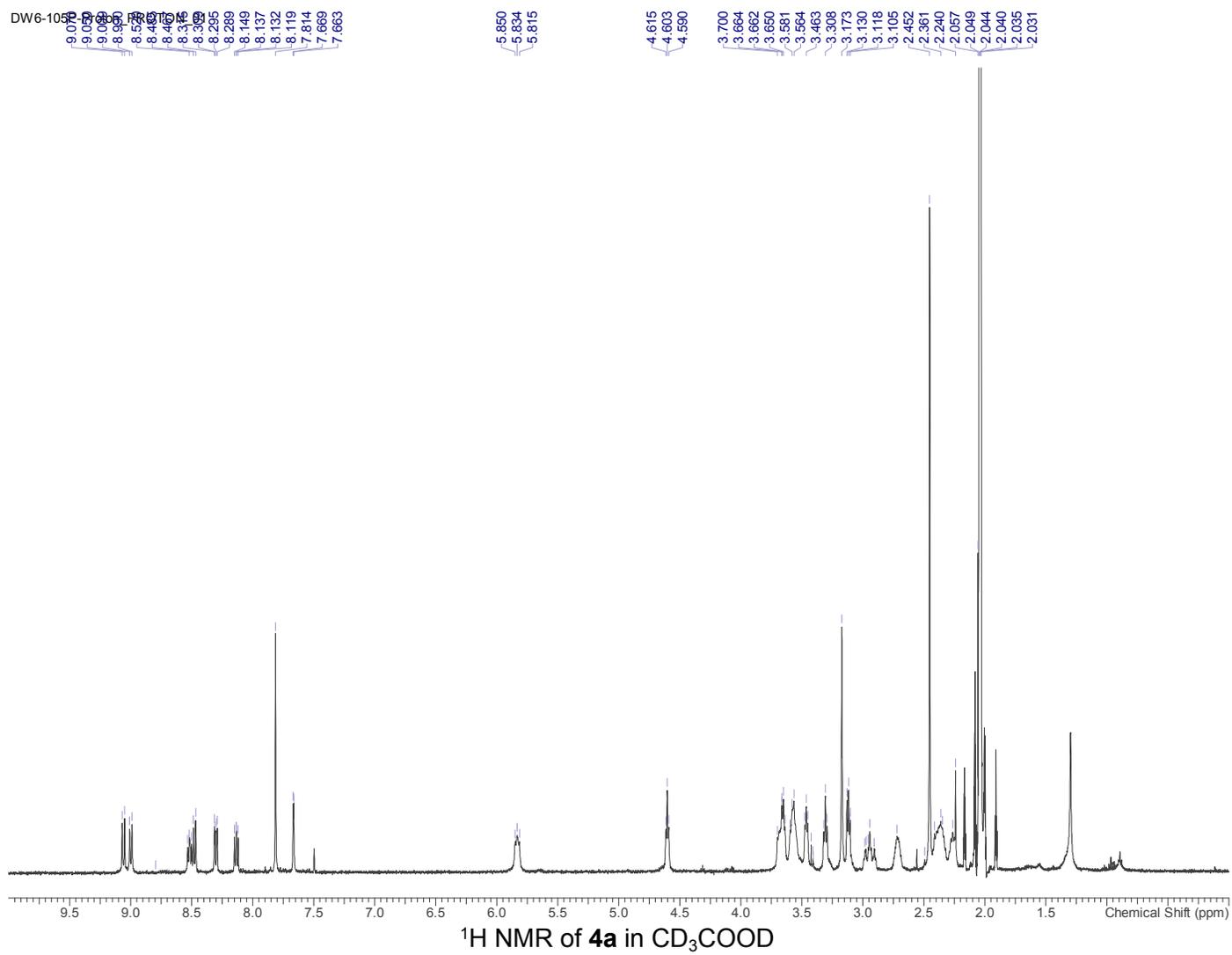
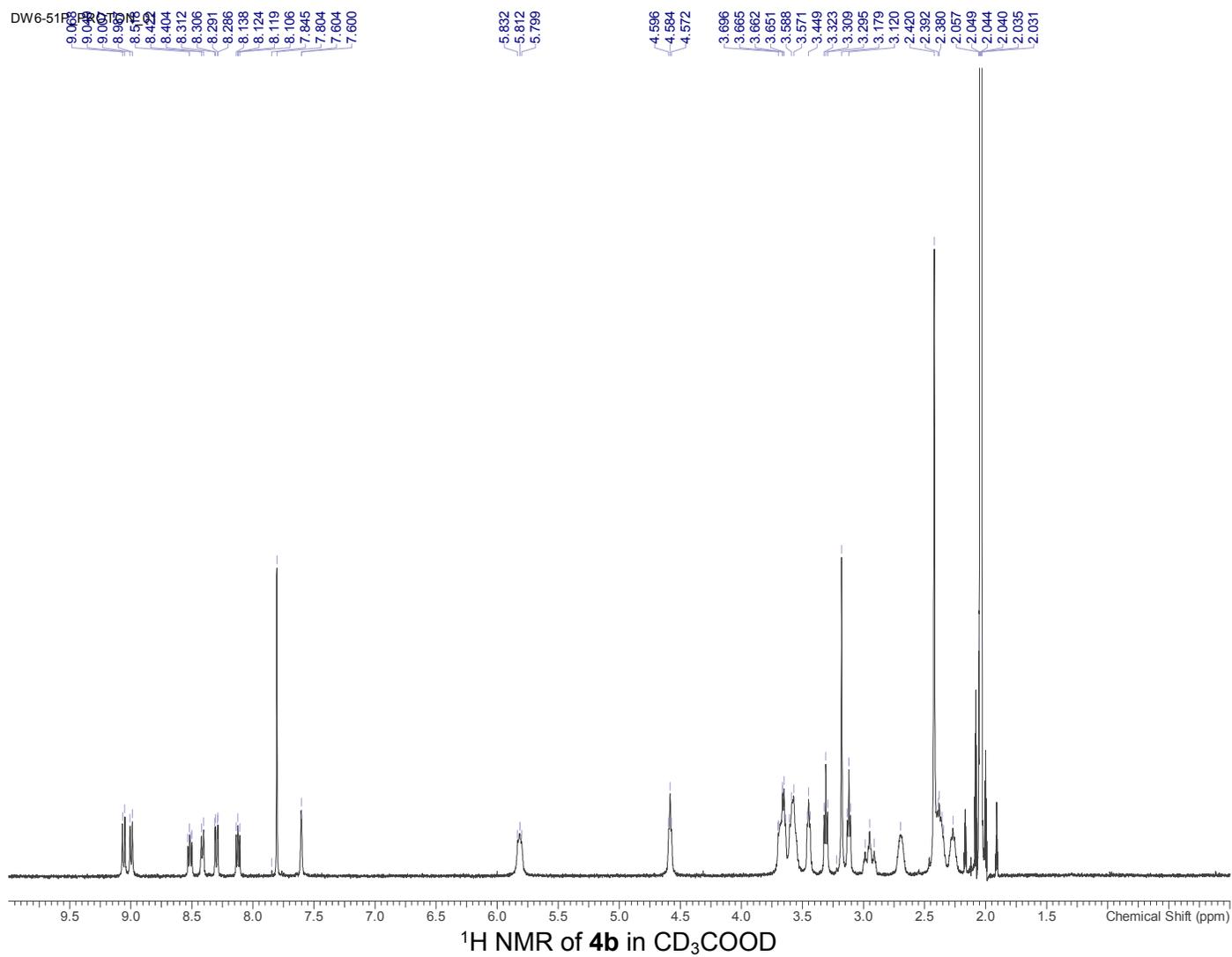


Figure S7. HPLC trace of compound **4a**. HPLC analysis was performed using a Phenomenex, Kinetex XB-C18, 2.6 micron, 4.6 x 50 mm column and a 10 minute gradient of 10 → 90% MeCN/water (each with 0.05% trifluoroacetic acid) followed by a 1.2 minute gradient of 90 → 100% MeCN/water (each with 0.05% trifluoroacetic acid) followed by 100% MeCN (with 0.05% trifluoroacetic acid) for 3.8 minutes.









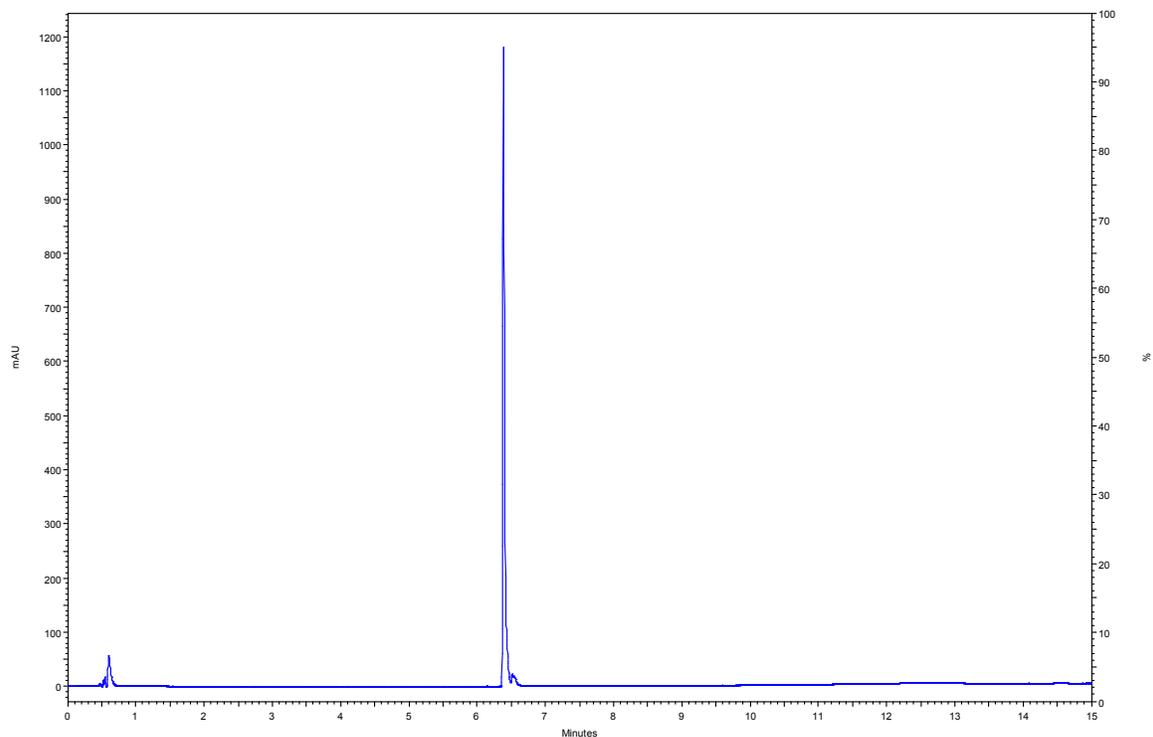
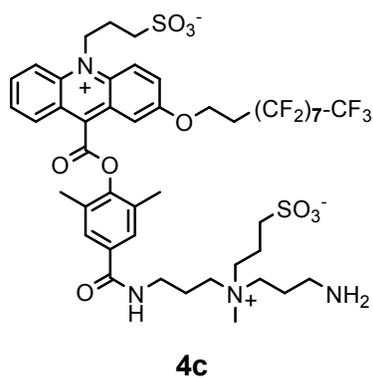
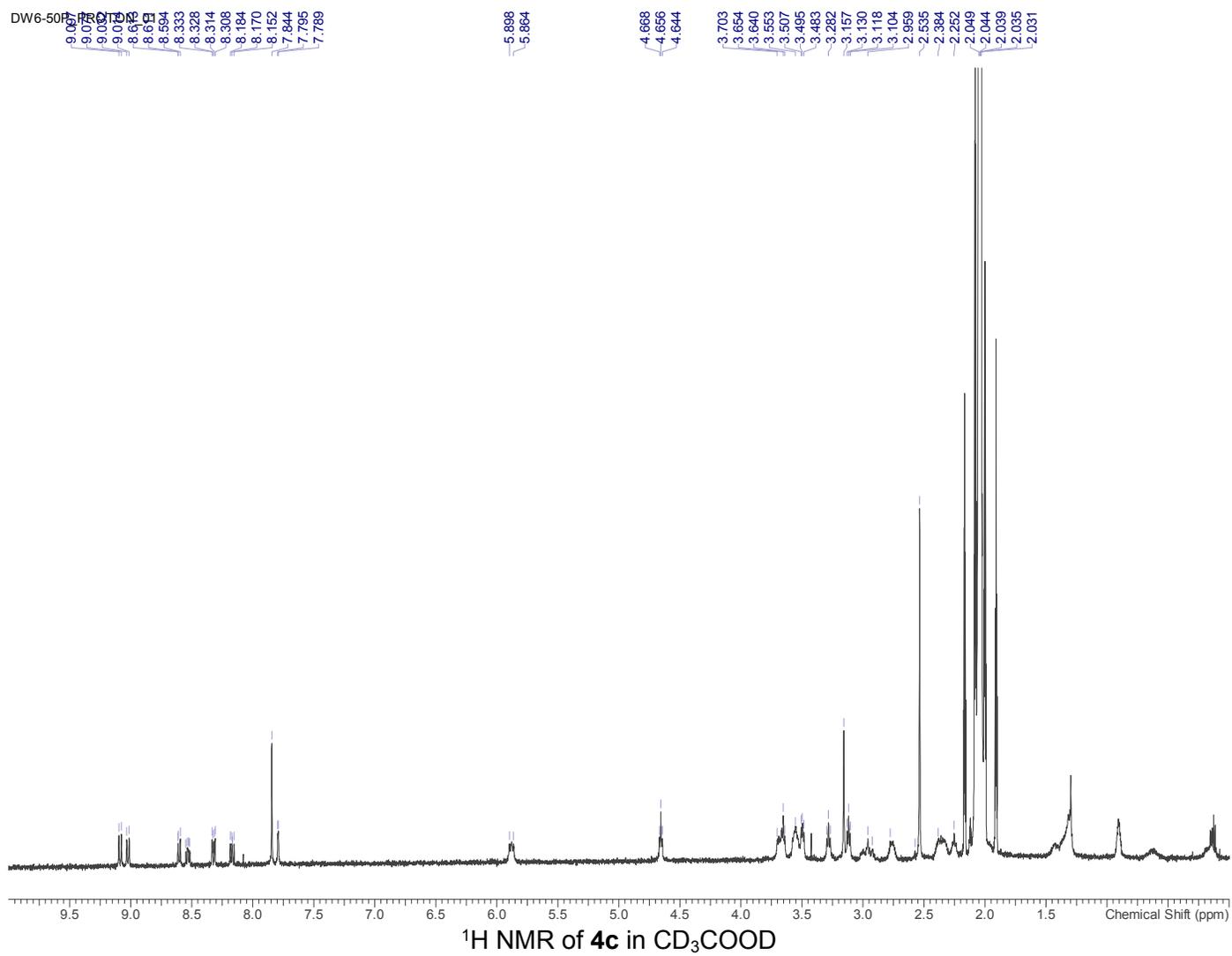


Figure S9. HPLC trace of compound **4c**. HPLC analysis was performed using a Phenomenex, Kinetex XB-C18, 2.6 micron, 4.6 x 50 mm column and a 10 minute gradient of 10 → 90% MeCN/water (each with 0.05% trifluoroacetic acid) followed by a 1.2 minute gradient of 90 → 100% MeCN/water (each with 0.05% trifluoroacetic acid) followed by 100% MeCN (with 0.05% trifluoroacetic acid) for 3.8 minutes.





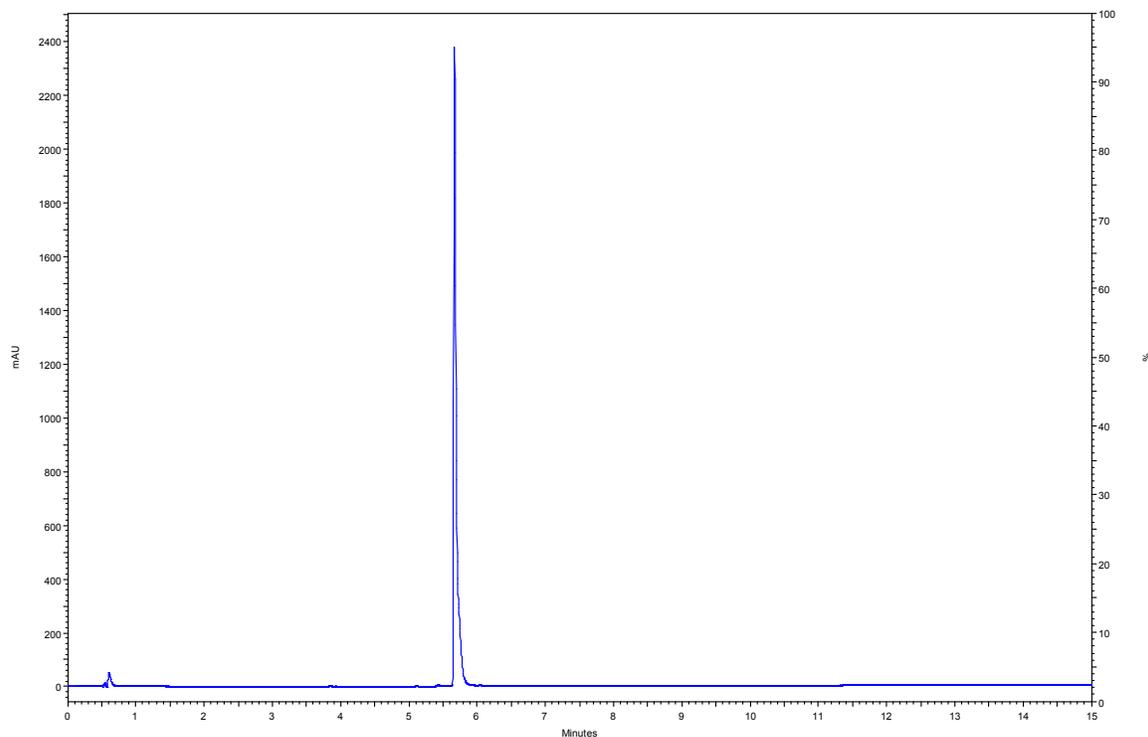
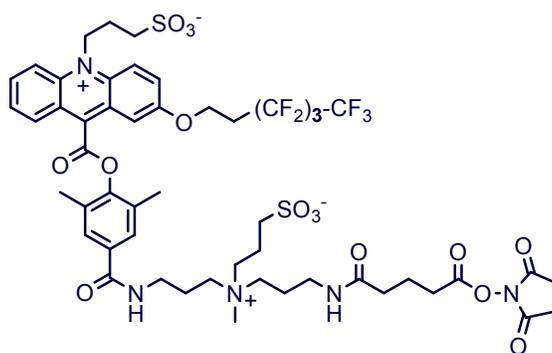
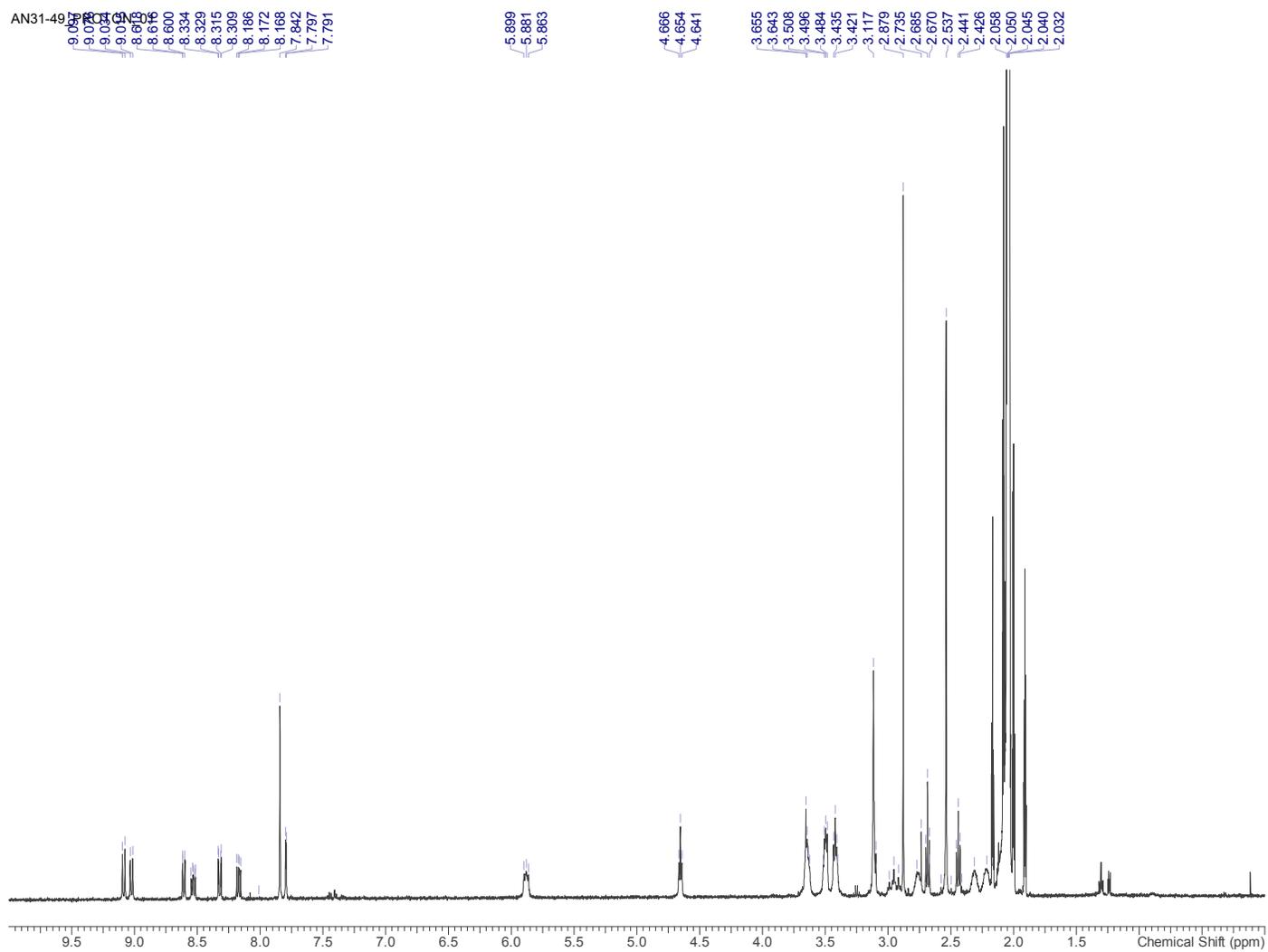


Figure S10. HPLC trace of compound **5a**. HPLC analysis was performed using a Phenomenex, Kinetex XB-C18, 2.6 micron, 4.6 x 50 mm column and a 10 minute gradient of 10 → 90% MeCN/water (each with 0.05% trifluoroacetic acid) followed by a 1.2 minute gradient of 90 → 100% MeCN/water (each with 0.05% trifluoroacetic acid) followed by 100% MeCN (with 0.05% trifluoroacetic acid) for 3.8 minutes.

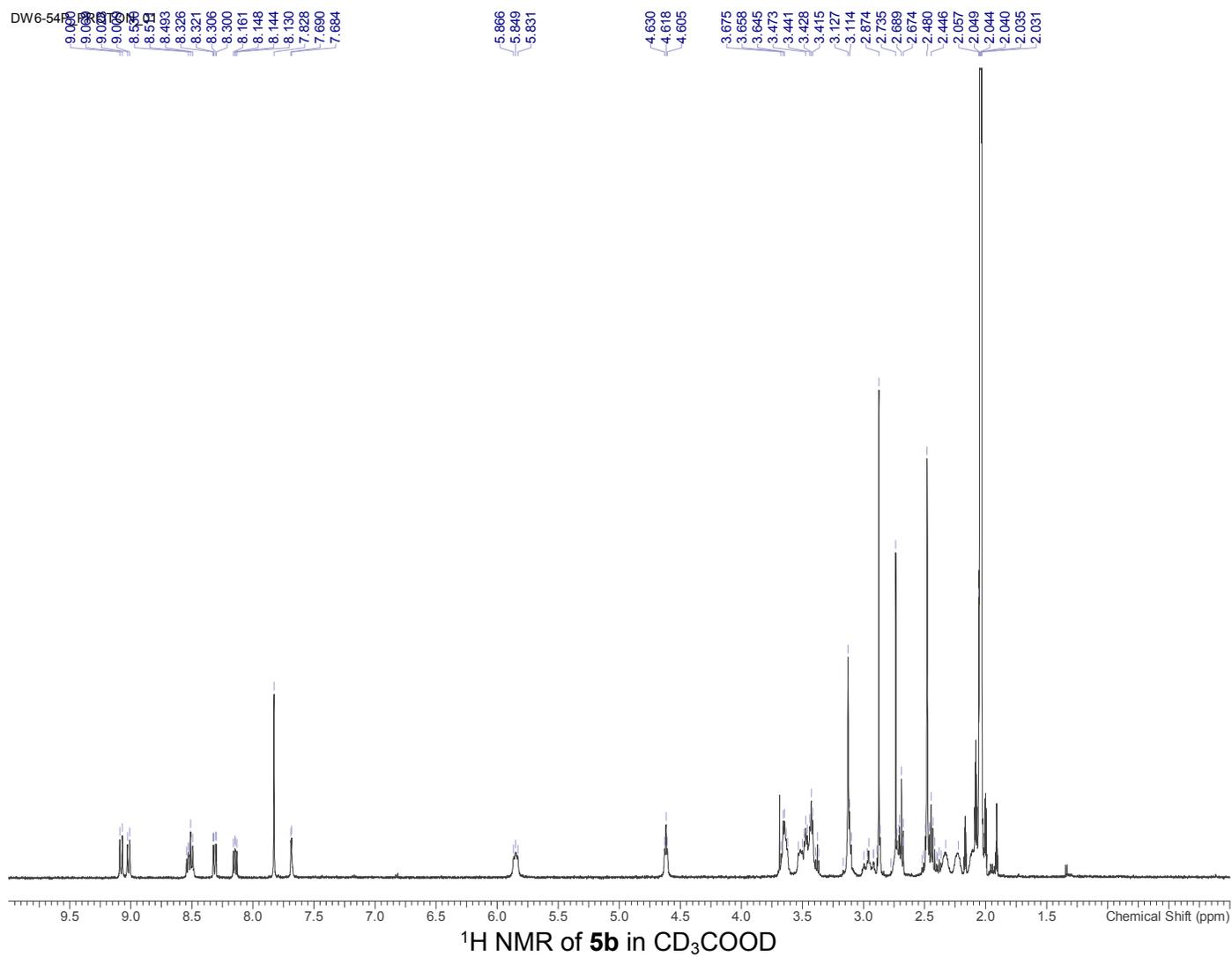


**5a**



$^1\text{H}$  NMR of **5a** in  $\text{CD}_3\text{COOD}$





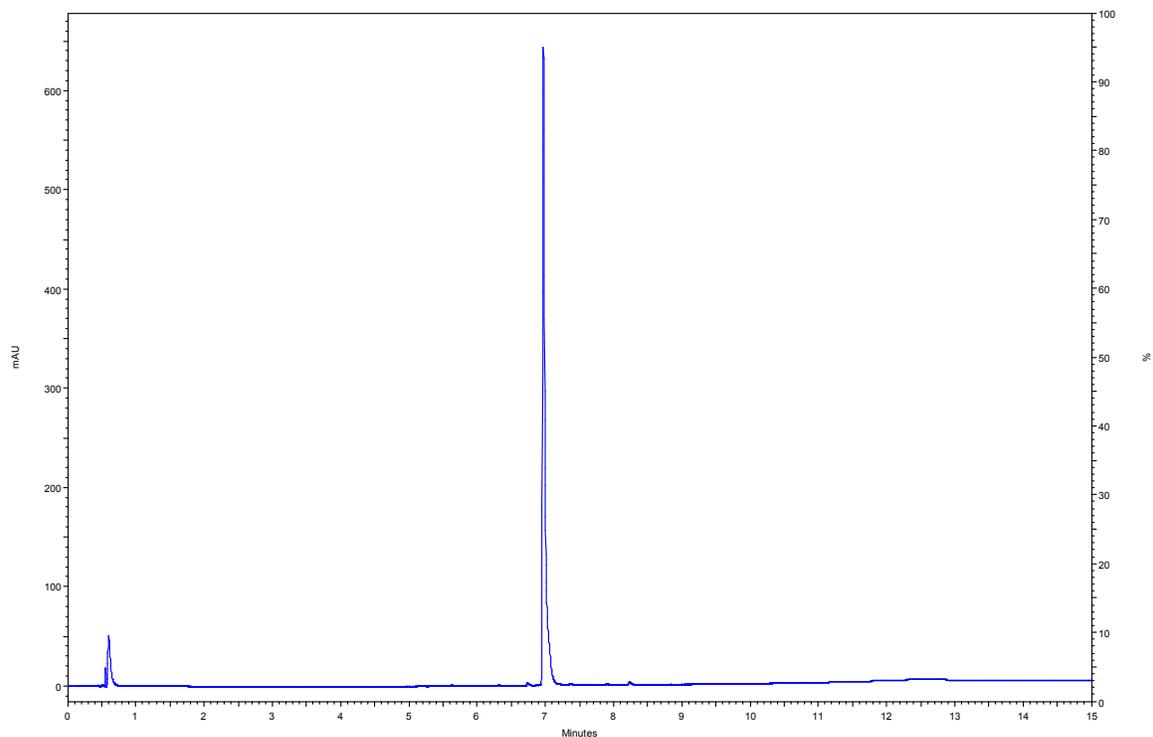
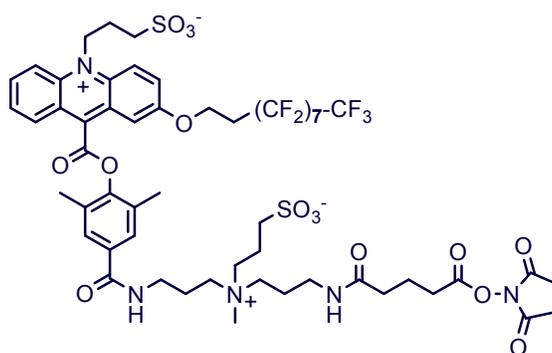
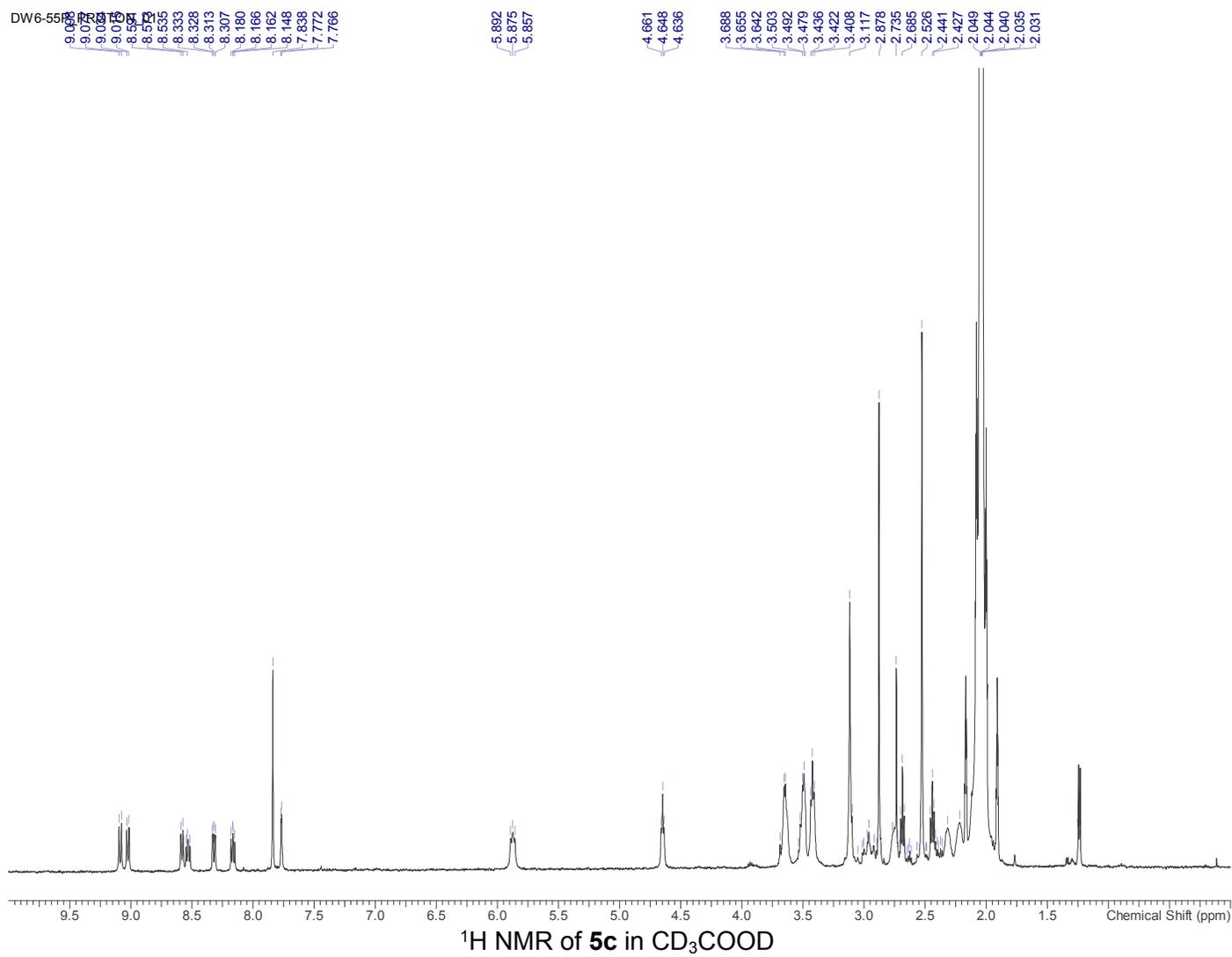


Figure S12. HPLC trace of compound **5c**. HPLC analysis was performed using a Phenomenex, Kinetex XB-C18, 2.6 micron, 4.6 x 50 mm column and a 10 minute gradient of 10 → 90% MeCN/water (each with 0.05% trifluoroacetic acid) followed by a 1.2 minute gradient of 90 → 100% MeCN/water (each with 0.05% trifluoroacetic acid) followed by 100% MeCN (with 0.05% trifluoroacetic acid) for 3.8 minutes.



**5c**



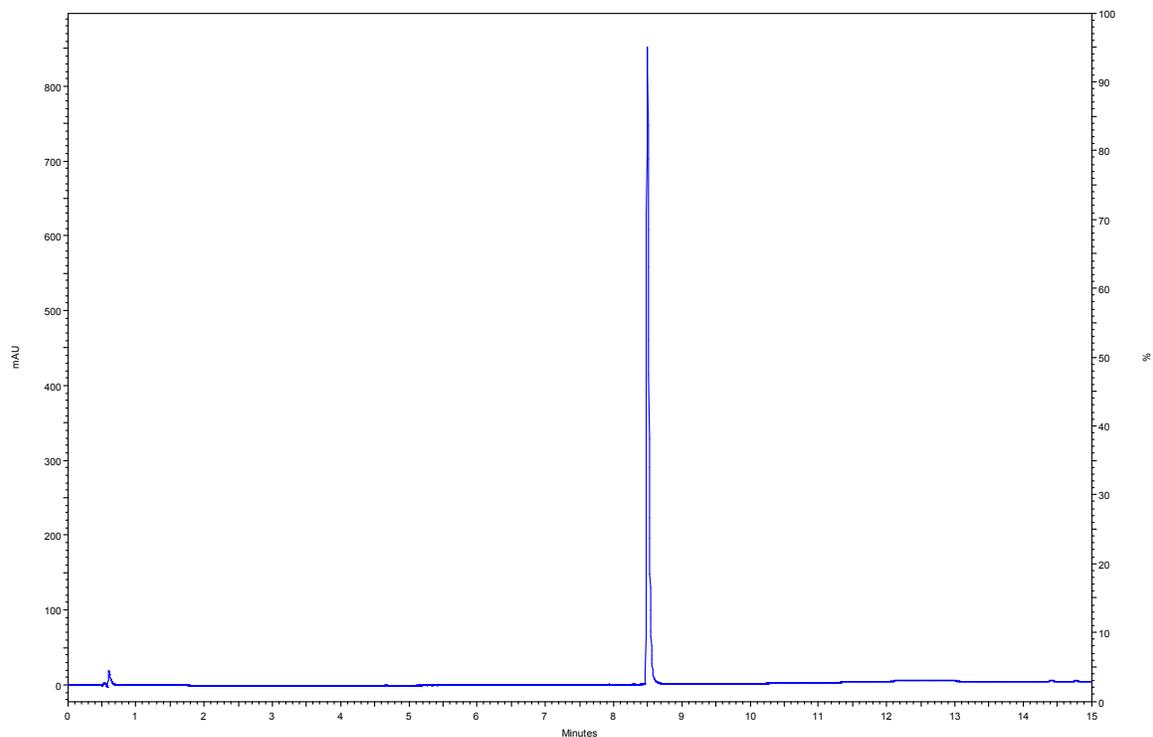
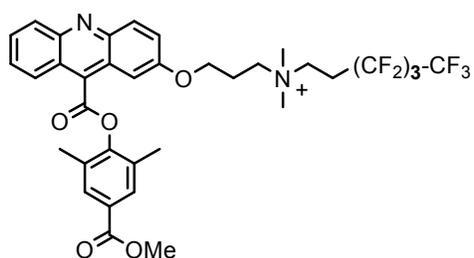


Figure S13. HPLC trace of compound **7a**. HPLC analysis was performed using a Phenomenex, Kinetex XB-C18, 2.6 micron, 4.6 x 50 mm column and a 10 minute gradient of 10 → 90% MeCN/water (each with 0.05% trifluoroacetic acid) followed by a 1.2 minute gradient of 90 → 100% MeCN/water (each with 0.05% trifluoroacetic acid) followed by 100% MeCN (with 0.05% trifluoroacetic acid) for 3.8 minutes.



**7a**



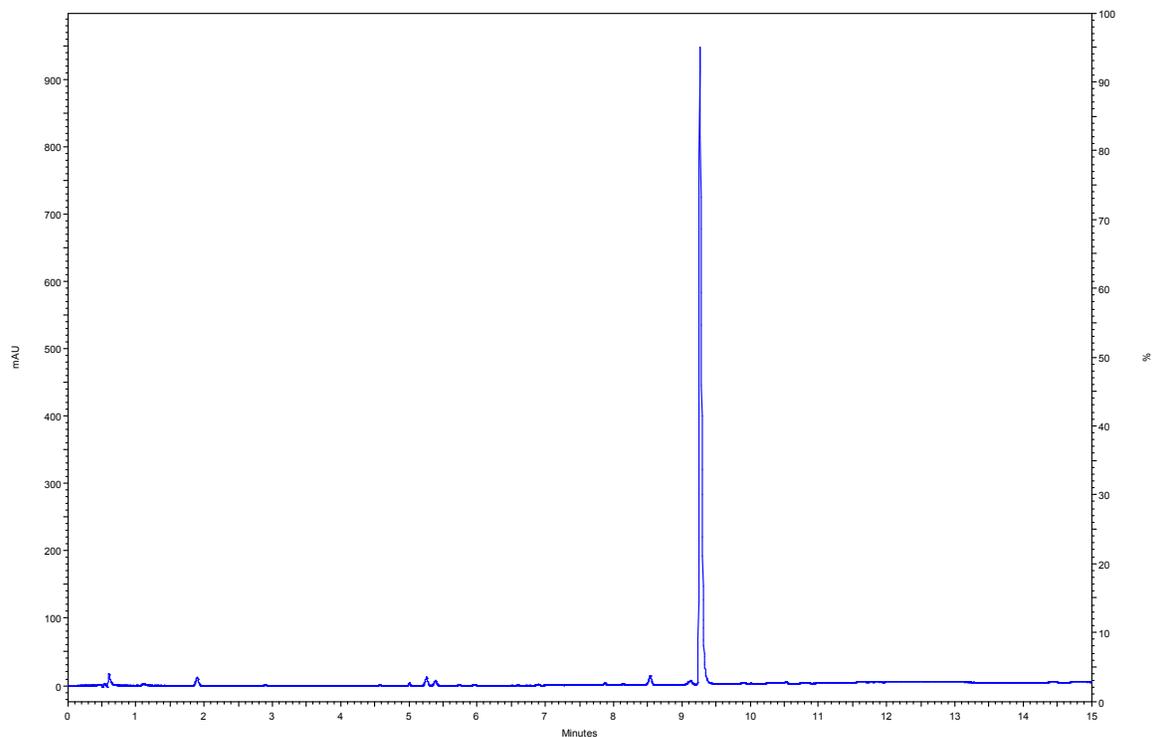
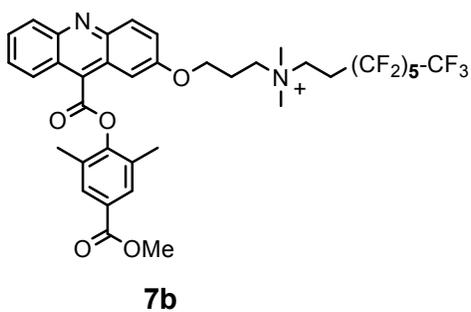
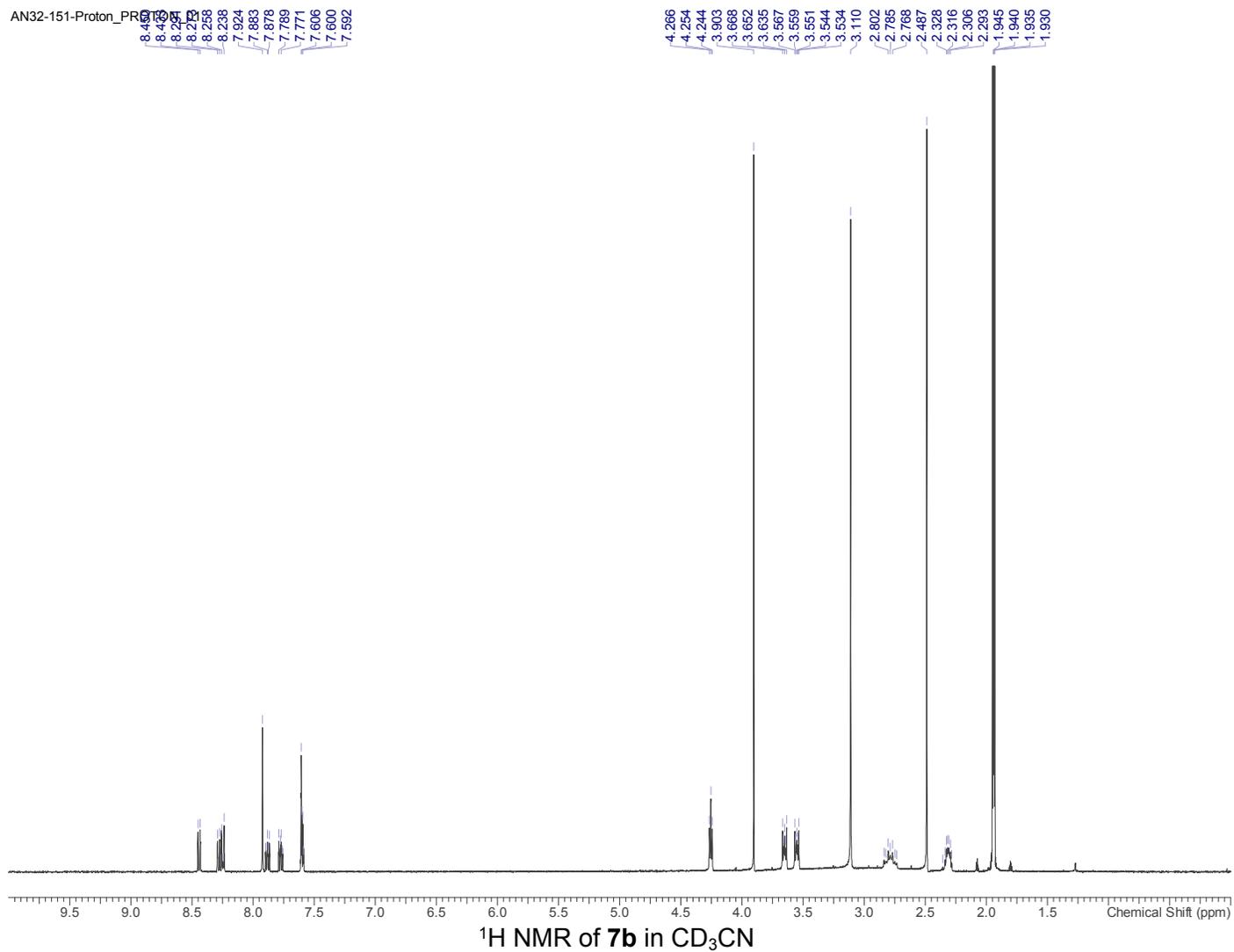


Figure S14. HPLC trace of compound **7b**. HPLC analysis was performed using a Phenomenex, Kinetex XB-C18, 2.6 micron, 4.6 x 50 mm column and a 10 minute gradient of 10 → 90% MeCN/water (each with 0.05% trifluoroacetic acid) followed by a 1.2 minute gradient of 90 → 100% MeCN/water (each with 0.05% trifluoroacetic acid) followed by 100% MeCN (with 0.05% trifluoroacetic acid) for 3.8 minutes.





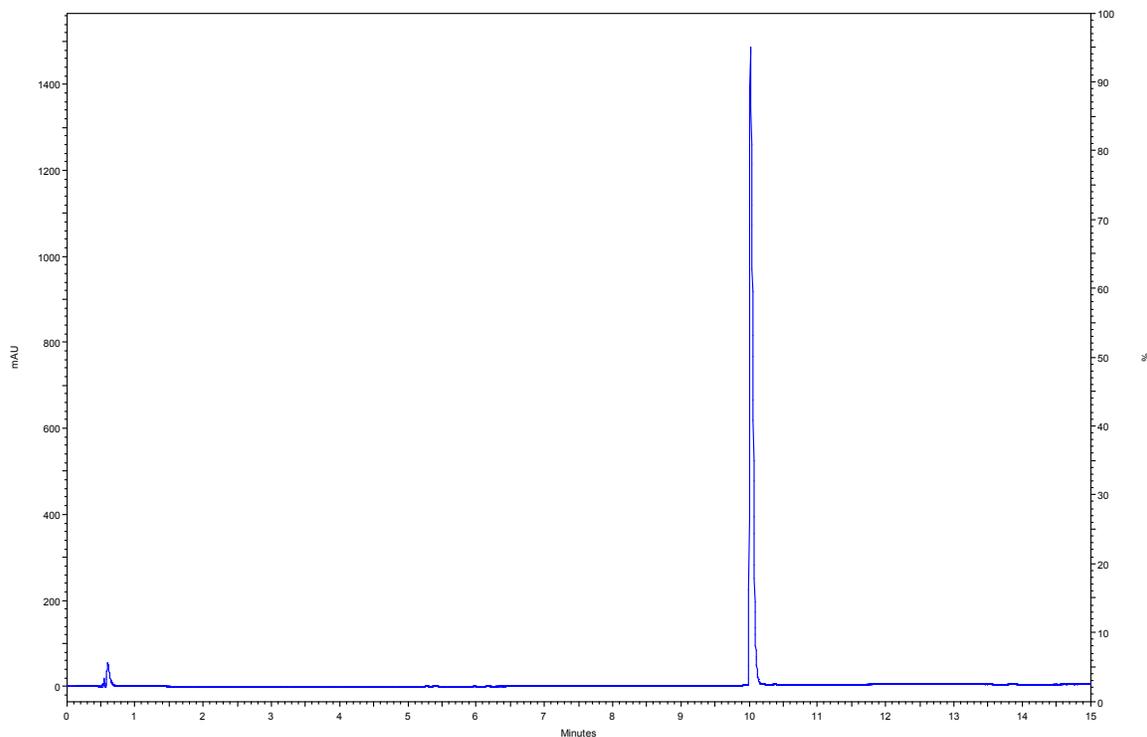
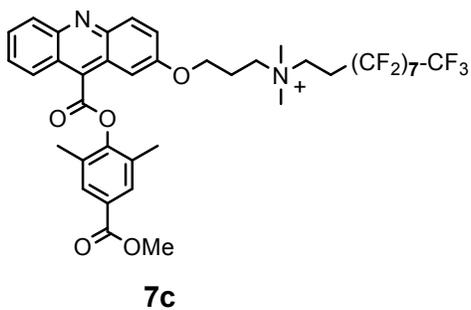
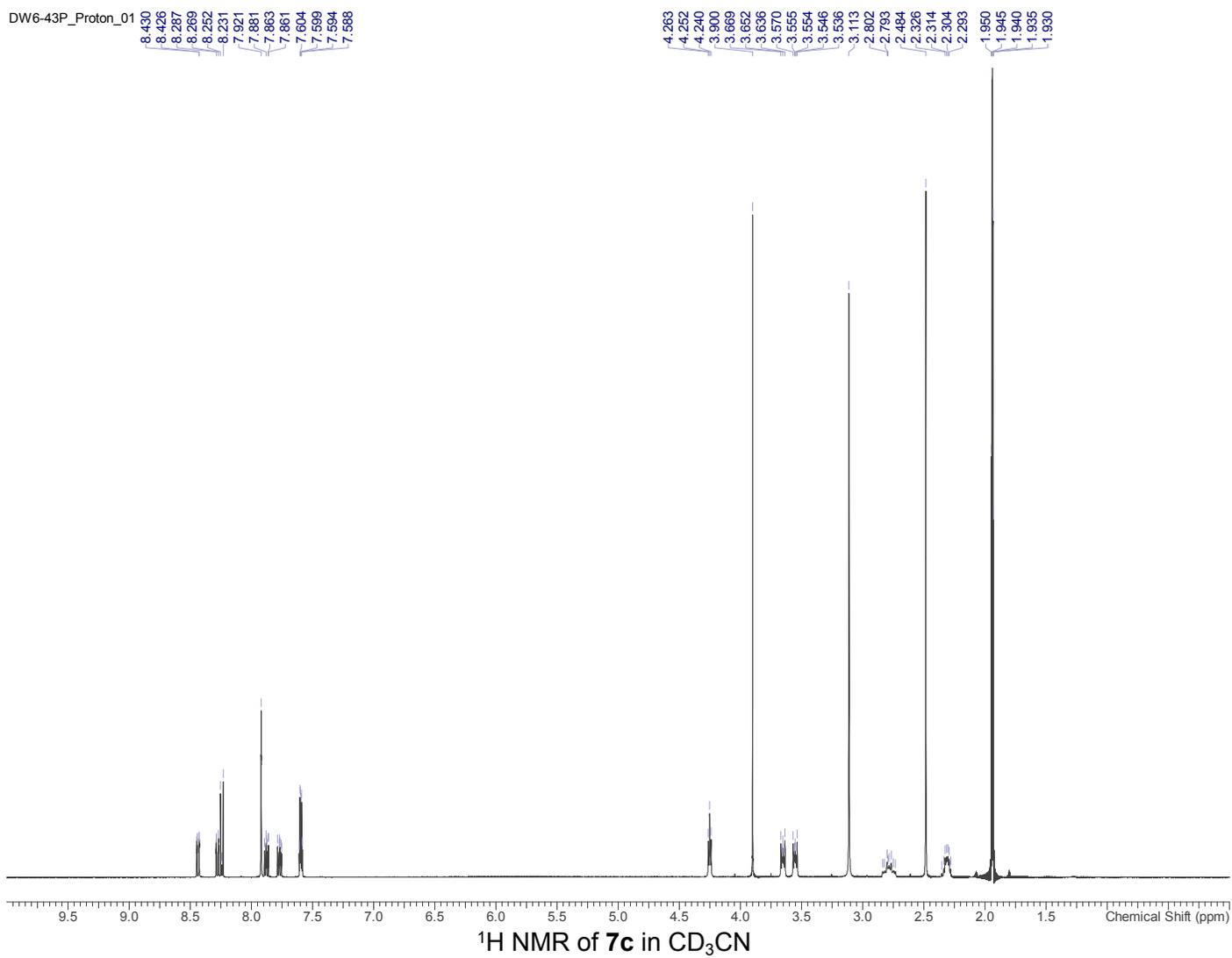


Figure S15. HPLC trace of compound **7c**. HPLC analysis was performed using a Phenomenex, Kinetex XB-C18, 2.6 micron, 4.6 x 50 mm column and a 10 minute gradient of 10 → 90% MeCN/water (each with 0.05% trifluoroacetic acid) followed by a 1.2 minute gradient of 90 → 100% MeCN/water (each with 0.05% trifluoroacetic acid) followed by 100% MeCN (with 0.05% trifluoroacetic acid) for 3.8 minutes.



DW6-43P\_Proton\_01



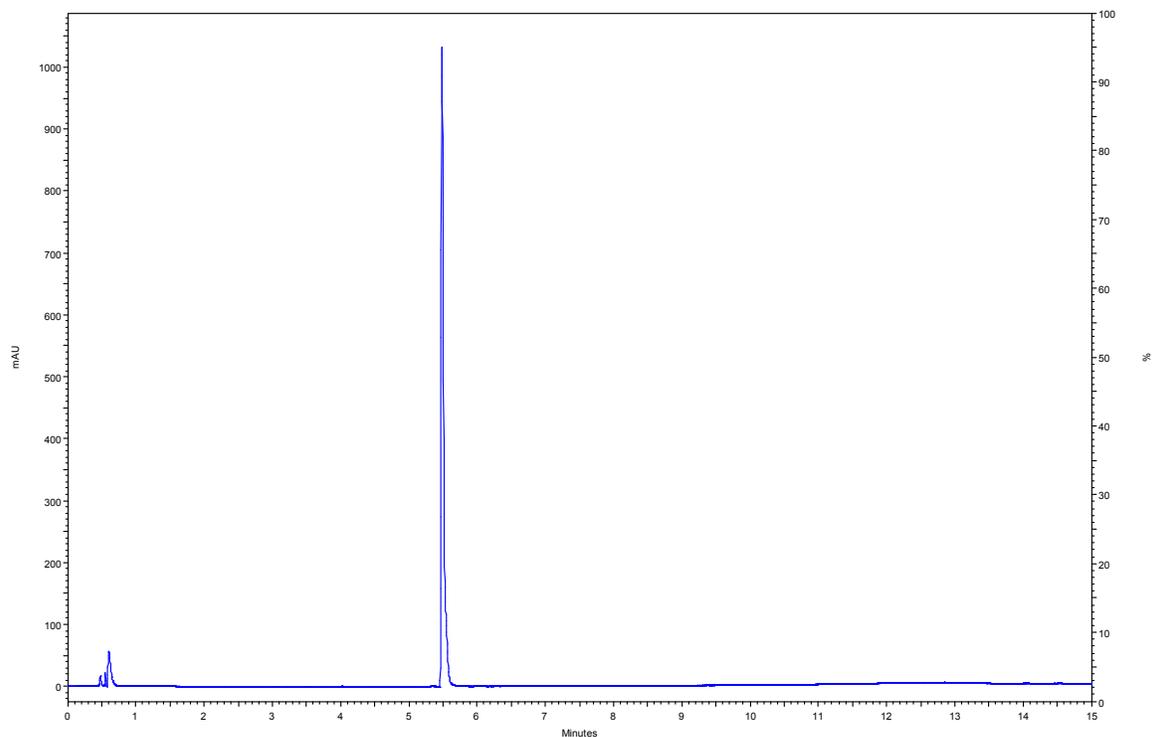
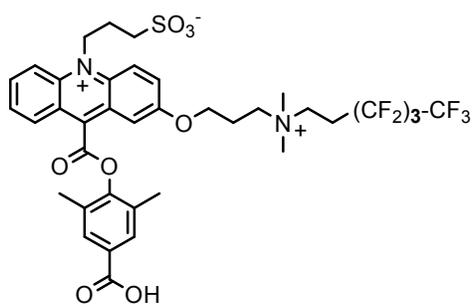
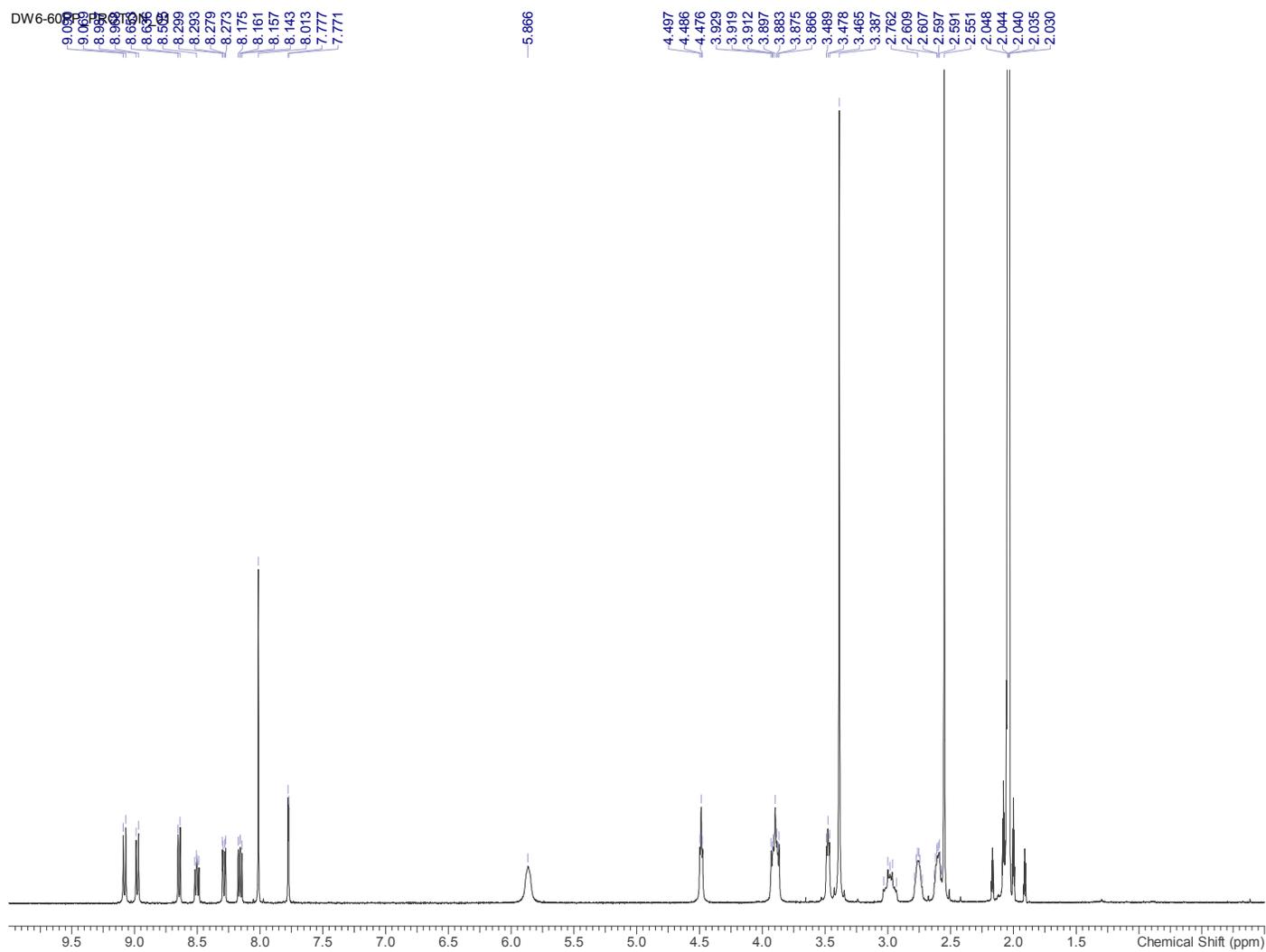


Figure S16. HPLC trace of compound **8a**. HPLC analysis was performed using a Phenomenex, Kinetex XB-C18, 2.6 micron, 4.6 x 50 mm column and a 10 minute gradient of 10 → 90% MeCN/water (each with 0.05% trifluoroacetic acid) followed by a 1.2 minute gradient of 90 → 100% MeCN/water (each with 0.05% trifluoroacetic acid) followed by 100% MeCN (with 0.05% trifluoroacetic acid) for 3.8 minutes.



**8a**



$^1\text{H}$  NMR of **8a** in  $\text{CD}_3\text{COOD}$

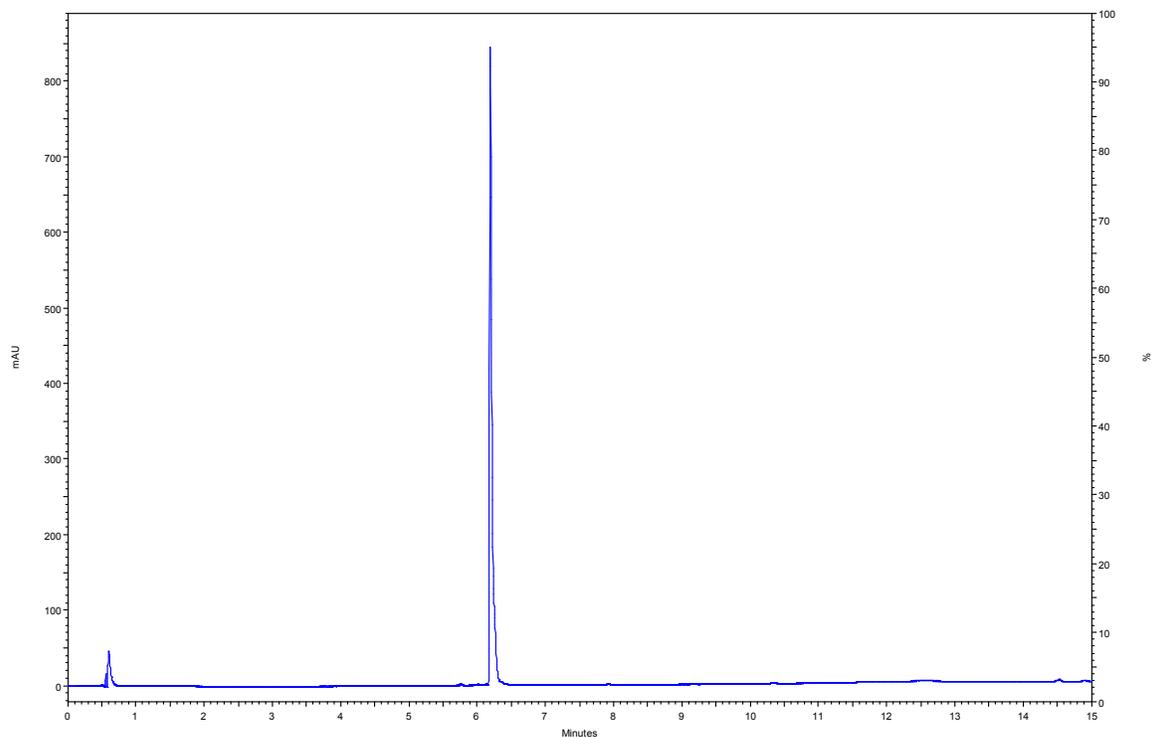
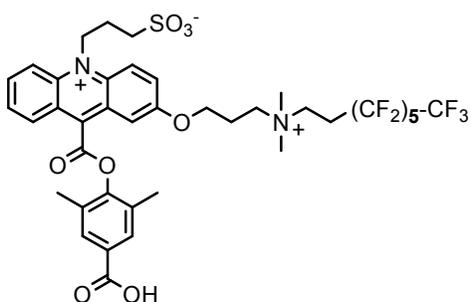
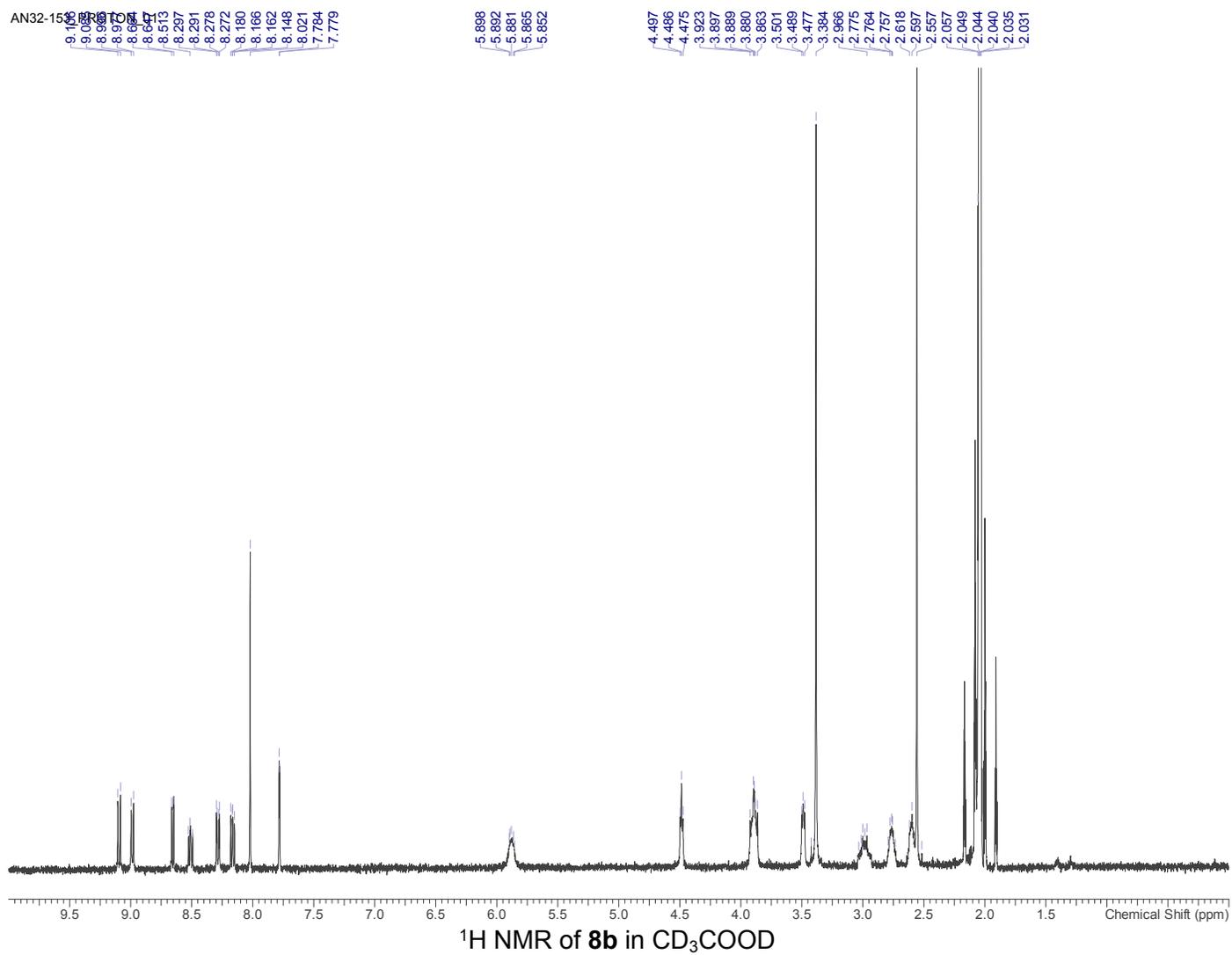


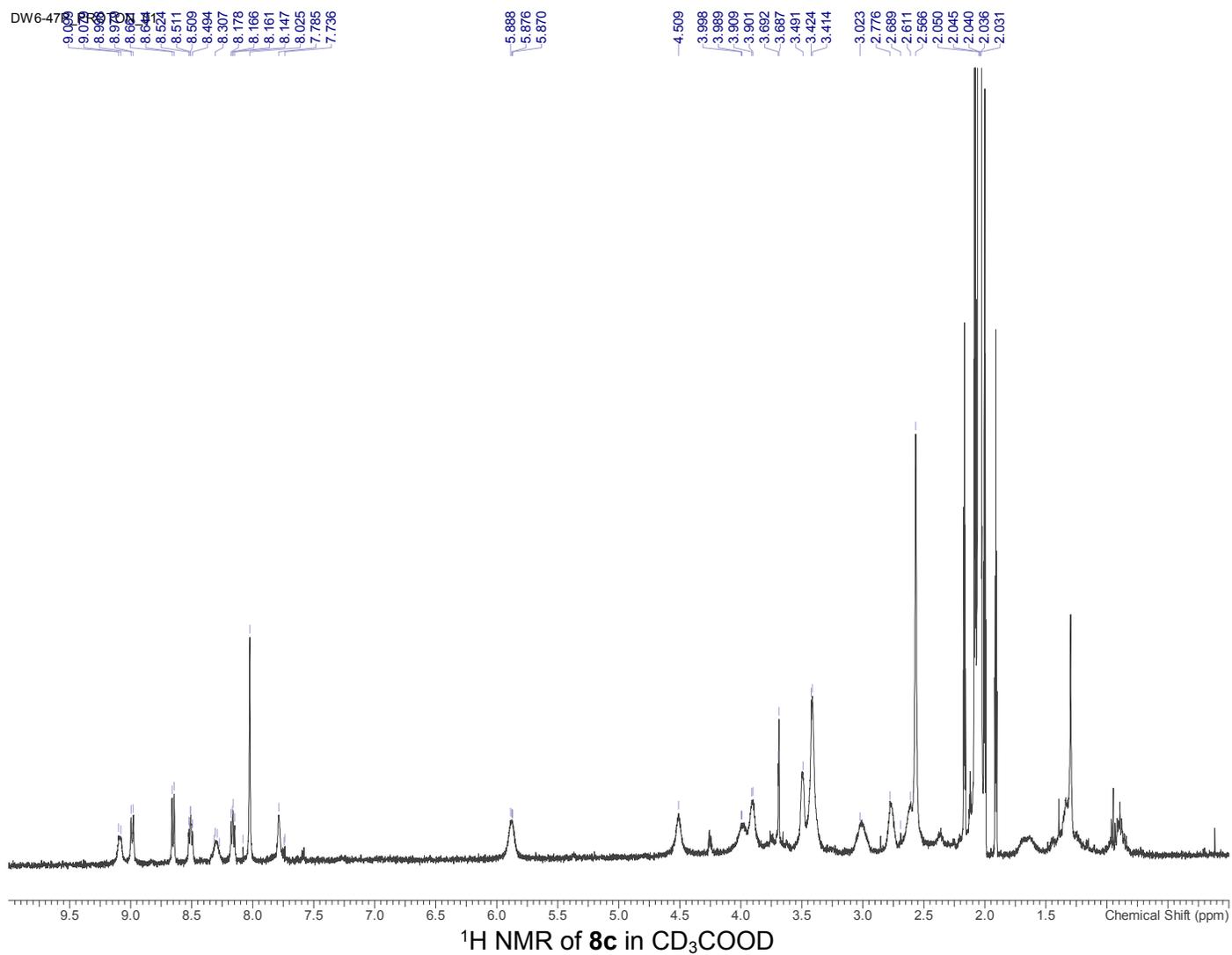
Figure S17. HPLC trace of compound **8b**. HPLC analysis was performed using a Phenomenex, Kinetex XB-C18, 2.6 micron, 4.6 x 50 mm column and a 10 minute gradient of 10 → 90% MeCN/water (each with 0.05% trifluoroacetic acid) followed by a 1.2 minute gradient of 90 → 100% MeCN/water (each with 0.05% trifluoroacetic acid) followed by 100% MeCN (with 0.05% trifluoroacetic acid) for 3.8 minutes.



**8b**







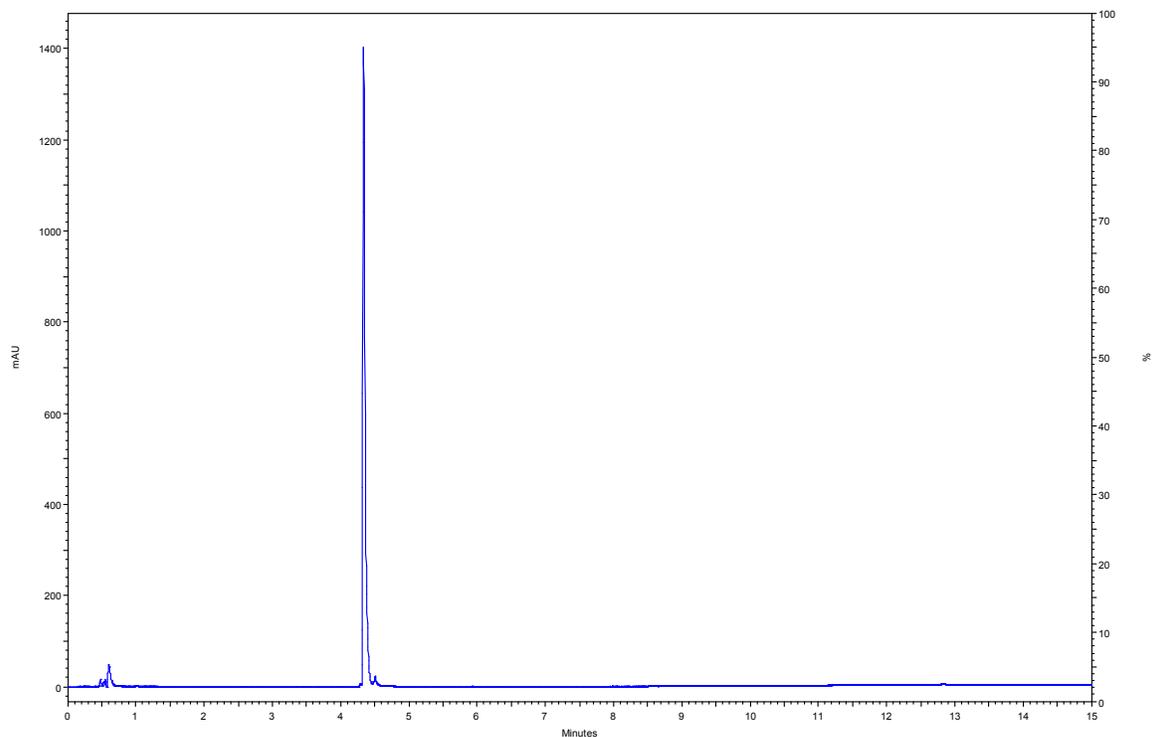
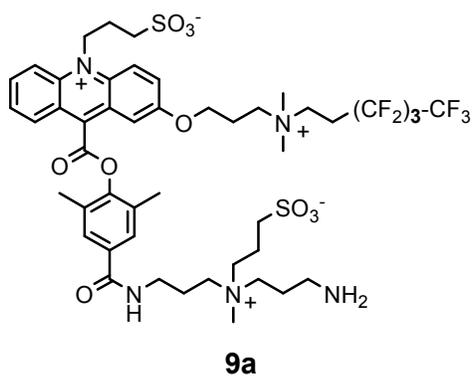
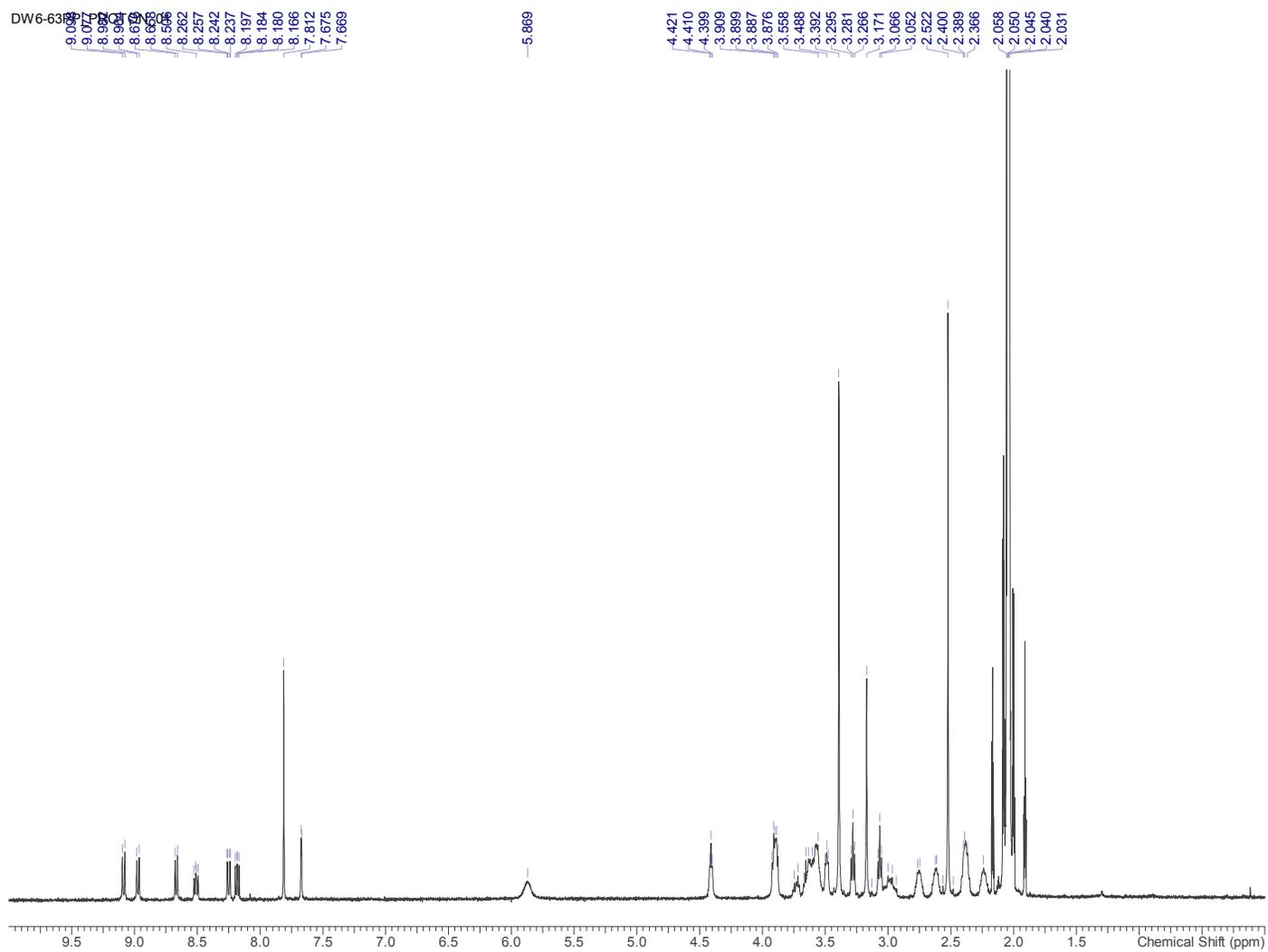


Figure S19. HPLC trace of compound **9a**. HPLC analysis was performed using a Phenomenex, Kinetex XB-C18, 2.6 micron, 4.6 x 50 mm column and a 10 minute gradient of 10 → 90% MeCN/water (each with 0.05% trifluoroacetic acid) followed by a 1.2 minute gradient of 90 → 100% MeCN/water (each with 0.05% trifluoroacetic acid) followed by 100% MeCN (with 0.05% trifluoroacetic acid) for 3.8 minutes.





$^1\text{H}$  NMR of **9a** in  $\text{CD}_3\text{COOD}$

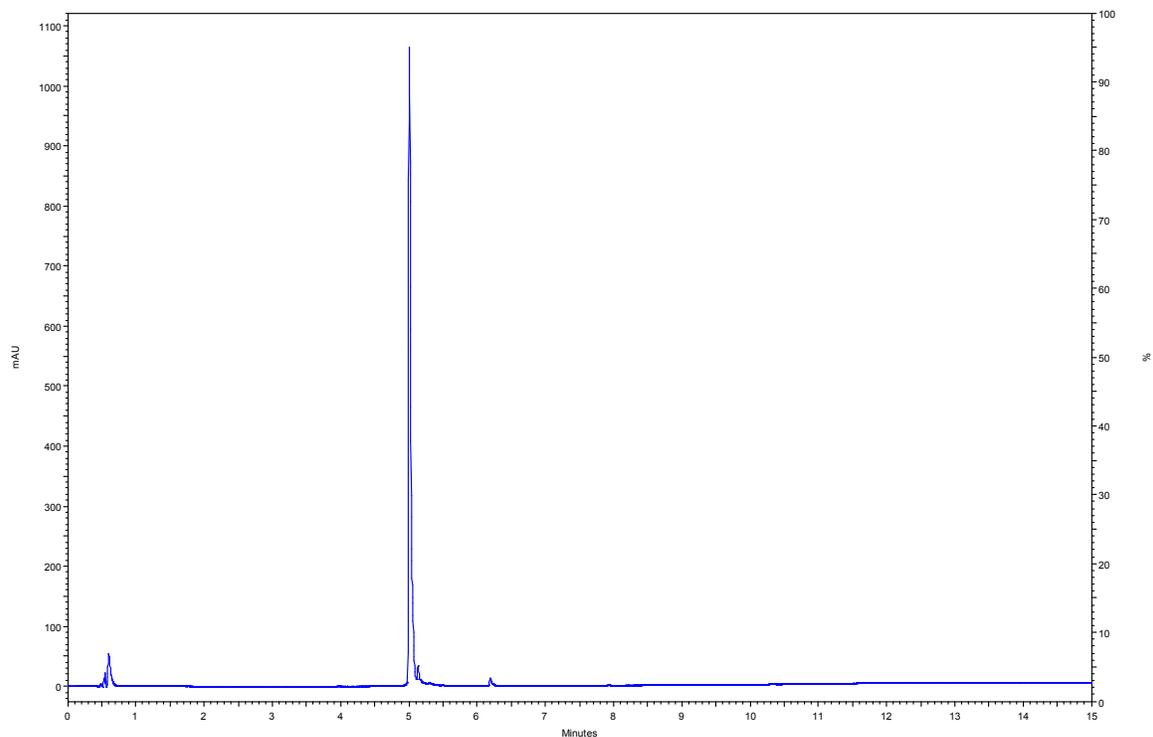
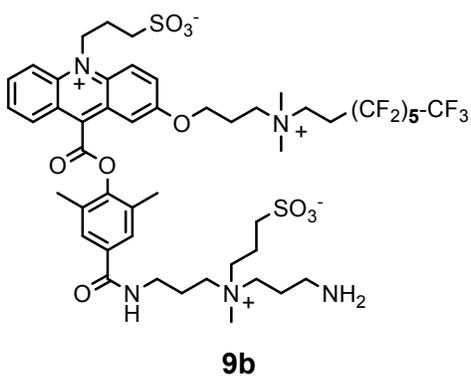
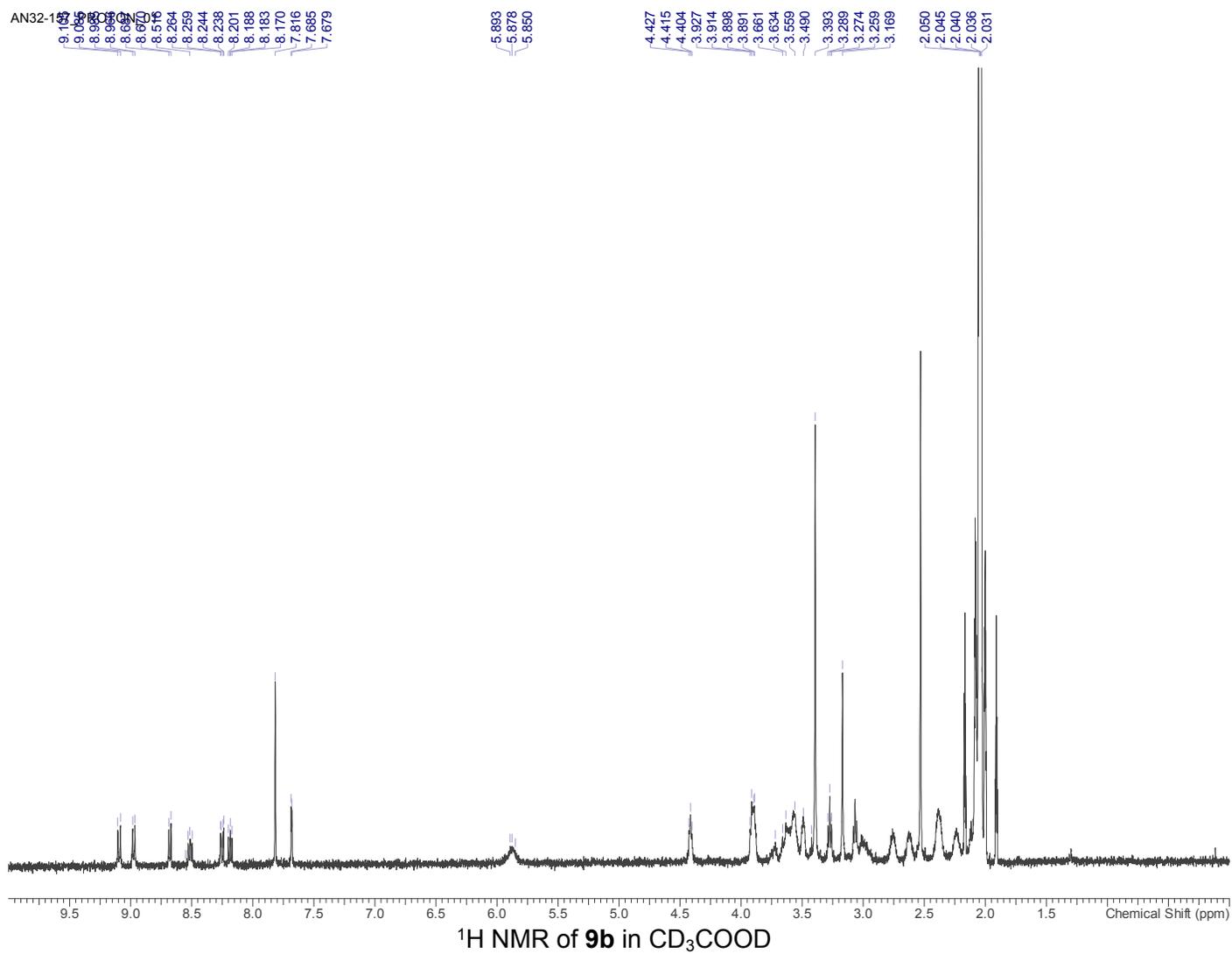


Figure S20. HPLC trace of compound **9b**. HPLC analysis was performed using a Phenomenex, Kinetex XB-C18, 2.6 micron, 4.6 x 50 mm column and a 10 minute gradient of 10 → 90% MeCN/water (each with 0.05% trifluoroacetic acid) followed by a 1.2 minute gradient of 90 → 100% MeCN/water (each with 0.05% trifluoroacetic acid) followed by 100% MeCN (with 0.05% trifluoroacetic acid) for 3.8 minutes.





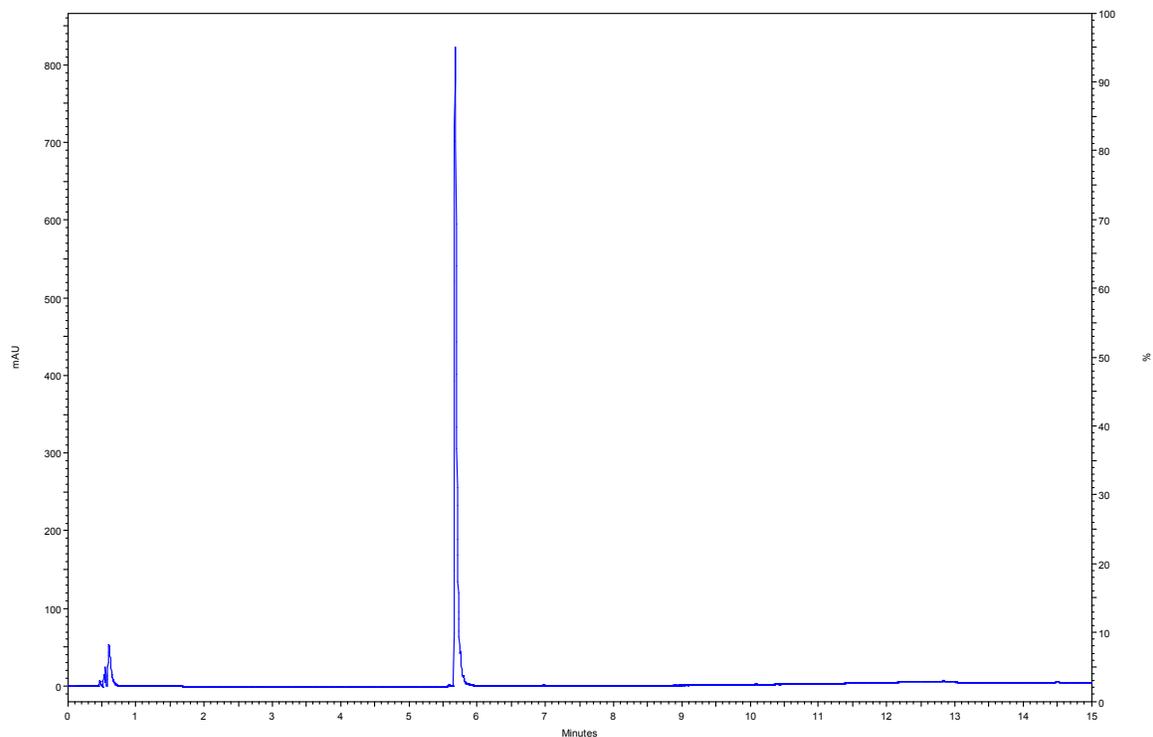
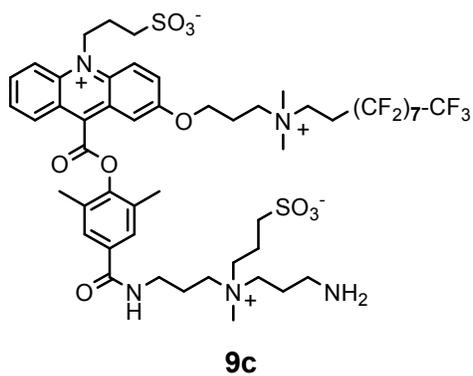
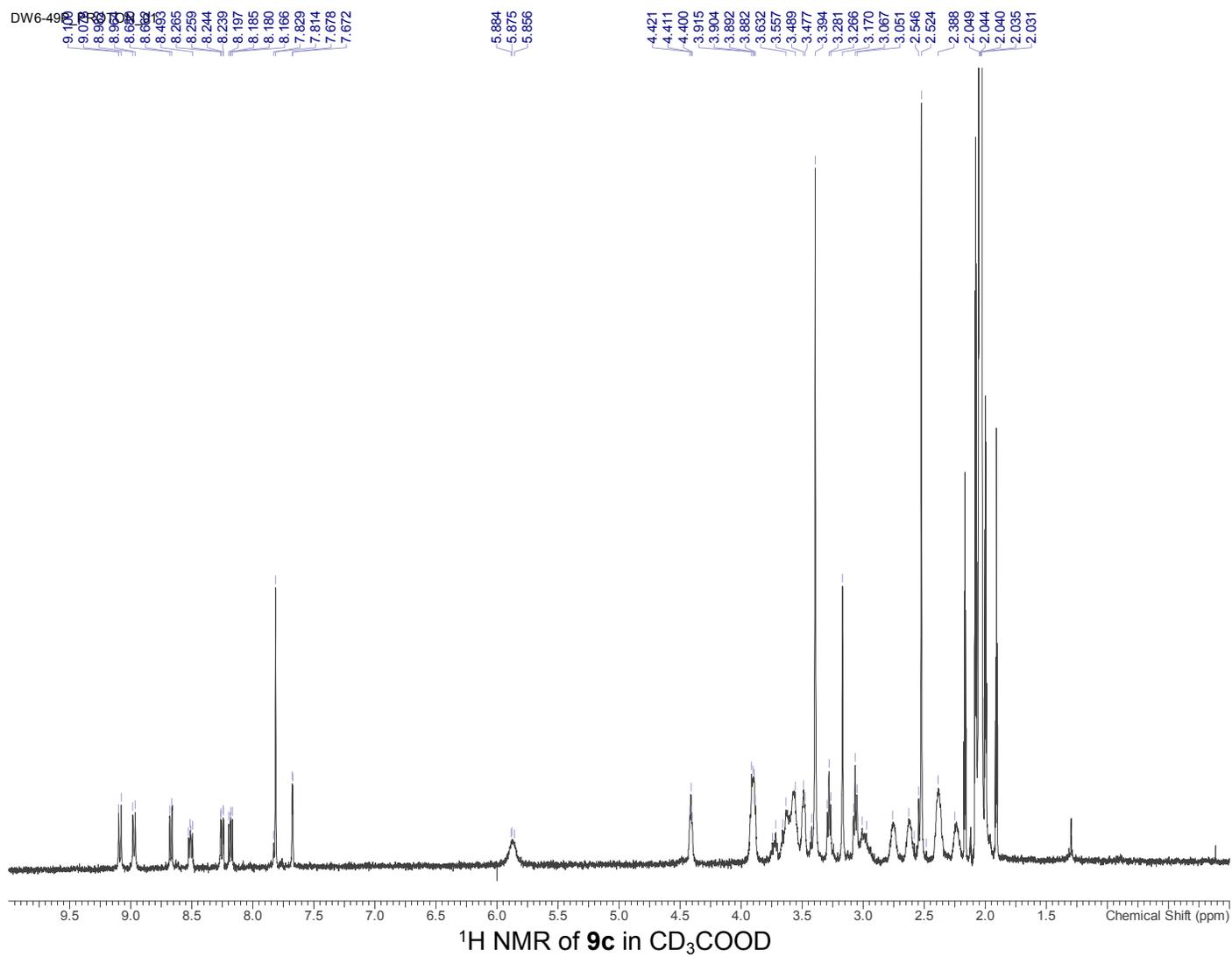
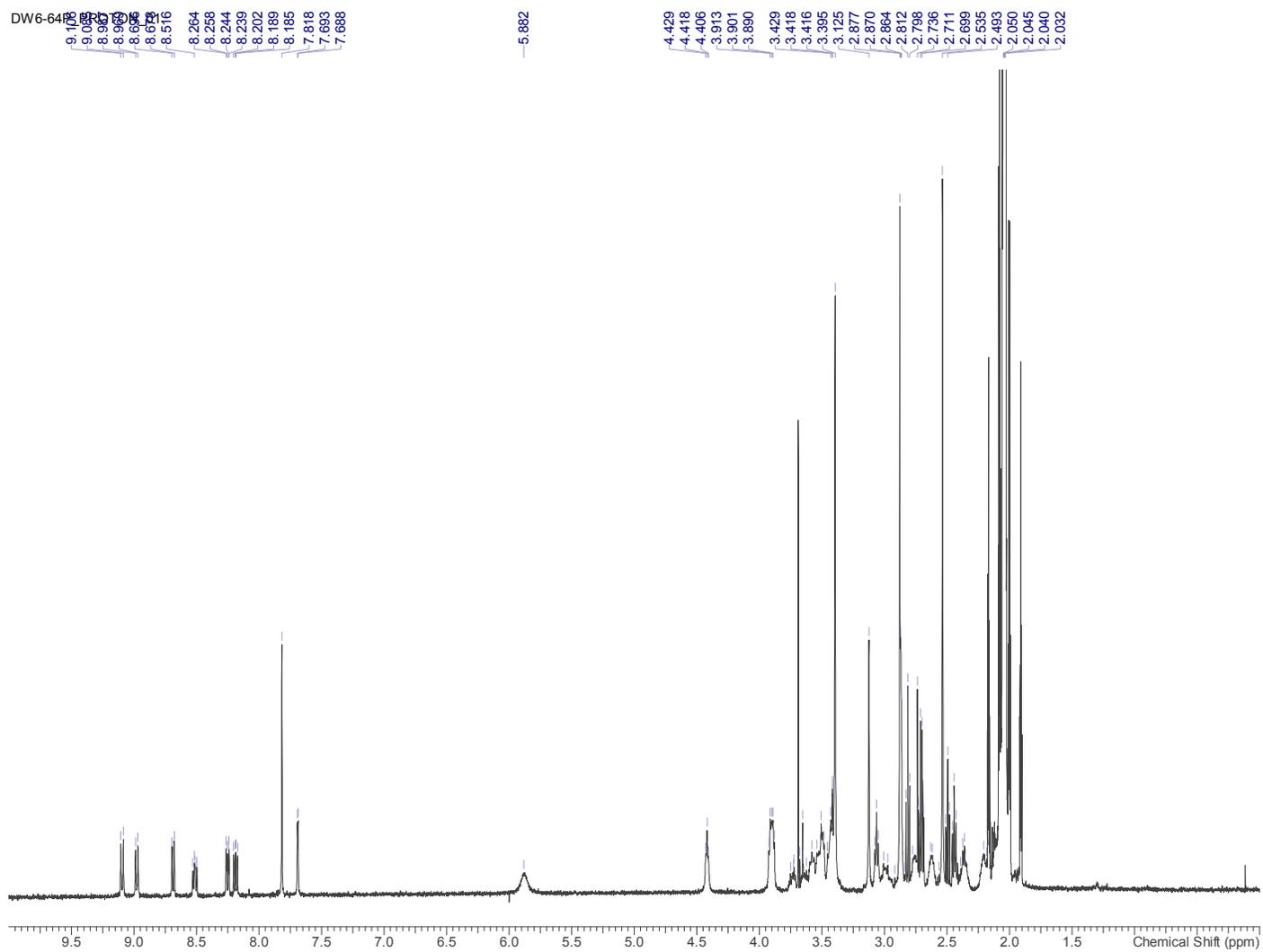


Figure S21. HPLC trace of compound **9c**. HPLC analysis was performed using a Phenomenex, Kinetex XB-C18, 2.6 micron, 4.6 x 50 mm column and a 10 minute gradient of 10 → 90% MeCN/water (each with 0.05% trifluoroacetic acid) followed by a 1.2 minute gradient of 90 → 100% MeCN/water (each with 0.05% trifluoroacetic acid) followed by 100% MeCN (with 0.05% trifluoroacetic acid) for 3.8 minutes.









$^1\text{H}$  NMR of **10a** in  $\text{CD}_3\text{COOD}$

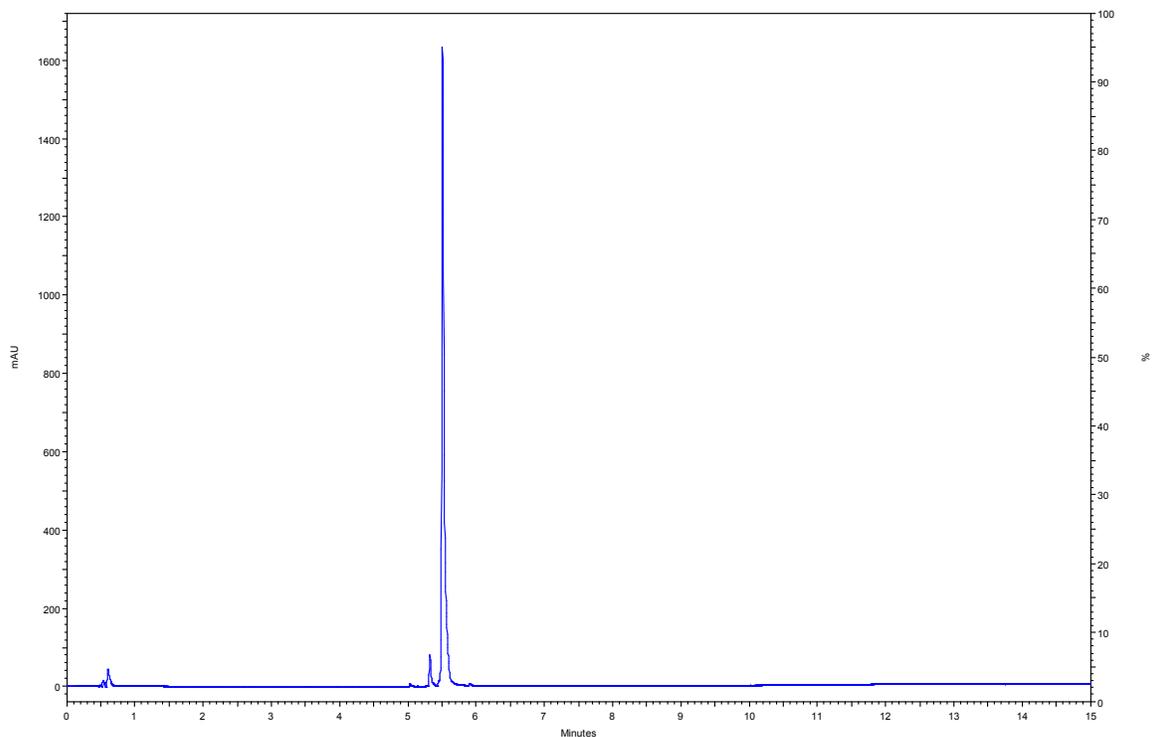
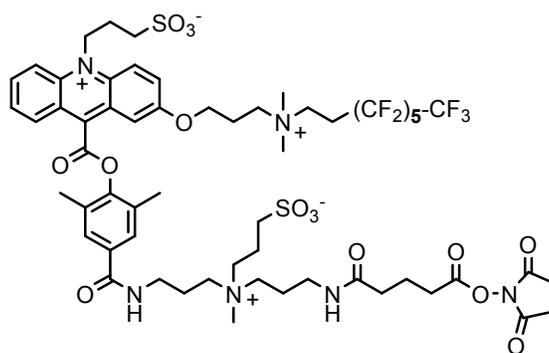
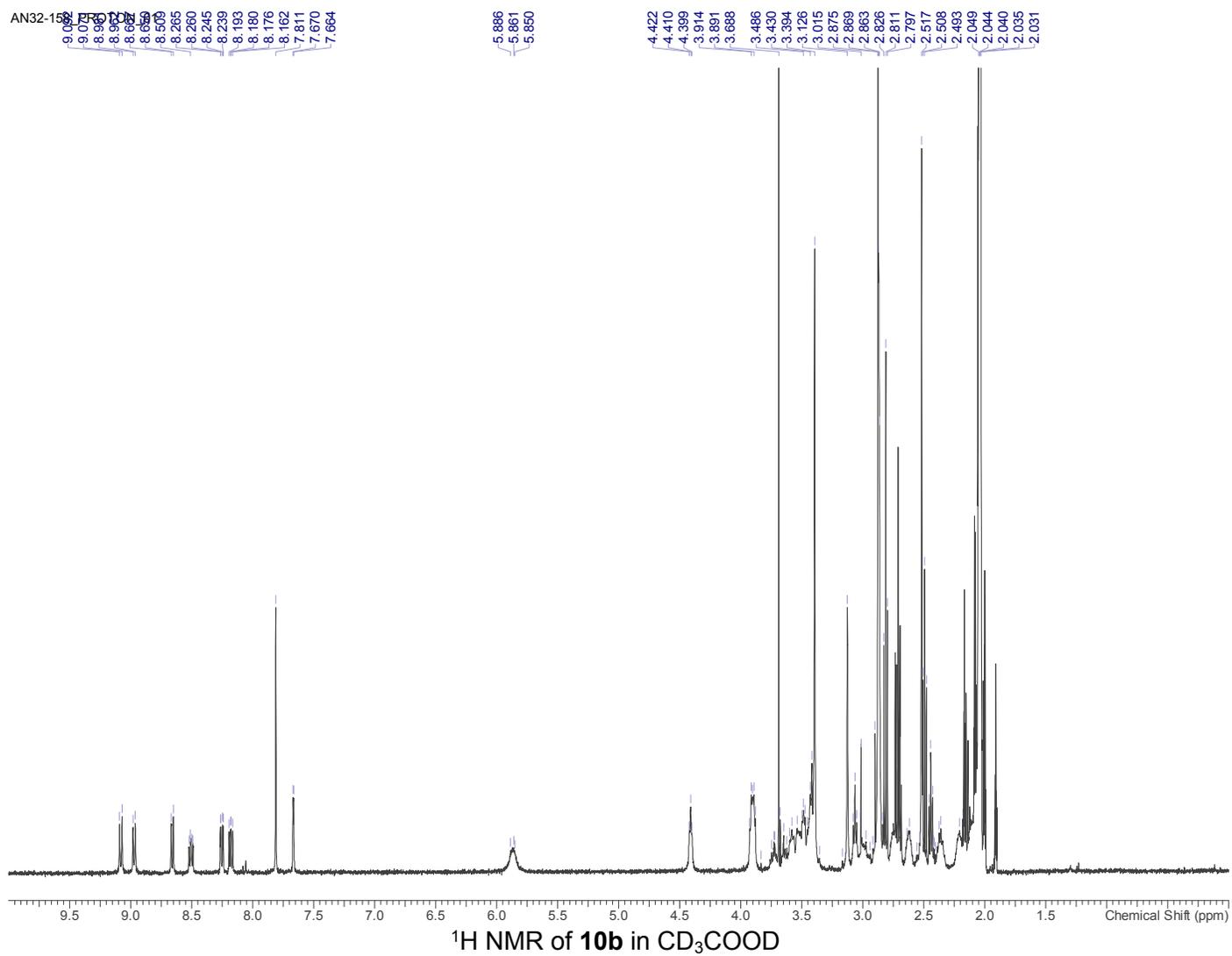


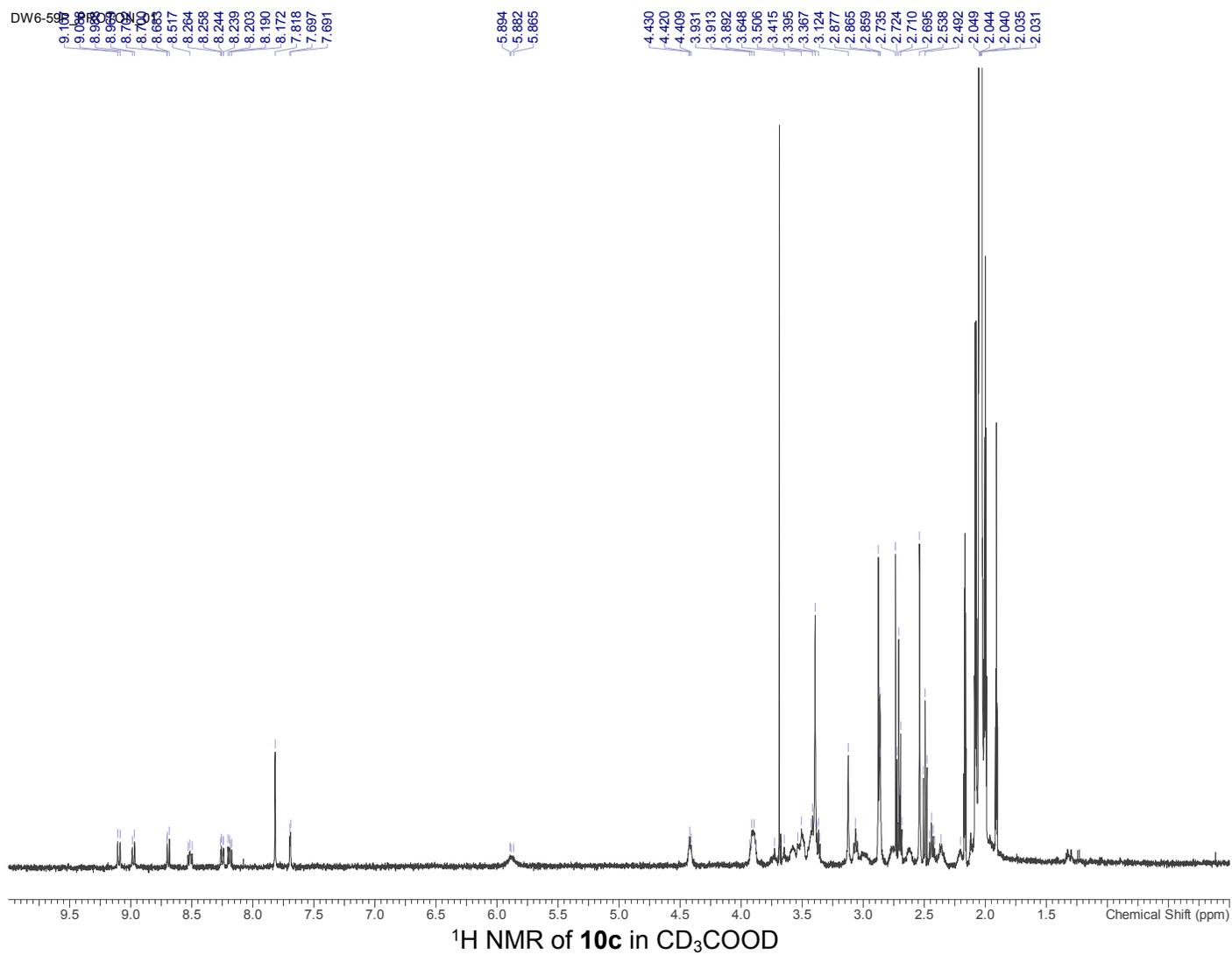
Figure S23. HPLC trace of compound **10b**. HPLC analysis was performed using a Phenomenex, Kinetex XB-C18, 2.6 micron, 4.6 x 50 mm column and a 10 minute gradient of 10 → 90% MeCN/water (each with 0.05% trifluoroacetic acid) followed by a 1.2 minute gradient of 90 → 100% MeCN/water (each with 0.05% trifluoroacetic acid) followed by 100% MeCN (with 0.05% trifluoroacetic acid) for 3.8 minutes.



**10b**







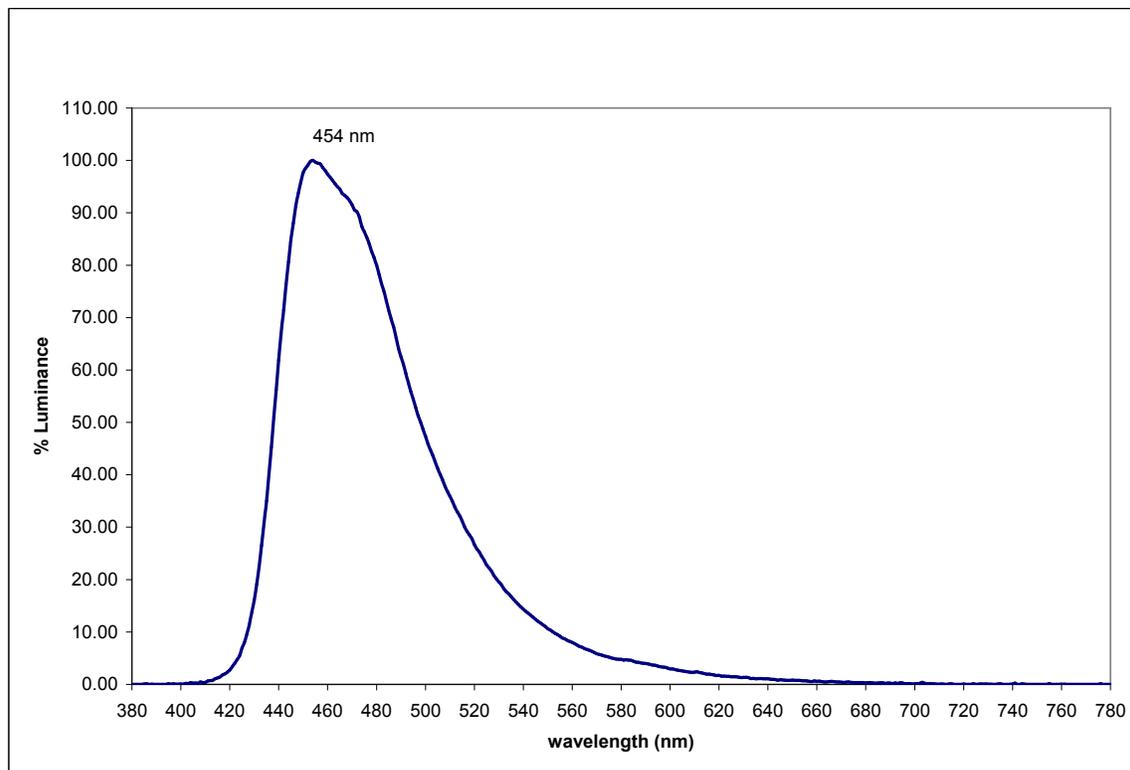
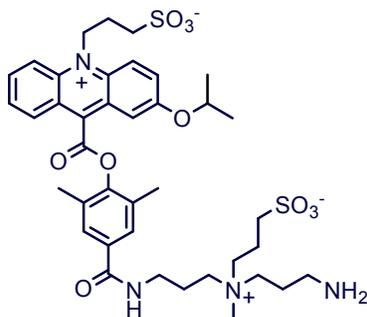


Figure S25. Emission spectrum of amine derivative of compound **11**.





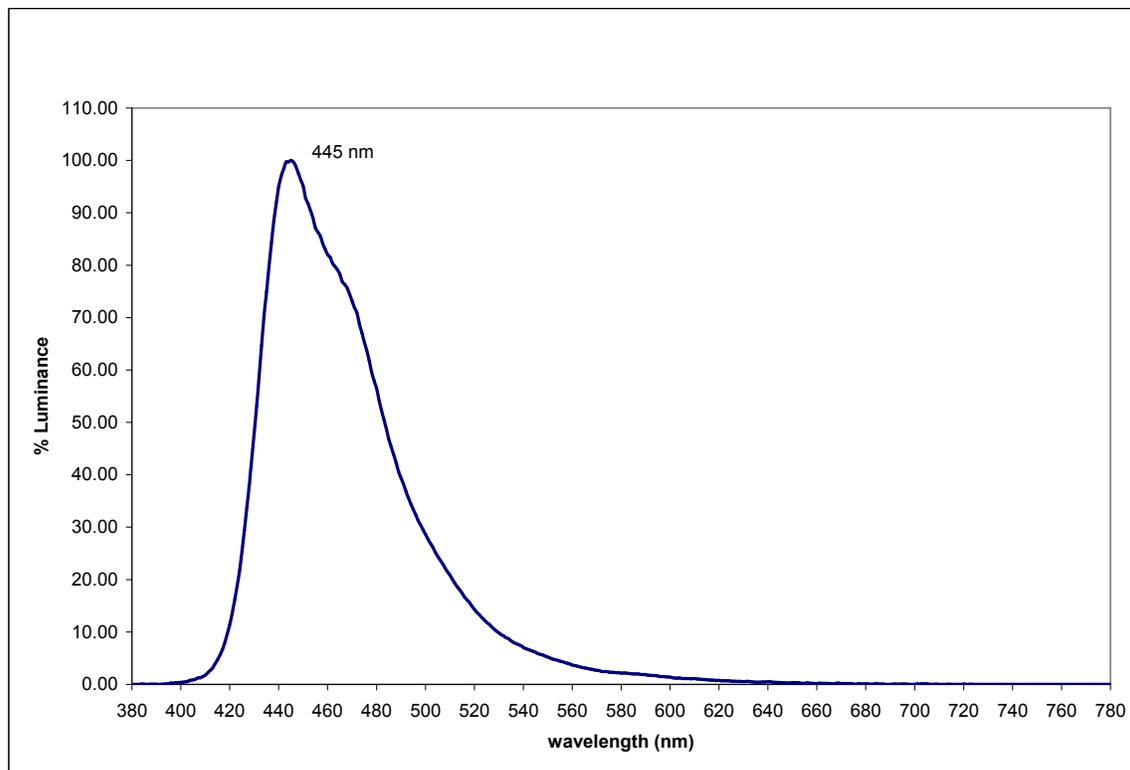
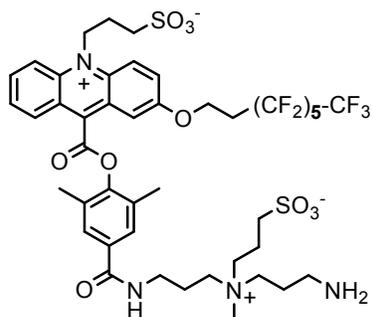


Figure S27. Emission spectrum of compound **4b**.





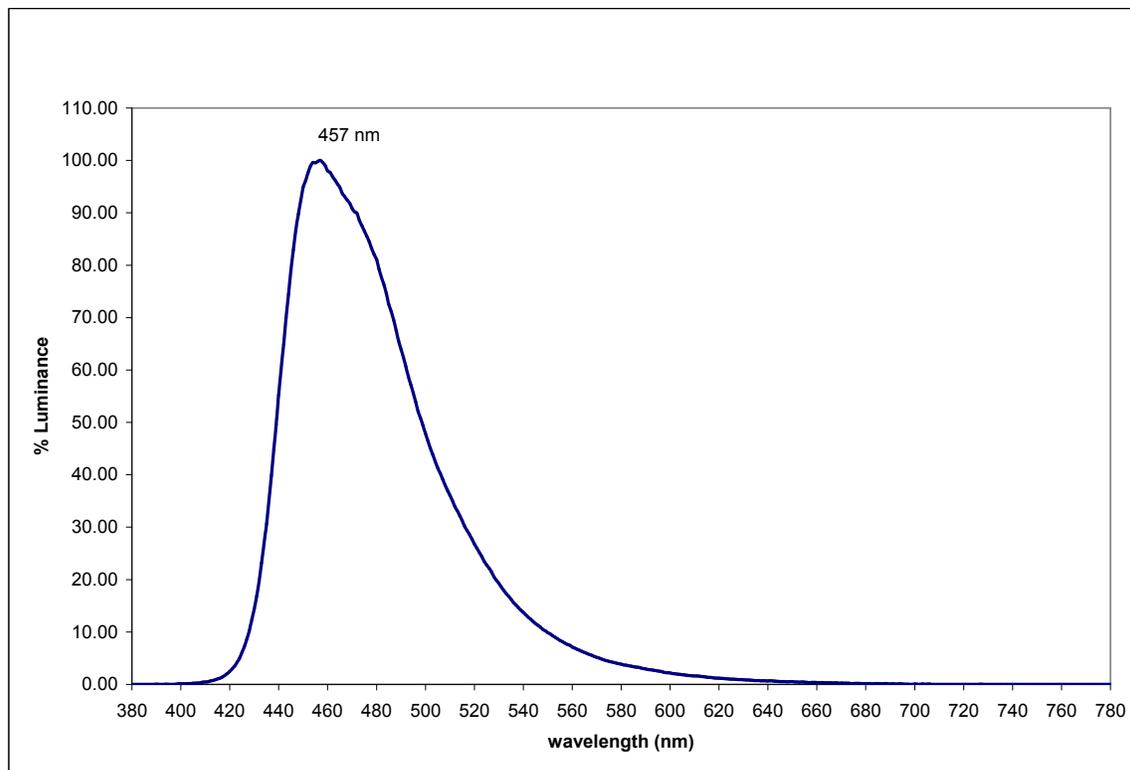
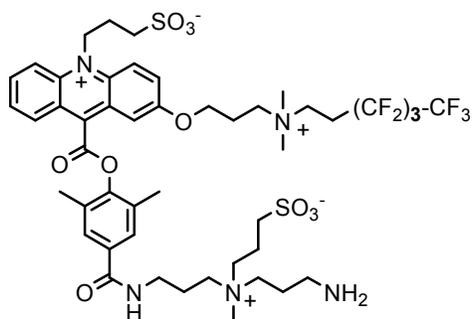


Figure S29. Emission spectrum of compound **9a**.





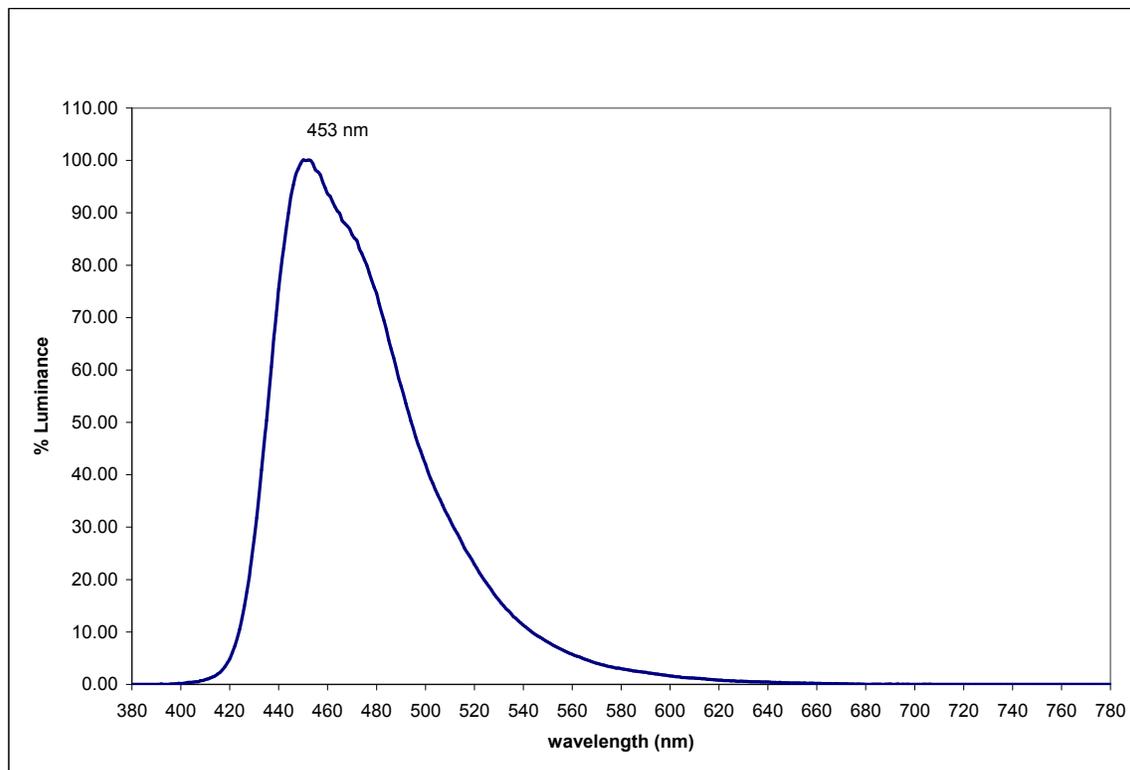
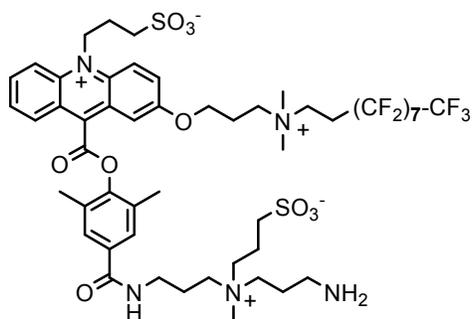


Figure S31. Emission spectrum of compound **9c**.



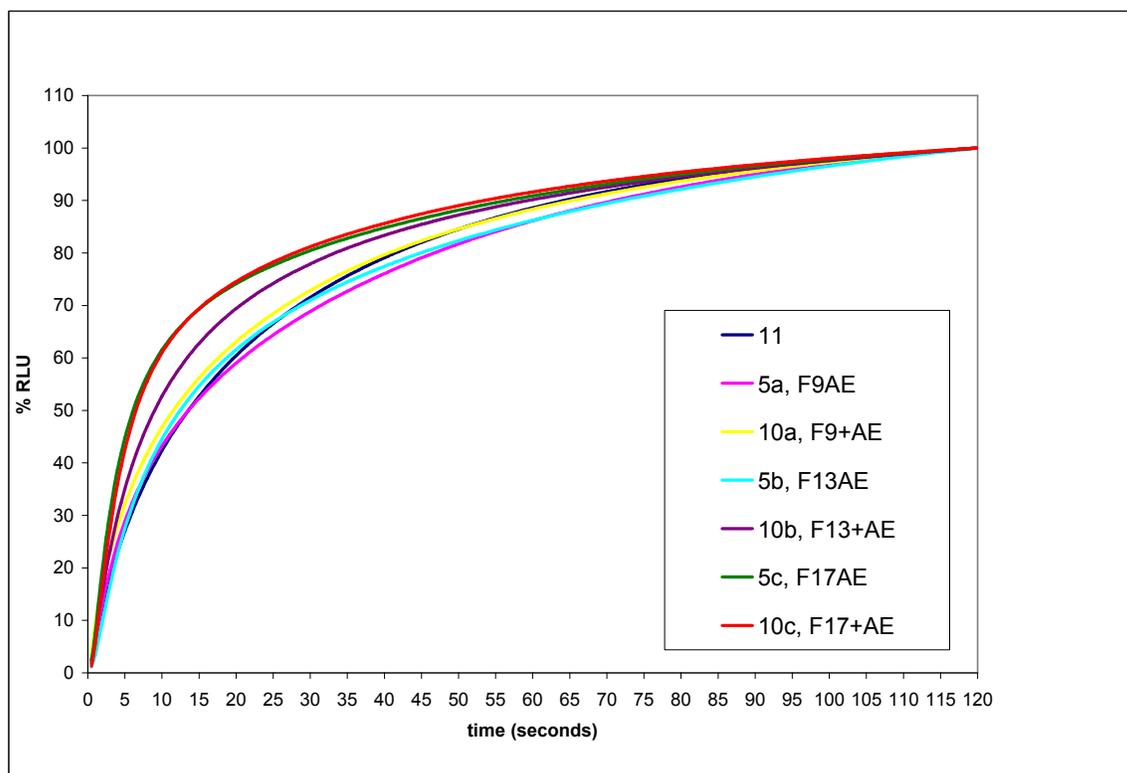


Figure S32. Chemiluminescence emission profiles of BSA conjugates in the absence of surfactant. Reagent 1 = 0.1 M nitric acid + 0.5% hydrogen peroxide; reagent 2 = 0.25 M NaOH. Light emission was triggered by the sequential additional of 0.3 mL reagent 1 followed by 0.3 mL reagent 2. Light was collected for 120 seconds integrated at 0.5 second intervals.

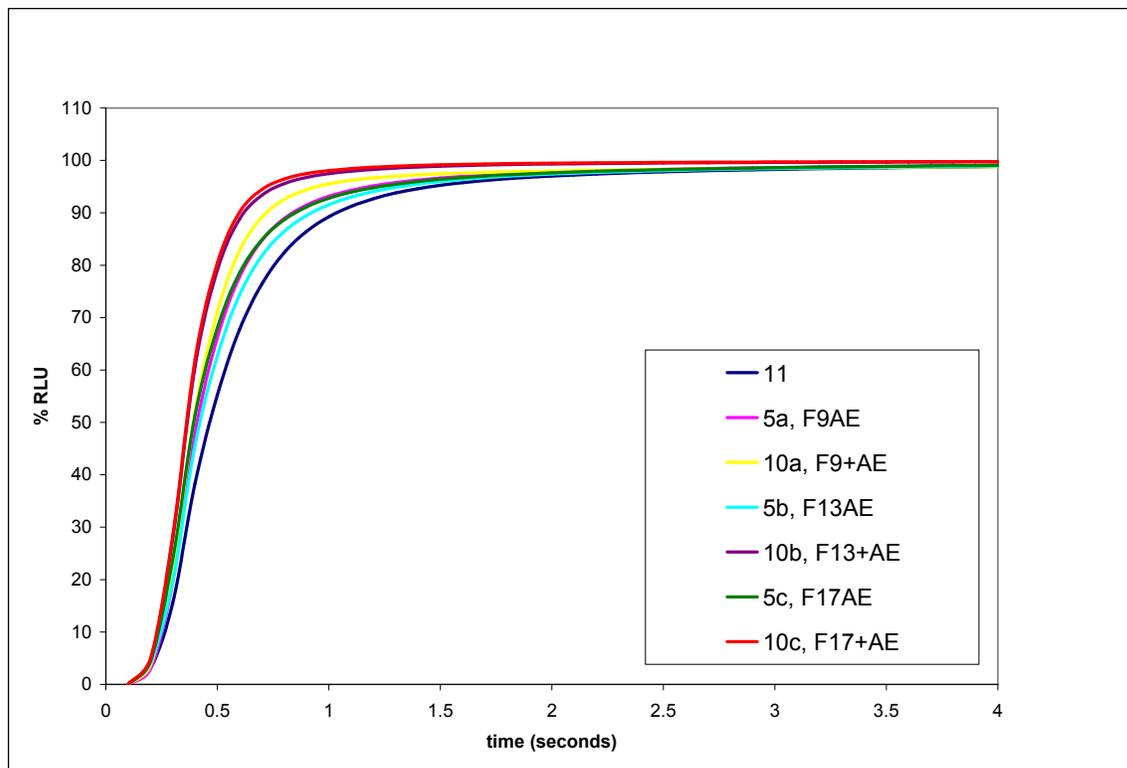


Figure S33. Chemiluminescence emission profiles of BSA conjugates in the presence of CTAC. Reagent 1 = 0.1 M nitric acid + 0.5% hydrogen peroxide; reagent 2 = 7 mM CTAC in 0.25 M NaOH. Light emission was triggered by the sequential additional of 0.3 mL reagent 1 followed by 0.3 mL reagent 2. Light was collected for 20 seconds integrated at 0.1 second intervals.

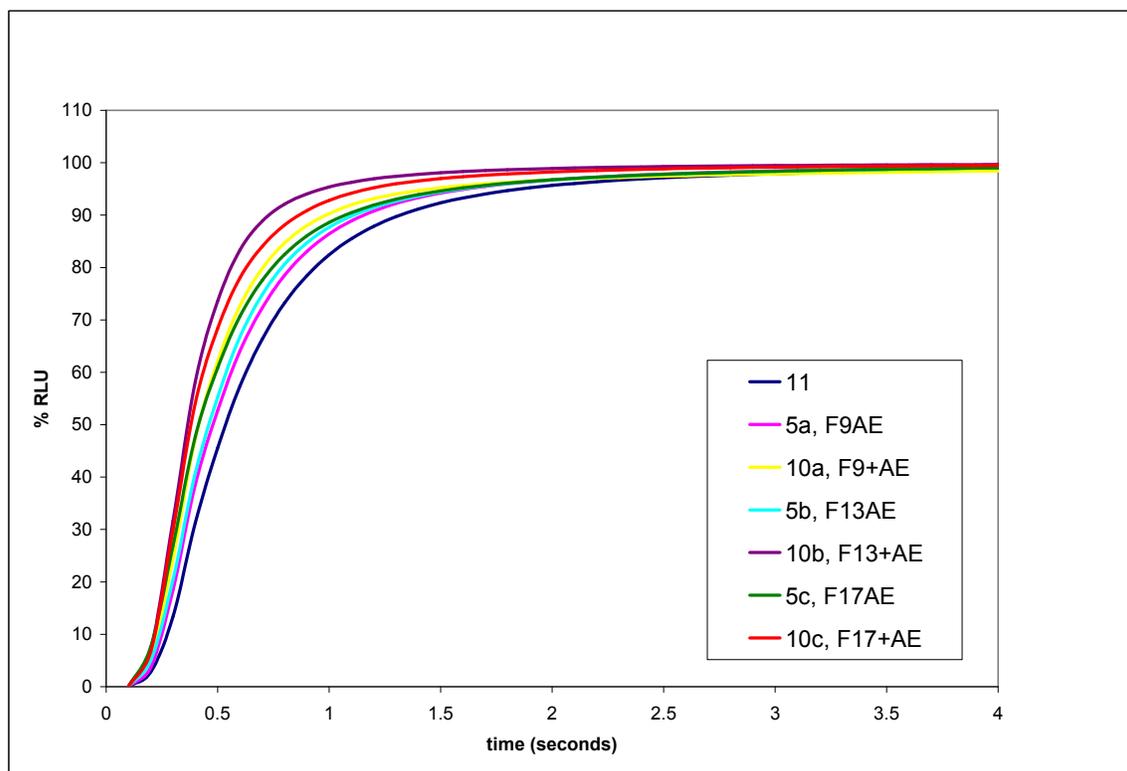


Figure S34. Chemiluminescence emission profiles of BSA conjugates in the presence of CTAC : SPFO = 10:1. Reagent 1 = 0.1 M nitric acid + 0.5% hydrogen peroxide; reagent 2 = 7 mM CTAC + 0.7 mM SPFO in 0.25 M NaOH. Light emission was triggered by the sequential additional of 0.3 mL reagent 1 followed by 0.3 mL reagent 2. Light was collected for 20 seconds integrated at 0.1 second intervals.

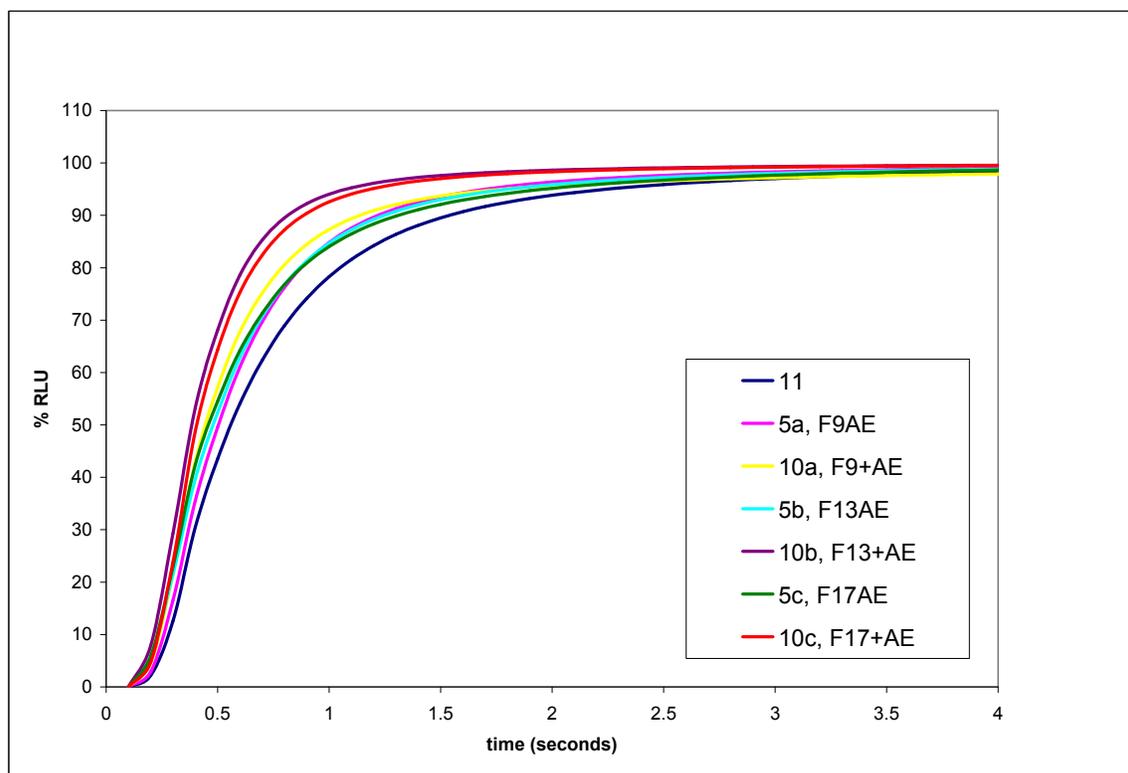


Figure S35. Chemiluminescence emission profiles of BSA conjugates in the presence of CTAC : SPFO = 5:1. Reagent 1 = 0.1 M nitric acid + 0.5% hydrogen peroxide; reagent 2 = 7 mM CTAC + 1.4 mM SPFO in 0.25 M NaOH. Light emission was triggered by the sequential additional of 0.3 mL reagent 1 followed by 0.3 mL reagent 2. Light was collected for 20 seconds integrated at 0.1 second intervals.

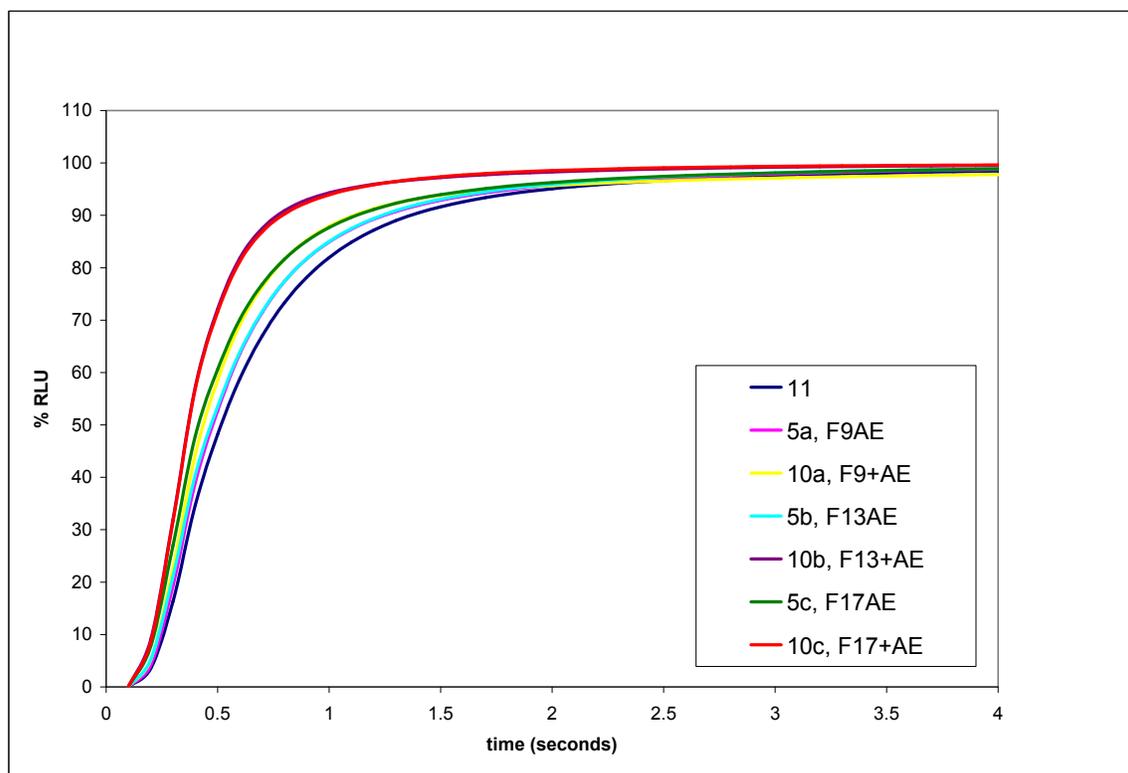


Figure S36. Chemiluminescence emission profiles of BSA conjugates in the presence of CTAC : SPFO = 3.3:1. Reagent 1 = 0.1 M nitric acid + 0.5% hydrogen peroxide; reagent 2 = 7 mM CTAC + 2.1 mM SPFO in 0.25 M NaOH. Light emission was triggered by the sequential additional of 0.3 mL reagent 1 followed by 0.3 mL reagent 2. Light was collected for 20 seconds integrated at 0.1 second intervals.

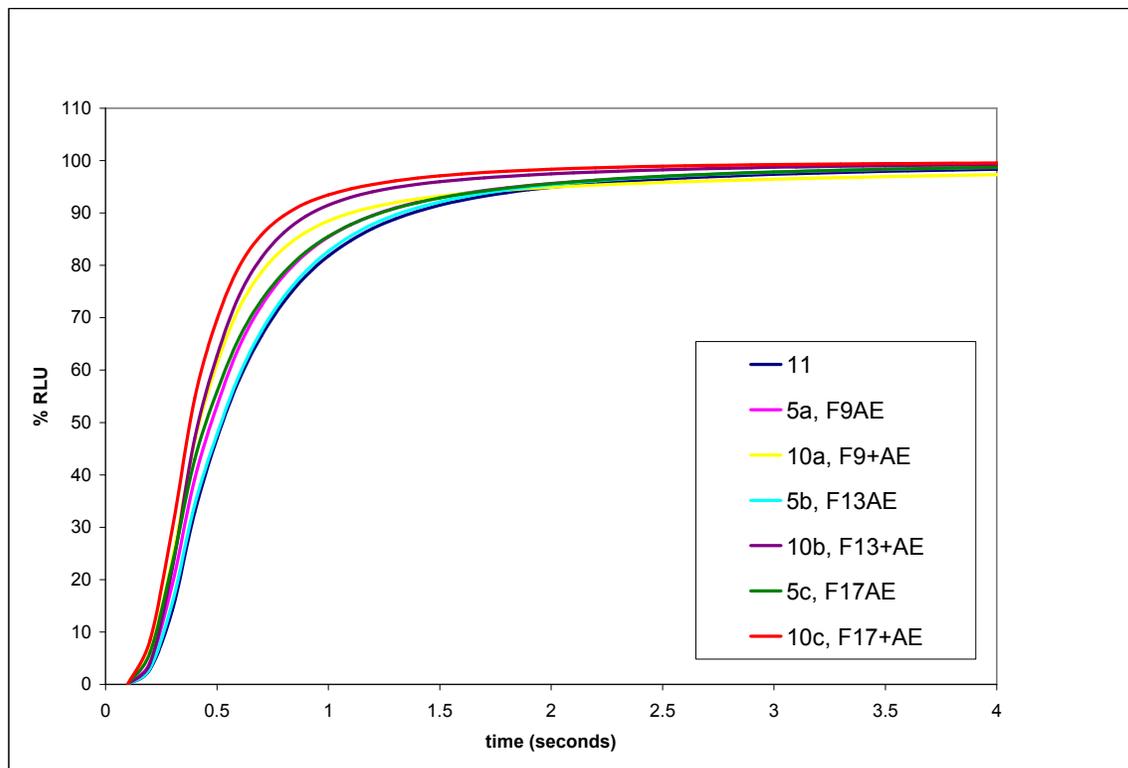


Figure S37. Chemiluminescence emission profiles of BSA conjugates in the presence of CTAC : SPFO = 2.5:1. Reagent 1 = 0.1 M nitric acid + 0.5% hydrogen peroxide; reagent 2 = 7 mM CTAC + 2.8 mM SPFO in 0.25 M NaOH. Light emission was triggered by the sequential additional of 0.3 mL reagent 1 followed by 0.3 mL reagent 2. Light was collected for 20 seconds integrated at 0.1 second intervals.

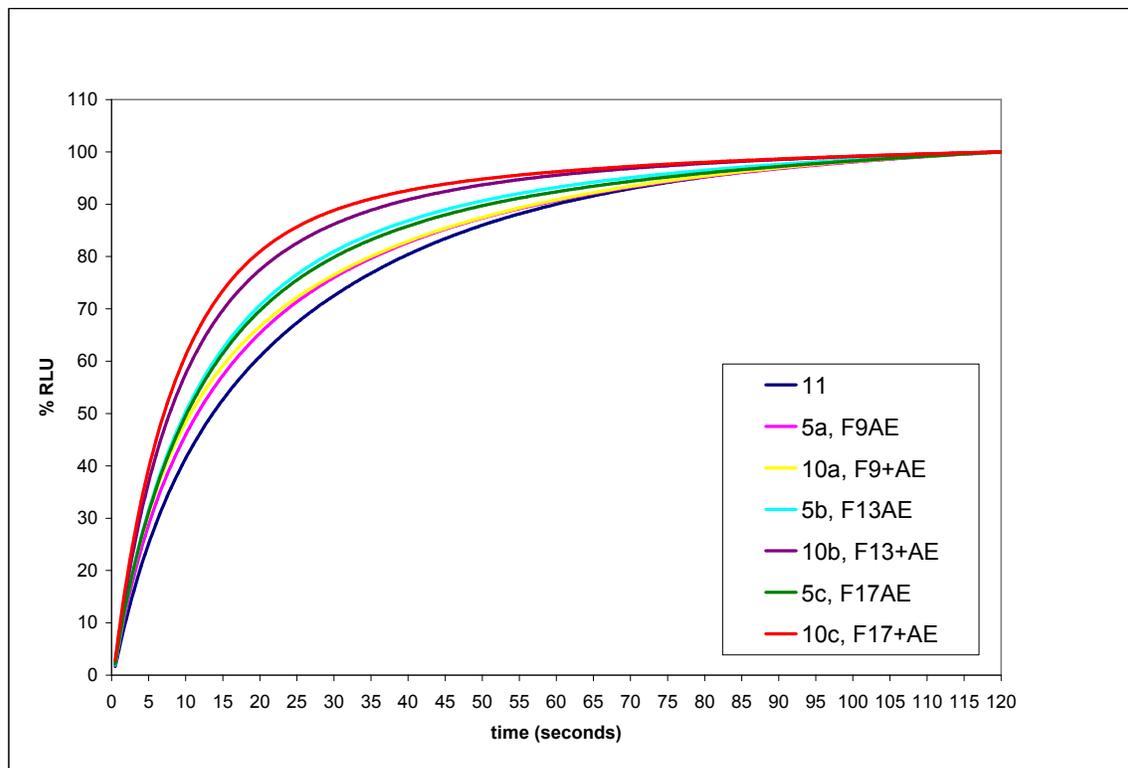


Figure S38. Chemiluminescence emission profiles of BSA conjugates in the presence of SPFO. Reagent 1 = 0.1 M nitric acid + 0.5% hydrogen peroxide; reagent 2 = 75 mM SPFO in 0.25 M NaOH. Light emission was triggered by the sequential additional of 0.3 mL reagent 1 followed by 0.3 mL reagent 2. Light was collected for 120 seconds integrated at 0.5 second intervals.

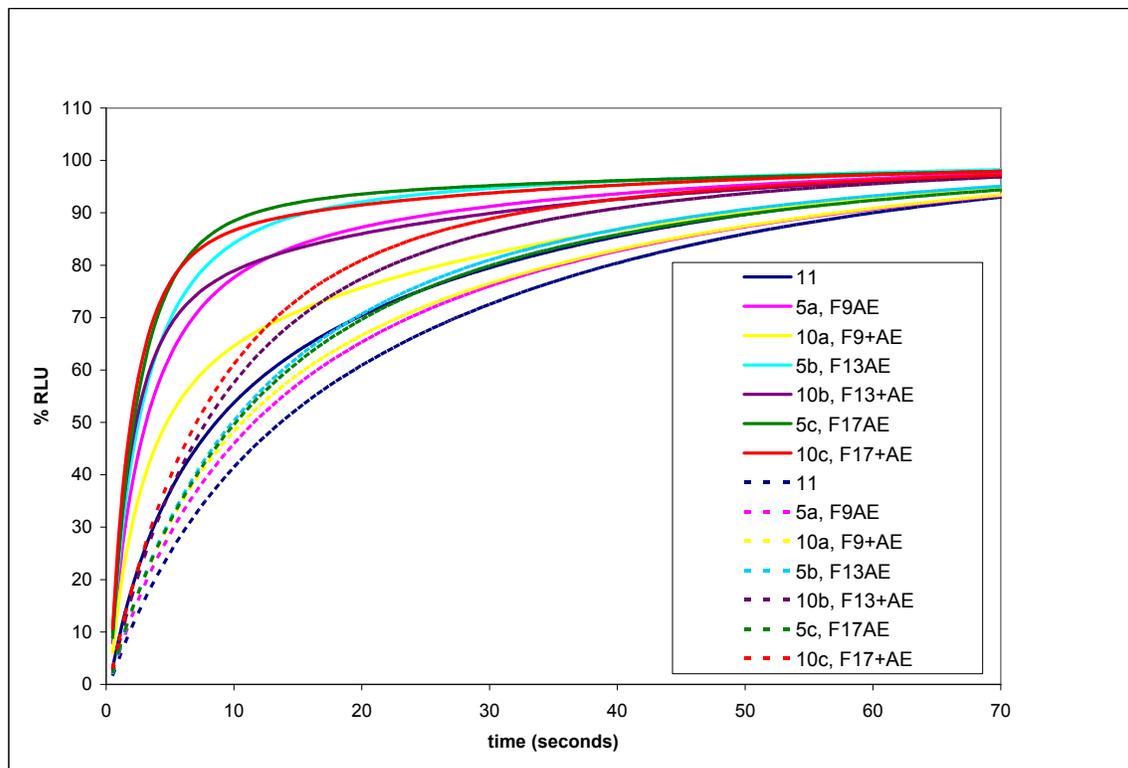


Figure S39. Comparison of the chemiluminescence emission profiles of BSA conjugates in the absence (solid lines) or presence (hatched lines) of SPFO. Reagent 1 = 0.1 M nitric acid + 0.5% hydrogen peroxide; reagent 2 = 0.25 M NaOH versus 75 mM SPFO in 0.25 M NaOH. Light emission was triggered by the sequential additional of 0.3 mL reagent 1 followed by 0.3 mL reagent 2. Light was collected for 120 seconds integrated at 0.5 second intervals. A greater inhibition of emission kinetics was observed with increasing fluororous content of the acridinium esters. Compound **11** without any fluororous chain showed the least amount of change in the emission profile in the presence of SPFO in comparison to the no surfactant control.

*Synthesis of BSA conjugates of acridinium esters (Table 1)*

BSA (1 mg, 15 nanomoles, 0.2 mL of a 5 mg/mL solution) was diluted with 0.2 mL of 0.1 M sodium carbonate, pH 9. The protein solutions, in separate labeling reactions, were treated with five equivalents of various acridinium esters of Figure 4. The acridinium esters were dissolved in dimethyl sulfoxide to give 2 mg/mL solutions. For labeling with the various acridinium esters, 0.038 mL of **11**, 0.0458 mL of F9AE (**5a**), 0.049 mL of F9+AE (**10a**), 0.0495 mL of F13AE (**5b**), 0.053 mL of F13+AE (**10b**), 0.053 mL of F17AE (**5c**) and 0.0565 mL of F17+AE (**10c**) were added. The reactions were stirred at room temperature for 2 hours. The reactions were then then diluted with 0.5 mL de-ionized water and transferred to 4 mL Amicon filters from Millipore (MW 30,000 cutoff). The labeling reactions in the filters were further diluted with 3 mL de-ionized water. The solutions were concentrated to ~ 0.1 mL by centrifugation at 7000 G for 10 minutes. This dilution and concentration process was repeated three more times. The final conjugate solutions were transferred to vials and the solutions were frozen and lyophilized. The lyophilized conjugates were dissolved in 0.5 mL of de-ionized water. The conjugates were analyzed by MALDI-TOF mass spectrometry to measure acridinium ester incorporation. This entailed measuring the molecular weight of the unlabeled protein and the labeled protein. The acridinium ester label contributed to the observed difference in mass between these two measurements. By knowing the molecular weight of the specific acridinium ester label, the extent of label incorporation for that specific acridinium ester could thus be calculated (Table 1).

*Emission wavelength measurements (Table 2, Figures S25-S31)*

Visible wavelength emission spectra of the acridinium esters **10a-10d** were measured by FSSS (Fast Spectral Scanning System) using a PR-740 spectroradiometer (camera) from Photo Research Inc. The following instrument parameters were used: bandwidth slit: 2 nm; aperture: 2 degrees; exposure time: 5000 msec. In a typical

measurement, 0.05-0.1 mL of a 1 mg/mL solution of the acridinium ester in a 2:1 mixture of water/MeCN (with 0.05% TFA) was diluted with 0.3 mL of reagent 1 comprising 0.5% hydrogen peroxide in 0.1 M nitric acid. Just prior to the addition of reagent 2 comprising 7 mM CTAC in 0.25 M sodium hydroxide, the shutter of the camera was opened and light was collected for 5 seconds. The output of the instrument is a graph of light intensity versus wavelength.

*Chemiluminescence measurements (Tables 3 & 4, Figures S32-S39)*

Chemiluminescence of BSA conjugates of acridinium esters (Figure 4) was measured on an Autolumat LB953 Plus luminometer from Berthold Technologies. Protein conjugates, 2 mg/mL, were serially diluted  $10^5$ -fold for chemiluminescence measurements in an aqueous buffer of 10 mM disodium hydrogen phosphate, 0.15 M NaCl, 8 mM sodium azide and 0.015 mM bovine serum albumin (BSA), pH = 8.0. A 0.010 mL volume of each diluted acridinium ester sample was dispensed into the bottom of a cuvette. Cuvettes were placed into the primed LB953 and the chemiluminescence reaction was initiated with the sequential addition of 0.3 mL of reagent 1, a solution of 0.5% hydrogen peroxide in 0.1 M nitric acid followed by the addition of 0.3 mL of reagent 2, a solution of 0.25 M sodium hydroxide with or without surfactant. For measurements with CTAC, 7 mM of this cationic surfactant was included in reagent 2. For measurements with CTAC/SPFO mixtures, the two surfactants were dissolved in reagent 2 at the indicated molar ratios (Tables 3 & 4). For measurements using SPFO, the fluorinated surfactant, 75 mM, was dissolved in reagent 2.

Each chemiluminescence flash curve was measured in 200 intervals of 0.1 second (20 seconds total time, CTAC and CTAC/SPFO mixtures) or 240 intervals of 0.5 seconds (2 minutes total time, no surfactant and SPFO only) from the point of chemiluminescence initiation with the addition of 0.25 M NaOH. Each chemiluminescence reaction was carried out a minimum of three times, averaged and

converted to a percentage of the chemiluminescence accumulated up to each time interval. Chemiluminescence times were normalized for comparison to reactions without surfactant with reactions with the various surfactants. The output from the luminometer instrument was expressed as R.L.U.s (Relative Light Units)

*Chemiluminescence stability measurements (Figure 7)*

The acridinium ester labeled BSA conjugates were diluted to a concentration of 0.2 nM in a buffer of 0.10 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.15 M sodium chloride, 1.0 mM EDTA (ethylenediaminetetraacetic acid), 10 mM Triton X-100 (4-octylphenol polyethoxylate), 10 mM sodium azide and 0.10 mM BSA (bovine serum albumin), pH 7.4. Five milliliter volumes of the diluted conjugates were sealed in liquid-tight polyethylene vials and incubated at 4°C and 37°C for four weeks. Chemiluminescence of a 0.01 mL sample (five replicates) of the diluted conjugates was periodically measured as a function of time. Chemiluminescence was measured on an AutoLumat Plus LB953 luminometer. The averaged results were calculated as residual chemiluminescence percentages with respect to values determined just prior to the start of incubation ( $t_0$ ). At both 4°C and 37°C the chemiluminescence stabilities of the fluorinated acridinium ester labels were better (higher) or equivalent to that of the reference label **11**.

*Measurement of non-specific binding (Table 5)*

Acridinium ester-labeled conjugates were diluted to a concentration of 10 nM in a buffer of 0.10 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.15 M sodium chloride, 1.0 mM EDTA (ethylenediaminetetraacetic acid), 10 mM sodium azide and 0.10 mM BSA (bovine serum albumin), pH 7.4. Paramagnetic particles, covalently derivatized with anti-fluorescein monoclonal antibody, were diluted to a concentration of 0.1 g/L in this same buffer. The fractional nonspecific binding assay was run in 5 mL polystyrene test tubes by mixing 0.10 mL (per test) of horse serum with 0.10 mL (per

test) of the 10 nM acridinium ester solution and 0.20 mL (per test) of the 0.1 g/L paramagnetic particle-suspension. The assay reactions were incubated at room temperature for 1 h, after which the paramagnetic particles were washed twice with either of three wash solutions: wash buffer alone, wash buffer with 0.8 mM Tween-20, and wash buffer with both 0.8 mM Tween-20 and 50 mM SPFO. The wash buffer itself being 10 mM potassium phosphate, 0.14 M sodium chloride, 14 mM sodium azide, and 1.0 mM EDTA, pH 7.2 in water. Residual chemiluminescence from each tube containing the washed paramagnetic particles was measured on the Autolumat Plus LB953 luminometer for 5 s. For each of the three wash conditions five replicates were run for each acridinium ester conjugate. The mean chemiluminescence of five replicates was divided by the total input chemiluminescence to calculate the fractional nonspecific binding for each acridinium ester conjugate with each of the three wash conditions.