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9-Aryl-9-Xanthenols: A Convenient Platform for the Design of Fluorimetric and Colorimetric pH Indicators

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General Methods. All organic solvents were dried and freshly distilled before use. Flash chromatography was performed using 40-75 μm silica gel. All NMR spectra were recorded in $CDCl_3$ (unless otherwise noted) using 400 MHz instrument. Absorption spectra were recorded on CARY 300 Bio UV-Visible spectrometer. Buffer solutions for kinetic experiments were prepared using literature pKa values of the buffer acids and activity coefficient recommended by Bates.[1] 2,2',4,4'–Tetrahydroxybenzophenone, phenylmagnesium bromide and o-tolylmagnesium bromide were purchased from TCI America and used as received. 3-Hydroxy-6-methoxy-9H-xanthen-9-one (**4**),[2] 3,6-dihydroxy-9H-xanthen-9-one (**3**),[3] and 3,6-bis(allyloxy)-9H-xanthen-9-one (**7**)[4] were prepared following literature procedures.

Kinetic experiments. The temperature of the sample solutions was kept constant at 25 ± 0.1 °C using a thermostat. Rate constants for the formation of xanthylium cation **2b** were obtained by least-squares fitting of experimental data to a single exponential function using Origin 8.1 software. All measurements were performed in triplicates. Rate measurements for specific acid catalysis were performed in three series of solutions with varying buffer concentrations but constant buffer ratio in each series.

Fluorescence measurements were conducted in aqueous buffers or acid solutions containing 20% of acetonitrile at 1.4 μM concentration of **1b**. Fluorescence emission spectra were recorded with excitation 437 nm. The data analysis of the fluorimetric titrations were conducted based on the 477 nm emission band. The excitation source and the detector slit were set to 1 nm and 5 nm, respectively. The fluorescence quantum yield was determined using fluorescein in 0.1 N NaOH (Φ_{fl} = 0.95)[5] as the reference standard. All fluorescence spectra were recorded using spectrometer equipped with polarizer. The path length was 1 cm with a cell volume of 3 mL.

Cell Culture. NIH 3T3 mouse fibroblast cells were cultured at 37 °C (5% CO_2) in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% bovine serum, 4 mM L-glutamine, 200 unit/ml penicillin, and 200 μg/ml streptomycin. The culture medium was filtered through 0.2 μm filters. For staining experiment, cells were grown on glass cover slips to 80% confluency, washed with serum-free DMEM supplemented with 25 mM HEPES, and incubated with 5 μM xanthenol derivative **1c** diluted from a 10 mM DMSO stock solution, in serum-free DMEM for 1 hour at 37^0C (5% CO2). Cells were then washed with PBS and fixed with 3.7% formaldehyde for 15 minutes. After washing with PBS the cover slips were mounted on slides

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with ProLong gold antifade reagent (Invitrogen/Molecular Probes) for imaging. For colocalization studies, NIH 3T3 cells were transfected with Lamp1-RFP (Addgene) or pDsRed2-mito (Clontech) 48 hours before imaging using Turbofect transfection reagent (Fermentas). The cells were stained with **1c** and fixed with formaldehyde as described above.

Confocal Microscope Imaging. Fluorescence micrographs were acquired with a Zeiss LSM 510 confocal laser microscope fitted with a plan-apochromat 63x/1.4 oil objective. Compound **1c** was excited at 488 nm and emission collected with a band pass filter from 535-590 nm. Both red fluorescent proteins were excited at 543 nm and emission collected with a 565-615 nm band pass filter. To ensure proper channel separation, the emission path was additionally directed through a dichroic mirror (Ex/Em: 584/607 for RFP, and Ex/Em: 558/583 for DsRed2). For colocalization studies, images were acquired sequentially and checked for channel separation.

Materials

***3,6-Bis(allyloxy)-9-phenyl-9H-xanthen-9-ol* (1b)**. A solution of 3,6-*bis*(allyloxy)-9*H*-xanthen-9-one (**7**) 0.279 g, 0.905 mmol) in THF (5 mL) was added dropwise to a solution of phenylmagnesium bromide (1.1 mL, 1.1 mmol) in THF (4 mL) at 0 °C under nitrogen. The mixture was stirred for 2 h at rt, quenched with a saturated solution of NH_4Cl (2 mL), and the crude product extracted with ethyl acetate (3 x 25 mL). The solvent was evaporated and the product purified by silica gel chromatography eluting with 10 - 30 % EtOAc in hexanes (2% Et_3N) to afford 0.271 g (0.701 mmol, 77%) of 3,6-*bis*(allyloxy)-9-phenyl-9*H*-xanthen-9-ol (**1b**) as a pale yellow oil. ^{1}H NMR: 7.39 (d, J = 7.2 Hz, 2 H), 7.27 (t, J = 7.2, 8.4 Hz, 2 H), 7.19 – 7.15 (m, 4 H), 6.66 – 6.62 (m, 4 H), 6.09 – 5.99 (m, 2 H), 5.41 (dd, J = 17.2 , 1.6 Hz, 2 H), 5.29 (d, J = 10.4 Hz, 2 H), 4.53 (d, J = 5.2 Hz, 4 H), 2.58 (br, 1 H). ^{13}C NMR: 159.2, 150.9, 148. 5, 130.4, 128.1, 126.8, 126.7, 118.1, 112.0, 101.4, 70.4, 69.4. EI-MS m/z: 386(M^+, 4), 370 (11), 369 (M^+ - OH, 38), 328 (9), 310 (22), 309 (100), 268 (12), 240 (7), 202 (5), 105 (11). HRMS-ESI: calc. for [M - H]-; $C_{25}H_{21}O_4$: 385.1445, found 385.1449.

***3-Allyloxy-6-methoxy-9-(o-tolyl)-9H-xanthen-9-ol* (1c)**. A solution of 3-(allyloxy)-6-methoxy-9H-xanthen-9-one (**5**) (0.500 g, 1.77 mmol) in THF (5 mL) was added drop wise to a solution of *o*-tolylmagnesium bromide (2.1 mL, 2.1 mmol) in THF (4 mL) at 0 °C under nitrogen. The mixture was stirred for 2 h at rt, quenched with a saturated solution of NH_4Cl (2 mL), and the crude product extracted with ethyl acetate (3 x 25 mL). The solvent was evaporated and the

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product purified by silica gel chromatography eluting with 10 - 30 % EtOAc in hexanes (2% Et_3N) to afford 0.531 g (1.42 mmol, 80%) of 3-(allyloxy)-6-methoxy-9-(*o*-tolyl)-9*H*-xanthen-9-ol (**1c**) as a pale yellow oil. ^{1}H NMR: 8.30 (d, J = 7.6 Hz, 1H), 7.35 (t, J = 7.6 Hz, 1H), 7.22 (t, J = 8.8, 7.2 Hz, 1H), 6.98 (d, J = 7.2 Hz, 1H), 6.92 (d, J = 8.8 Hz ,2H), 6.64 (dd, J = 7.2, 2.4 Hz, 2H), 6.59 – 6.53 (m, 2H), 6.07 – 5.99 (m, 1H), 5.41 (d, J = 17.2 Hz, 1H-trans), 5.29 (d, J = 10.2 Hz, 2H), 4.51 (d, J = 5.2 Hz, 2H), 3.78 (s, 3H), 1.45 (s, 3H). ^{1}H NMR (CD_3OD): 8.24 (d, *J* = 7.8 Hz, 1H), 7.34 (t, *J* = 7.6 Hz, 1H), 7.22 – 7.18 (td, *J* = 7.4, 1.2 Hz, 1H), 6.97 (d, *J* = 7.4 Hz, 1H), 6.84 (d, *J* = 8.7 Hz, 2H), 6.72 – 6.70 (dd, *J* = 5.6, 2.5 Hz, 2H), 6.60 – 6.56 (td, *J* = 8.8, 7.2, 2.5 Hz, 2H), 6.11 – 6.01 (m, 1H), 5.43 – 5.38 (dd, *J* = 17.3, 1.6 Hz, 1H), 5.27 – 5.24 (dd, *J* = 10.6, 1.5 Hz, 1H), 4.57 – 4.55 (d, *J* = 5.1 Hz, 2H), 3.80 (s, 3H), 1.41 (s, 3H).^{13}C NMR: 160.3, 159.3, 151.2, 151.2, 144.2, 135.9, 132.1, 129.8, 127.8, 126.1, 125.4, 119.1, 118.9, 118.1, 111.8, 111.2, 101.4, 100.5, 69.7, 69.2, 55.6, 20.7. HRMS- ESI: calc. for $[M - H]^-$; $C_{24}H_{21}O_4$: 373.1445, found 373.1439

***3-(Allyloxy)-6-methoxy-9-(o-tolyl)xanthylium cation* (2c)**. CD_3OD (700 μL) was added to a 15 mL round bottom flask containing **1c** (10 mg, 0.03 mmol). The pale yellow solution was transferred to an NMR tube and the ^{1}H spectrum was recorded (S12, reported in **1c** above). A solution of DCl in D_2O (25 μL, 1.8 mmol) was added to the NMR tube and the ^{1}H and ^{13}C NMR spectra of the resulting intense yellow cation were obtained (S13 & S20). ^{1}H NMR (CD_3OD): 7.84 – 7.83 (d, *J* = 2.3 Hz, 2H), 7.70 – 7.64 (m, 3H), 7.62 – 7.58 (m, 1H), 7.54 (t, *J* = 7.5 Hz, 1H), 7.48 – 7.43 (td, *J* = 9.4, 2.4 Hz, 2H), 7.37 (d, *J* = 7.6 Hz, 1H), 6.23 – 6.13 (m, 1H), 5.59 – 5.55 (dd, *J* = 17.3, 1.3 Hz, 1H), 5.44 – 5.41 (dd, *J* = 10.6, 1.2 Hz, 1H), 5.04 – 5.03 (d, *J* = 5.4 Hz, 2H), 4.24 (s, 3H), 2.05 (s, 3H). ^{13}C NMR (300 MHz, CD_3OD): 173.6, 172.2, 169.5, 162.0, 161.8, 137.4, 133.8, 133.7, 132.7, 132.4, 132.4, 132.3, 130.3, 127.5, 122.5, 122.3, 120.1, 119.5, 119.5, 102.6, 101.7, 72.6, 58.8, 20.0.

***3,6-Dihydroxy-9H-xanthen-9-one* (3)**.[3] A suspension of 2,2',4,4'-tetrahydroxybenzophenone (7.0 g, 28.4 mmol) in 30 mL water and 10 mL of acetone was sealed in a pressure vessel and heated at 220 – 230 °C in an oven for 4 h. Upon cooling, the resulting solid was filtered out and washed with 100 mL of warm water at about 60° C to afford 6.4 g (28.0 mmol, 99 %) of 3,6-dihydroxy-9*H*-xanthen-9-one (**3**) as colorless needle like solid which did not melt below 350 °C. The spectral data are consistent with literature values.[3] ^{1}H NMR (DMSO-d6): δ 10.83 (s, 2H, -

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OH), 7.98 (d, J = 8.8 Hz, 2H), 6.86-(dd, J = 8.8, 2.0 Hz, 2H), 6.82 (d, J = 2.0 Hz, 2H). ^{13}C NMR (DMSO-d6): δ 174.8, 164.3, 158.4, 128.7, 114.9, 114.6, 103.0. DIP-EIMS: M^+ 228.

3-(Allyloxy)-6-methoxy-9H-xanthen-9-one **(5)**. Powered K_2CO_3 (0.519 g, 3.76 mmol) was added to a solution of 3-hydroxy-6-methoxy-9*H*-xanthen-9-one (4) (0.70 g, 2.89 mmol) in acetone (25.0 mL) followed by allyl bromide (0.375 ml, 4.33 mmol). The mixture was refluxed for 1 h and the solvent removed in vacuum. Purification by silica gel chromatography (20% EtOAc in hexanes) afforded 0.520 g (1.842 mmol, 63.7%) of 3-(allyloxy)-6-methoxy-9*H*-xanthen-9-one (**5**) as white fluffy solid, m.p 141 – 143 ˚C. ^{1}H NMR: δ 8.24 (d, J = 8.8 Hz, 2H), 6.95 (td, J = 8.8 Hz, 2.4 Hz, 2H), 6.87 (t, J = 2.4 Hz ,2H), 6.08 (m, 1H), 5.47 (d, J = 17.6 Hz, 1H-trans), 5.36 (d, J = 9.6 Hz, 1H-cis),4.66 (d, J = 5.2 Hz, 2H), 3.93 (s, 3H). ^{13}C NMR: δ 175.7, 164.9, 163.9, 158.3, 158.2, 132.5, 128.5, 118.7, 116.1, 113.5, 113.1, 101.4, 100.5, 69.5, 56.0. EIMS: M^+ 282, HRMS-ESI: calc. for $[M+ H]^+$; $C_{17}H_{15}O_4$: 283.0965, found 283.0969

3-(Allyloxy)-6,9-dimethoxy-9-(o-tolyl)-9H-xanthene **(6).** AcOH (5 drops) was added to a solution of 3-allyloxy-6-methoxy-9-(*o*-tolyl)-9*H*-xanthen-9-ol (*1b*) (0.230 g, 0.61 mmol) in MeOH (10 mL) and the mixture re-fluxed overnight. K_2CO_3, (about 100 mg) was added to a reaction mixture and the solvent evaporated. The residue was then adsorbed on silica gel and purified through a short plug of silica gel eluting with 5% EtOAc in hexanes to afford 0.226 g (0.58 mmol, 95%) of 3-(allyloxy)-6,9-dimethoxy-9-(*o*-tolyl)-9*H*-xanthene (**6**) as a pale yellow oil. ^{1}H NMR: δ 8.32 (d, J = 8 Hz, 1.3H), 7.34 (t, J = 7.6 Hz, 1.3H), 7.19 (t, J = 7.2 Hz, 1.3H), 6.96 (d, J = 7.6 Hz, 1.3H), 6.90 (d, J = 8.8 Hz, 2.6 H), 6.66 (m, 2.6 H), 6.60 (t, J = 8.4, 8.0 Hz, 2.6 H), 6.07 (m, 0.8 H), 5.44 (d, J = 17.2 Hz, 0.8 H), 5.31 (d, J = 10.4 Hz, 0.8 H), 4.55 (d, J = 5.2 Hz, 2.0 H), 3.83 (s, 5.50 H), 2.76 (s, 5.5 H). ^{13}C NMR: δ 160.3, 159.3, 153.4, 153.3, 145.2, 130.4, 127.5, 126.0, 125.3, 118.1, 111.3, 100.5, 99.6, 95.7, 74.5, 69.2, 55.6, 49.5, 20. 7. HRMS-ESI: calc. for $[M+Na]^+$; $C_{25}H_{24}NaO_4$: 411.1567, found 411.1561.

3,6-Diallyloxy-9H-xanthen-9-one **(7)**.[4] Allyl bromide (0.393 ml, 4.54 mmol) was added to a solution of Cs_2CO_3 (2.219 g, 6.81 mmol) and 3,6-dihydroxy-9*H*-xanthen-9-one (**3**) (0.520g, 2.28 mmol) in DMF (10 mL). The reaction mixture was stirred overnight at r.t. and quenched with 5% HCl solution. The product was extracted with ether (3 x 50 mL), washed with water (3 x 25 mL), brine (20 mL), and dried over MgSO4 to afford 0.66 g (2.14 mmol, 94%) of 3,6-*bis*(allyloxy)-9*H*-xanthen-9-one (7) as colorless crystals. m.p. = 137 -138 °C. Lit.[4] 140 °C. ^{1}H NMR: δ 8.23 (d, J

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= 8.8 Hz, 2H), 6.95 (dd, J = 8.8 Hz, 2.4 Hz, 2H), 6.86 (d, J = 2.0 Hz, 2H), 6.12 -6.04 (m, 2H), 5.47 (dd, J 17.2 Hz, 1.2 Hz, 2H), 5.36 (dd, J = 10.8 Hz, 1.2 Hz, 2H), 4.44 (d, J = 5.2 Hz, 4H). ^{13}C NMR: δ 175.6, 163.9, 158.2, 132.4, 128.4, 118.7, 116.2, 113.5, 101.4, 69.5. GCMS EI-MS m/z: 309, (15), 308 (M+, 65), 307 (13), 293 (14), 281 (11), 280 (31), 279 (18), 268 (19), 267 (100), 253 (22), 239 (39), 226 (15), 211 (10), 63 (14). HRMS-EI: calc. for $[M]^+$; calcd. for $C_{19}H_{14}O_4$ 308.1049, found 308.1046. It should be noted that the 1H NMR spectrum of this compound as well as many other structurally related compounds are unusual due to the existence of two rotamers that are not equilibrated on the NMR time scale.[6]

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Table S1: Spectrophotometric titration of **1c**

$[H_3O^+]$/M	Absorbance at 437 nm		
0.100	1.679	1.687	1.667
0.050	1.682	1.602	1.601
0.010	1.659	1.668	1.667
0.005	1.681	1.689	1.724
0.001	1.571	1.637	1.577
1.09E-04	1.522	1.646	1.648
8.16E-05	1.61	1.66	1.683
5.44E-05	1.599	1.613	1.644
2.72E-05	1.623	1.625	1.628
5.44E-06	1.539	1.545	1.57
2.72E-06	1.447	1.476	1.487
6.64E-07	1.231	1.216	1.23
4.98E-07	1.139	1.132	1.151
3.32E-07	0.948	0.931	0.948
1.66E-07	0.587	0.602	0.585
9.96E-08	0.394	0.386	0.395
3.32E-08	0.157	0.154	0.157
4.92E-09	0.0778	0.0839	0.0747
1.64E-09	0.0293	0.026	0.0234
2.42E-10	0.0102	0.0131	0.0124
2.42E-11	0.00437	0.00424	0.00428

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Table S2. Observed rates of formation of **2c**

$[H_3O^+]$/M	k_{obs} (s^{-1})		
8.16E-05	0.19257	0.16	0.16962
5.44E-05	0.1128	0.10694	0.10385
2.72E-05	0.06177	0.06046	0.05765
5.44E-06	0.01329	0.01319	0.01364
2.72E-06	0.00786	0.00701	0.00688
6.64E-07	0.00342	0.00348	0.00337
4.98E-07	0.00226	0.00231	0.00225
3.32E-07	0.0016	0.00159	0.00168
1.66E-07	0.00108	0.00112	0.00111
9.96E-08	0.00115	0.00112	0.00107
3.32E-08	7.85E-4	6.71E-4	7.54E-4
4.92E-09	1.31E-3	1.41E-3	1.26E-3
1.64E-09	5.01E-4	3.57E-4	4.03E-4
2.42E-10	1.63E-4	2.21E-4	2.02E-4
2.42E-11	7.40E-5	7.75E-5	7.93E-5

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Table S3. Fluorimetric titration of **1c**

$[H_3O^+]$/M	Normalized Emission at 477 nm		
0.0110	1.1739E6	1.25239E6	1.18546E6
0.0055	1.13924E6	1.16333E6	1.19046E6
0.0010	1.13727E6	1.22157E6	1.15483E6
7.24E-05	1.08131E6	1.08762E6	1.09299E6
5.37E-05	1.09779E6	1.0959E6	1.08928E6
3.39E-05	1.09699E6	1.08839E6	1.09339E6
1.86E-05	1.0795E6	1.07791E6	1.08016E6
2.88E-06	1.00653E6	992781	974744
1.41E-06	736741	762140	911389
1.86E-07	577759	593638	569816
1.62E-07	524653	523889	507737
1.1E-07	244963	233543	224643
5.37E-08	233068	225600	252962
3.8E-08	145512	138031	159371
2E-08	156907	133025	157652
1.38E-08	54326	52070	59378
5.25E-09	65370	62918	58804
2.34E-09	26141	22469	23233
1.51E-10	1208	1226	1295
3.72E-11	982	443	1112

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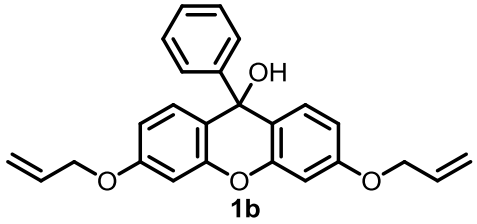
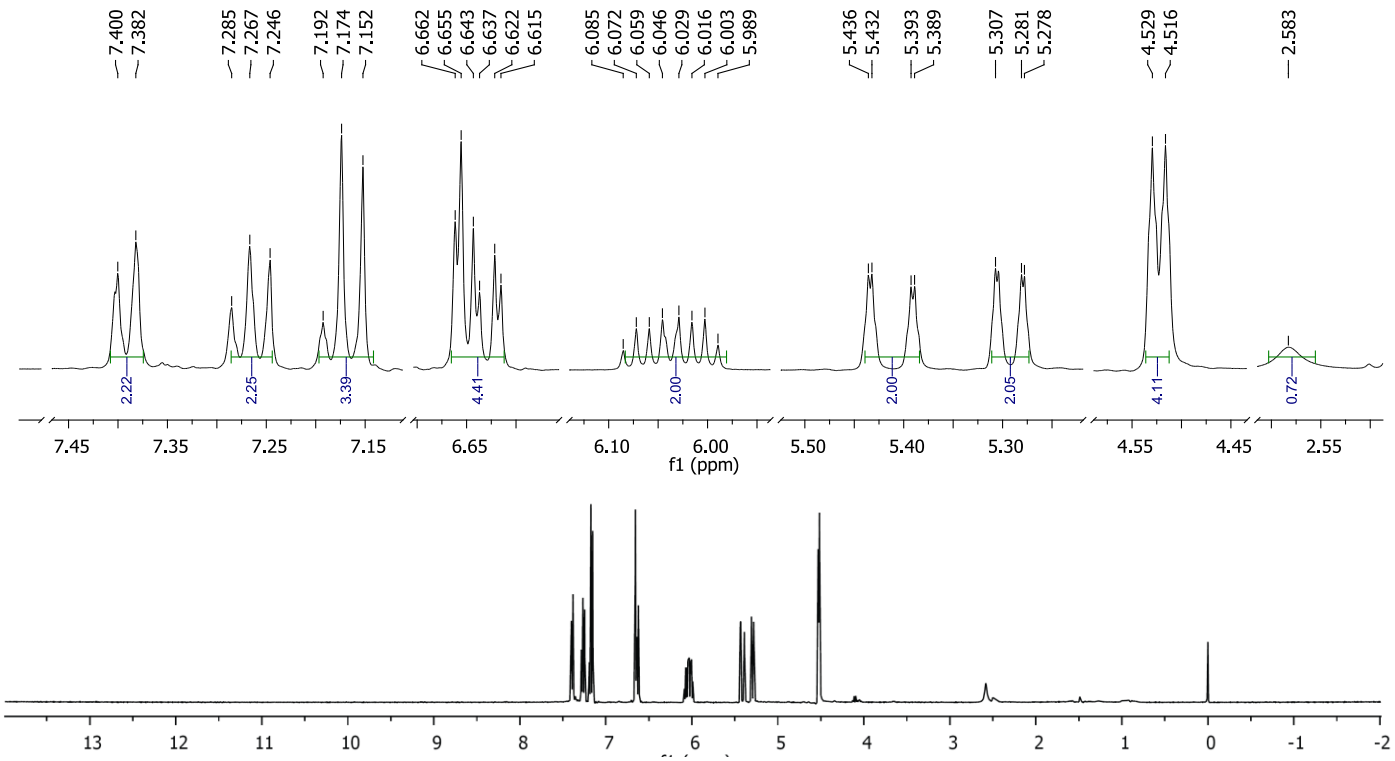
^{1}H NMR



1b



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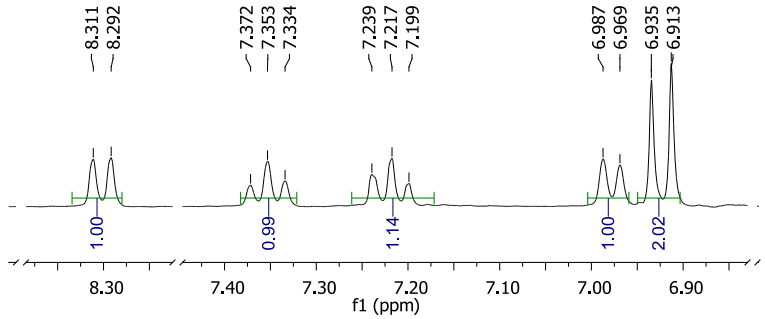

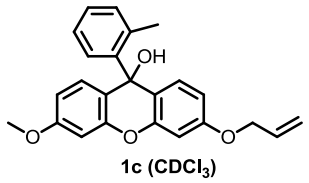

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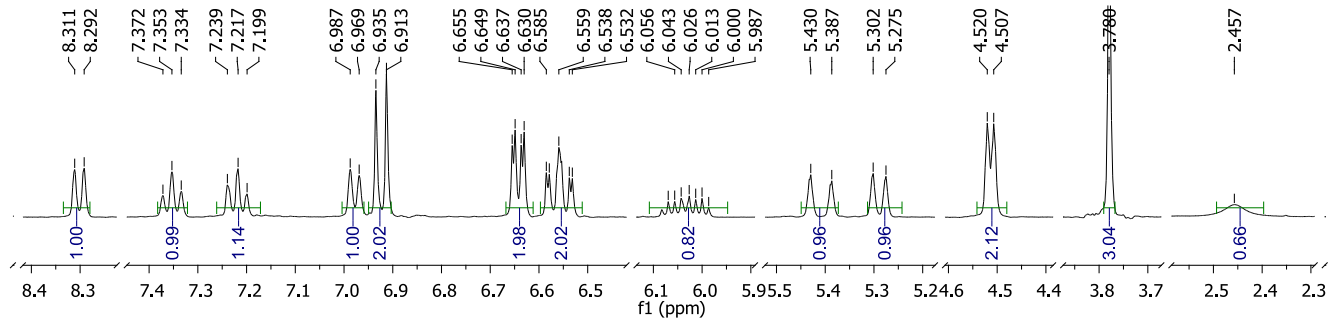

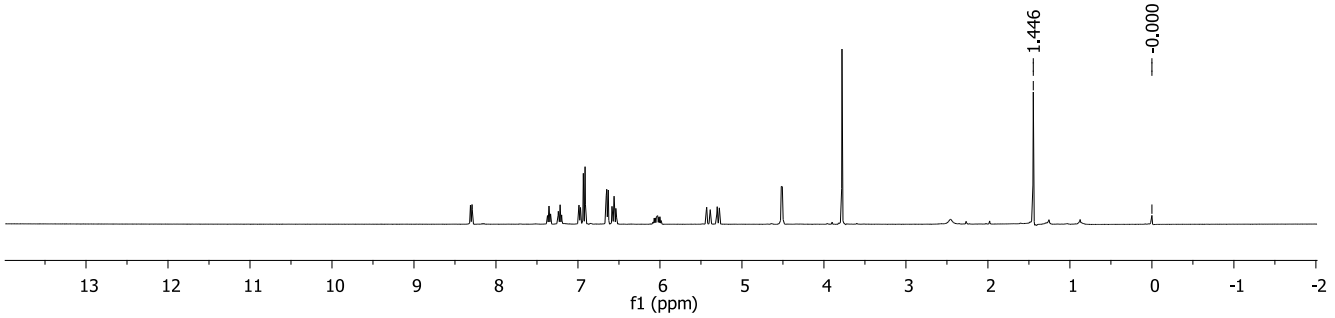

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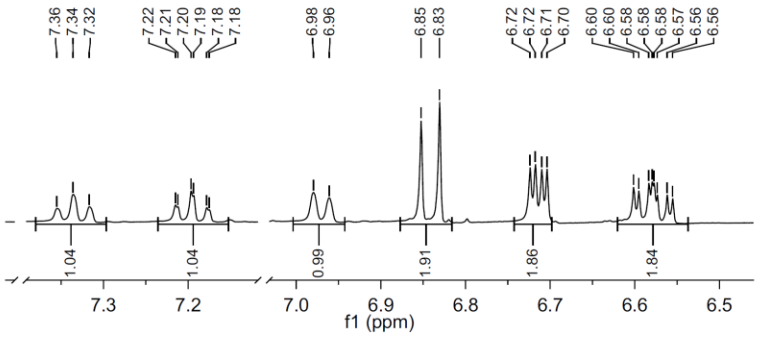

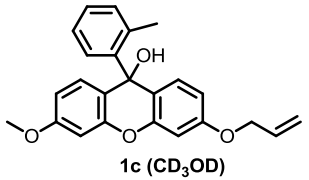

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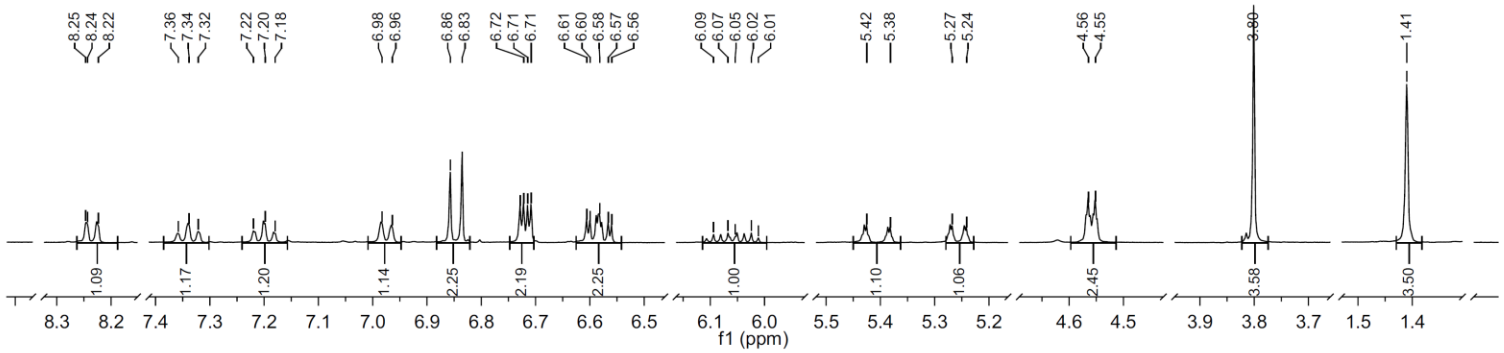

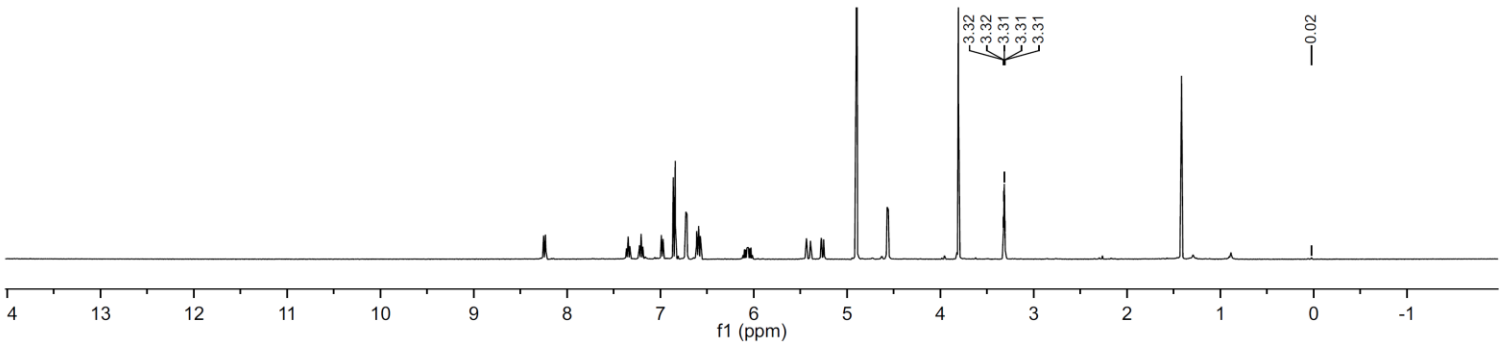

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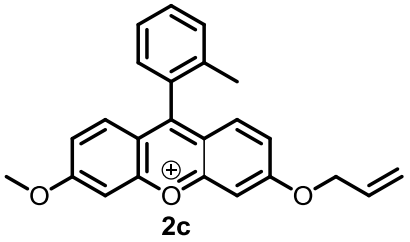

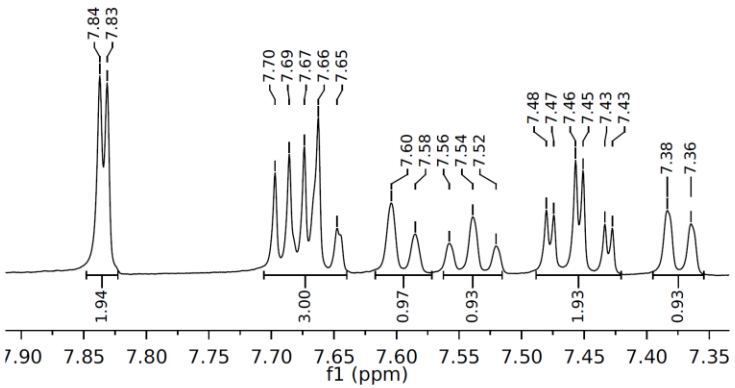

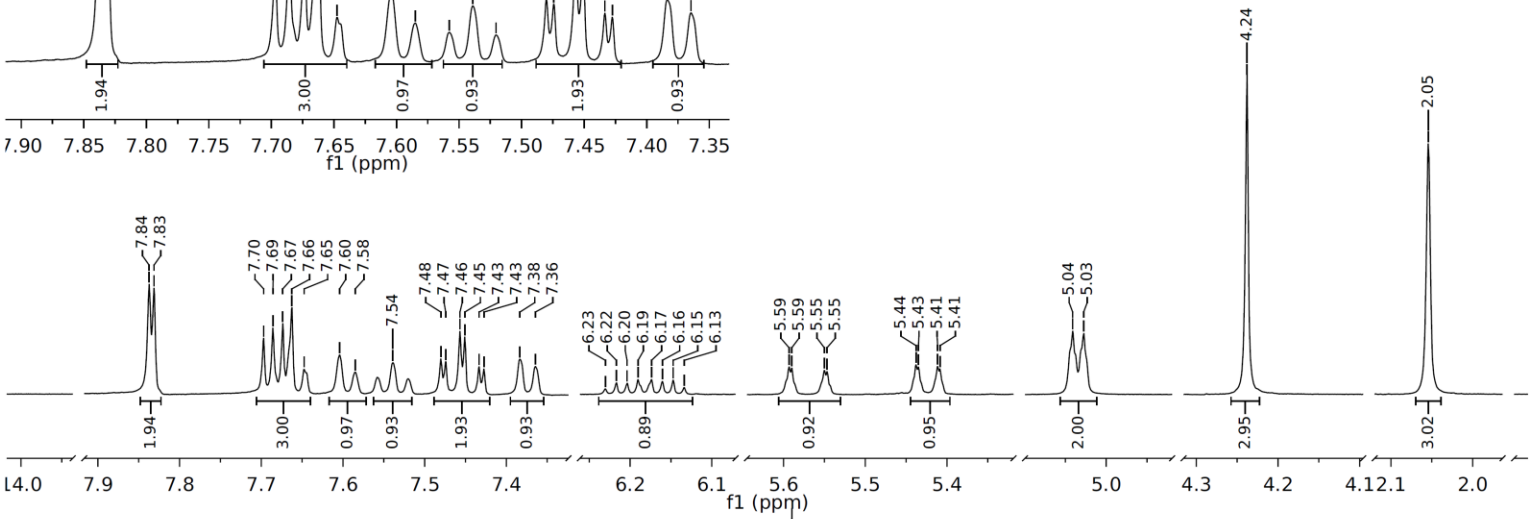

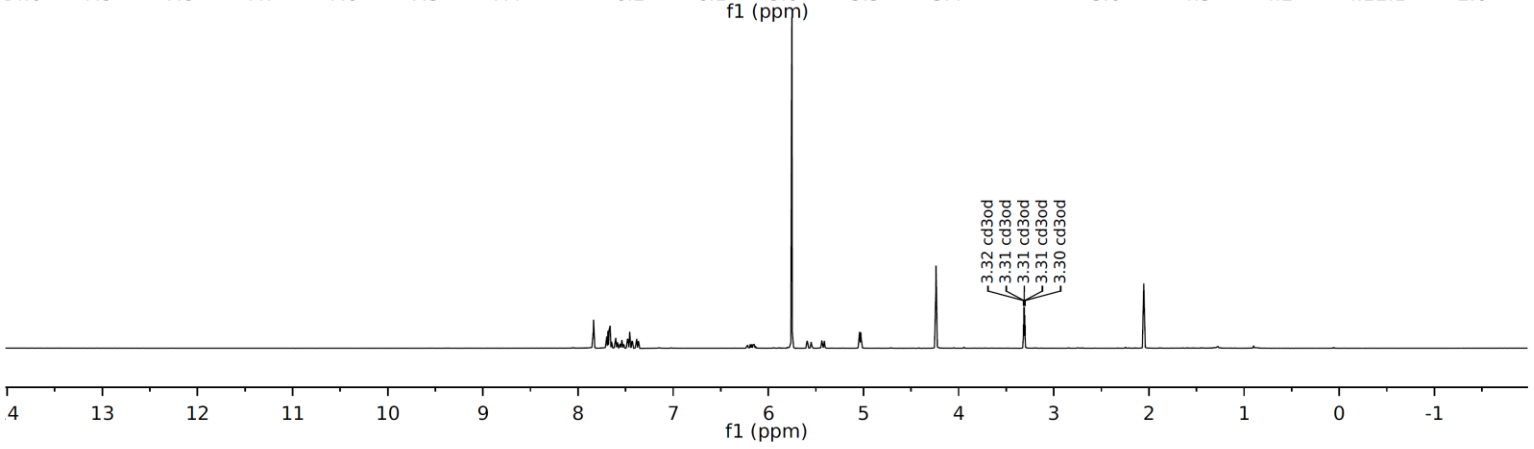

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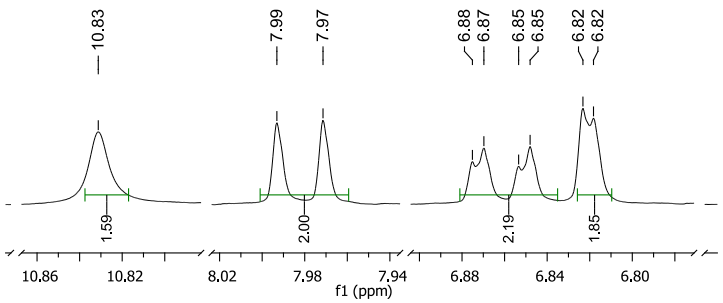

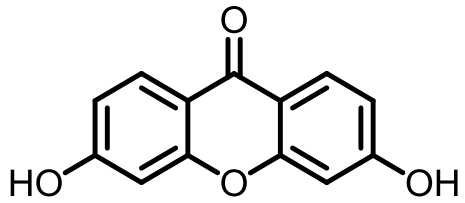

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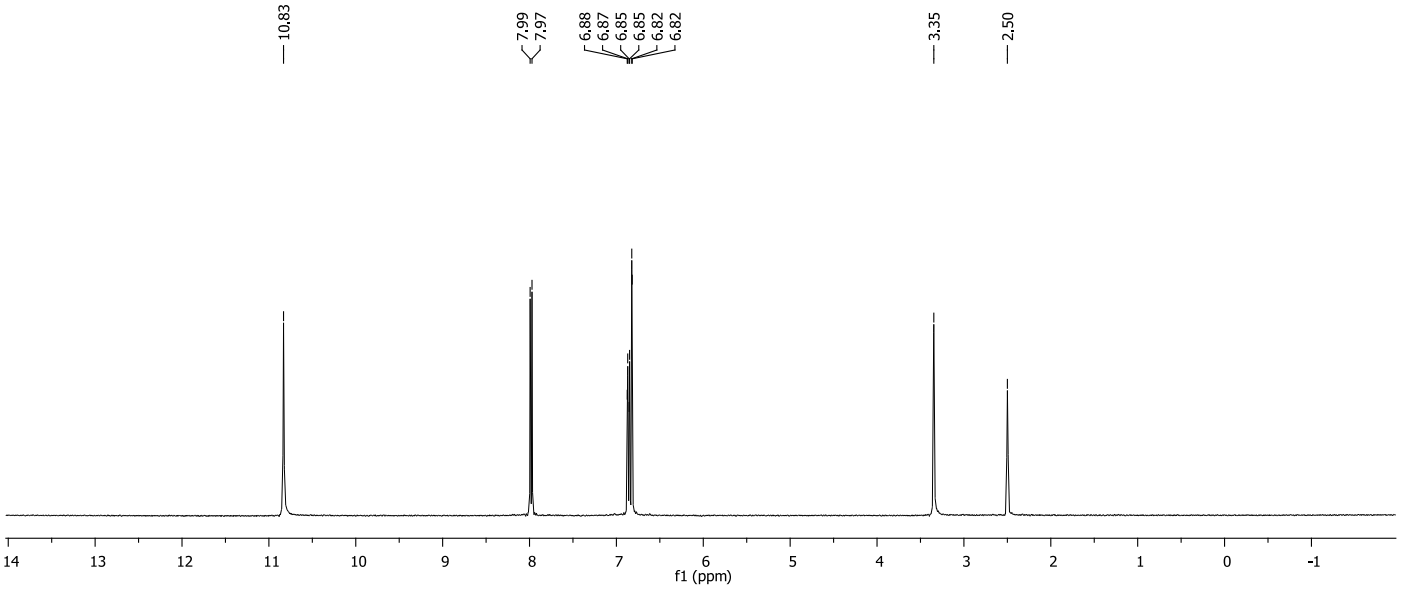

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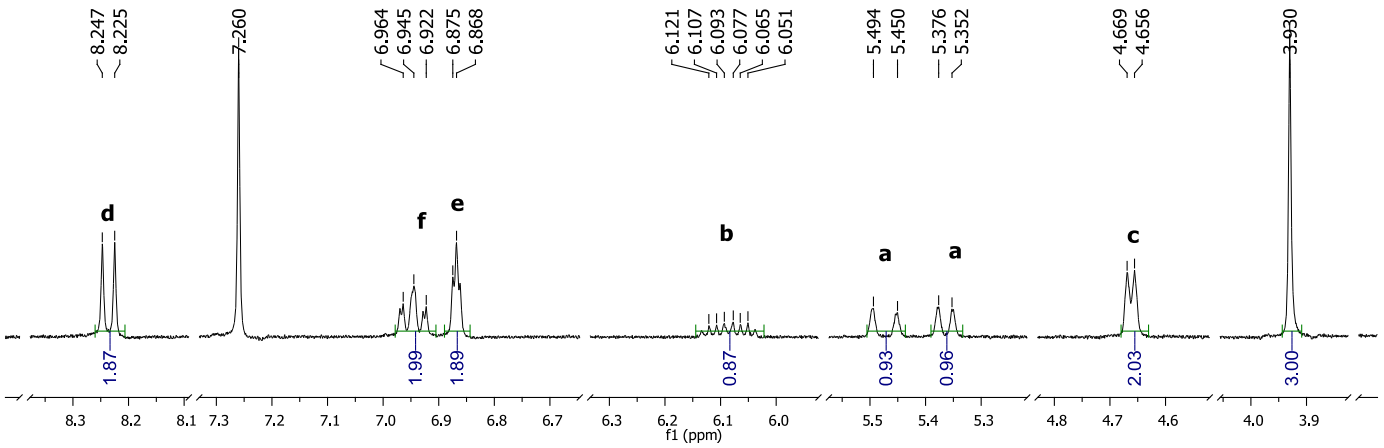

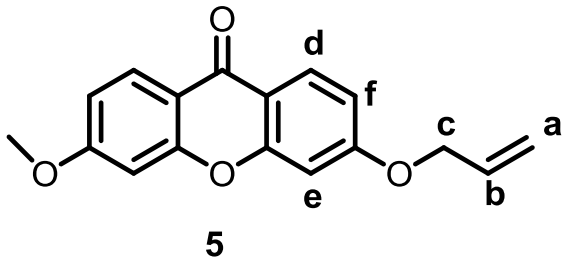

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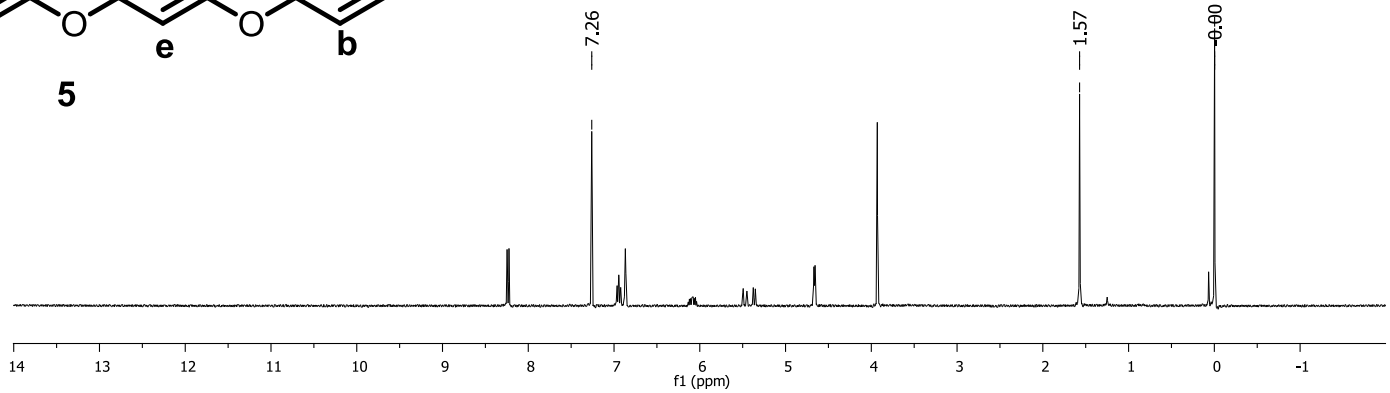

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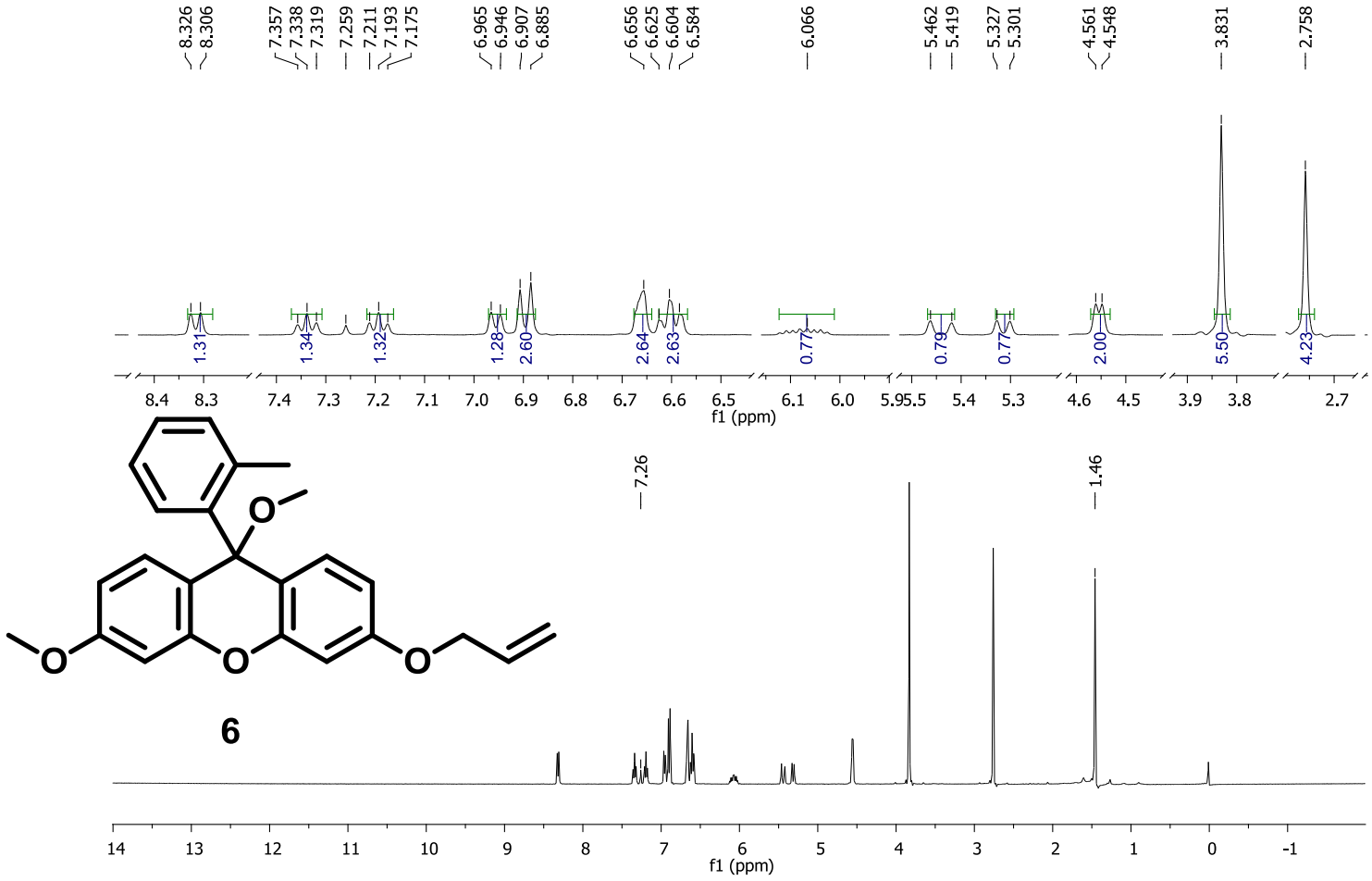

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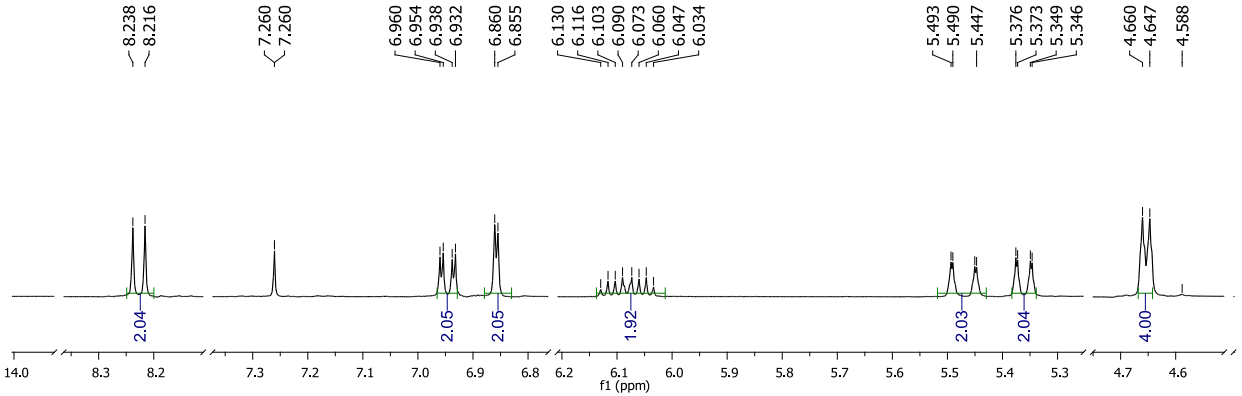

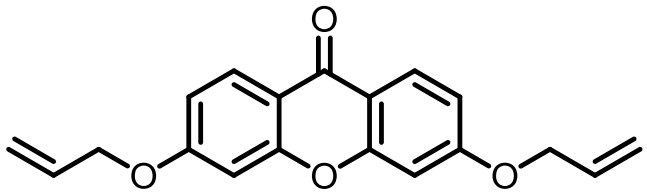

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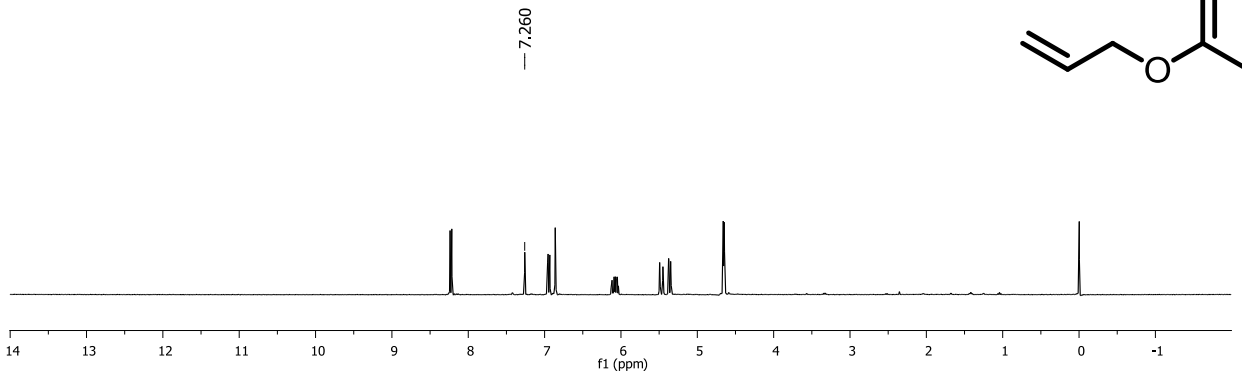

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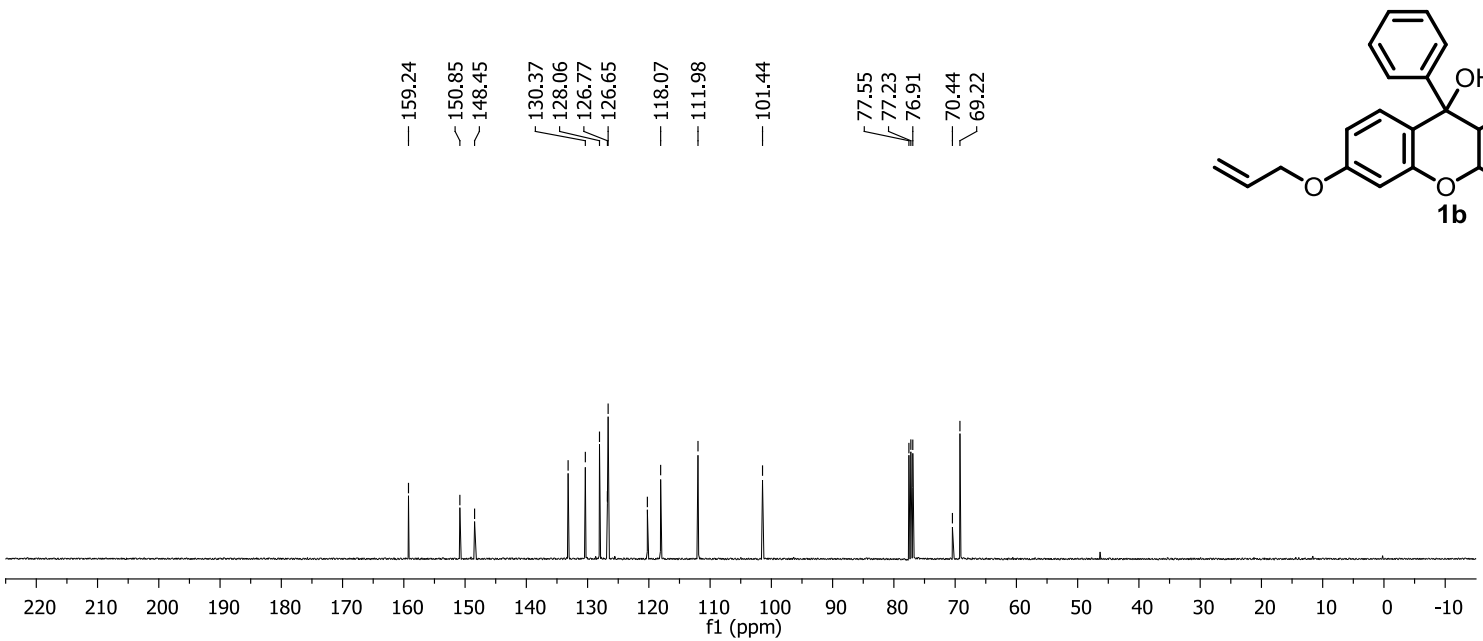
^{13}C NMR



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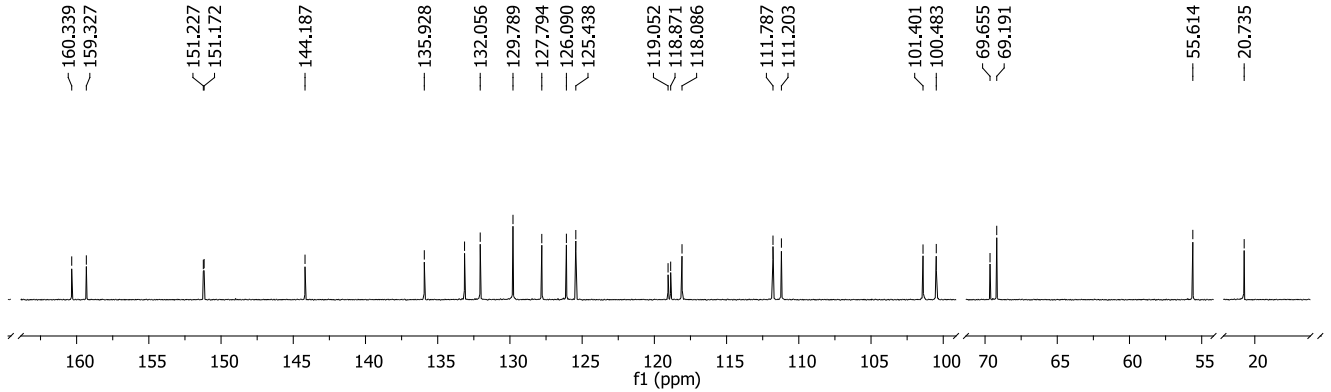

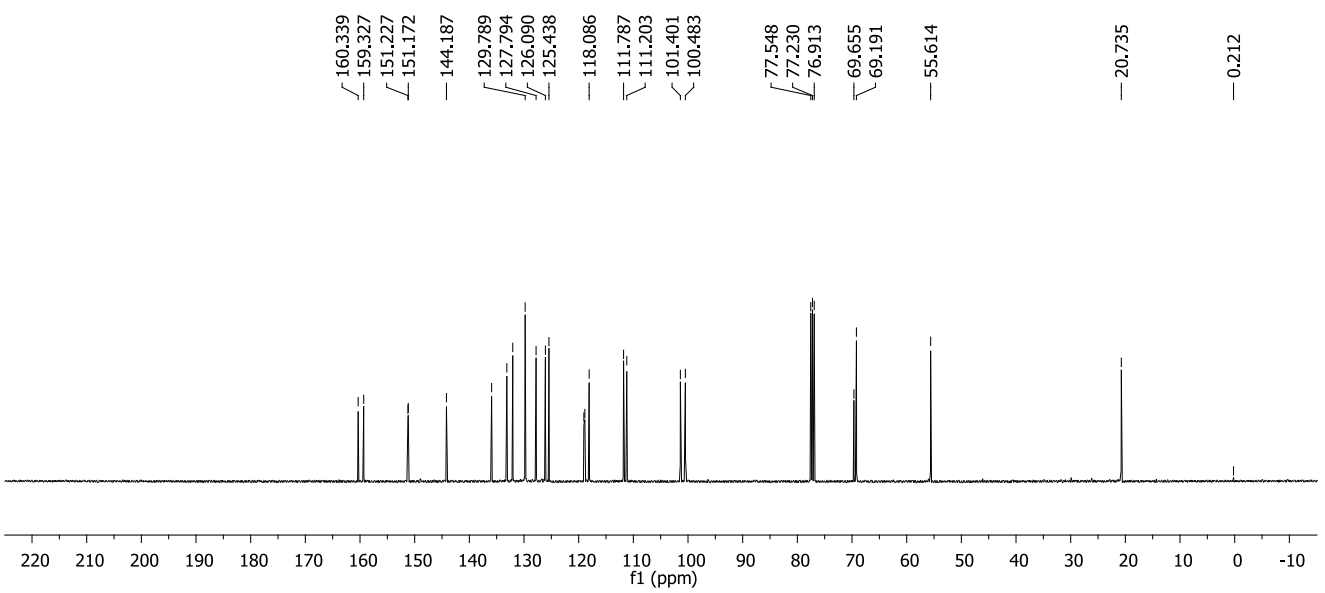

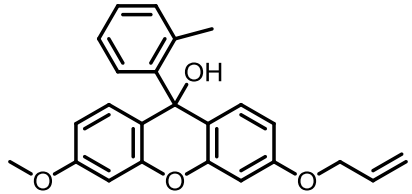

1c

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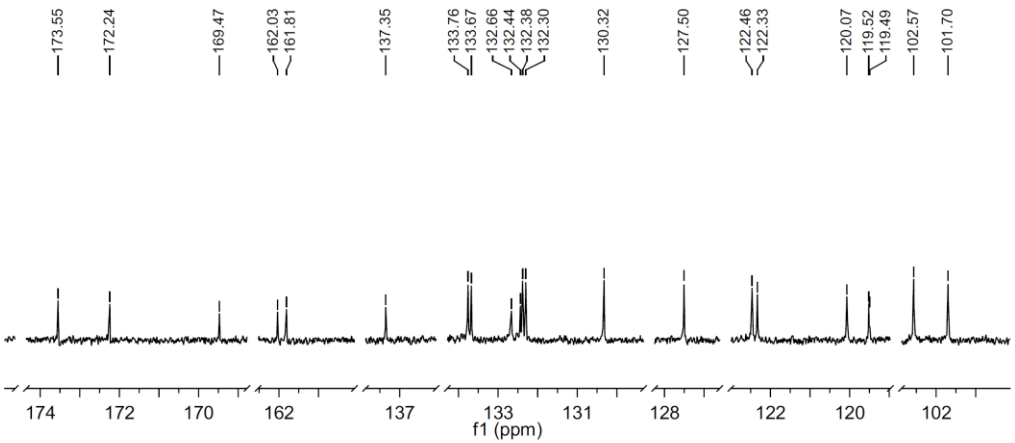
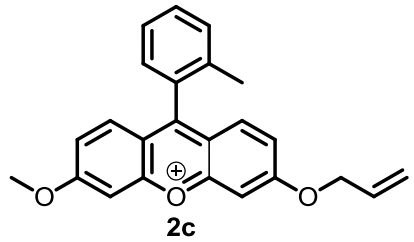
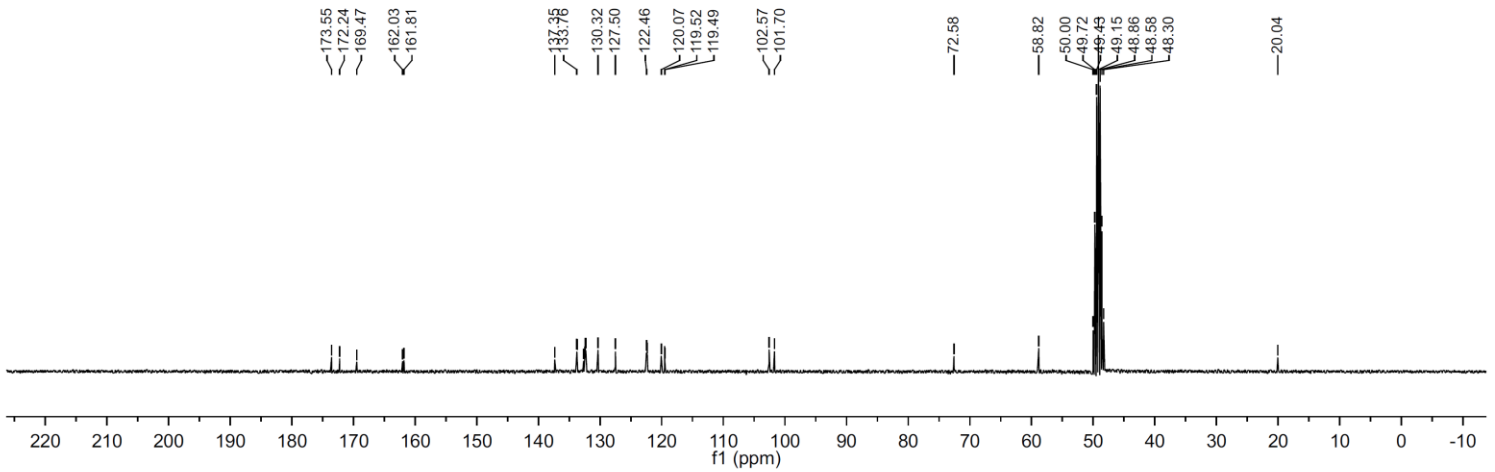




2c



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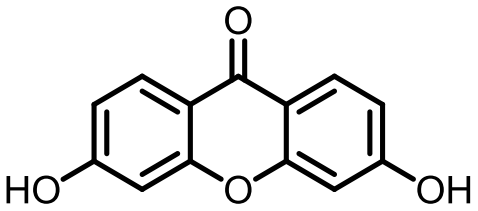

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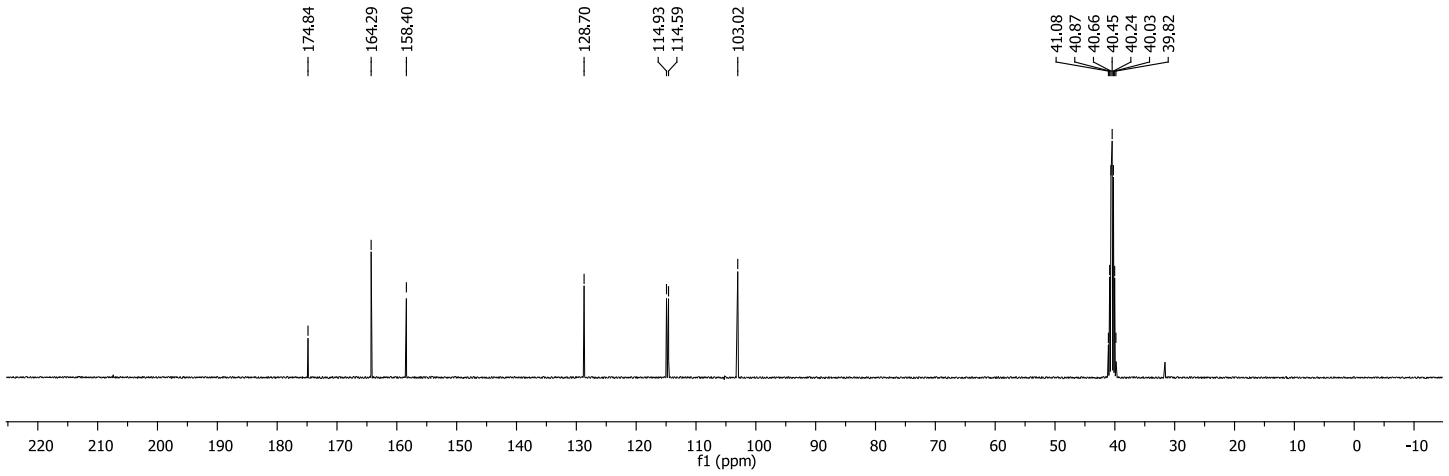

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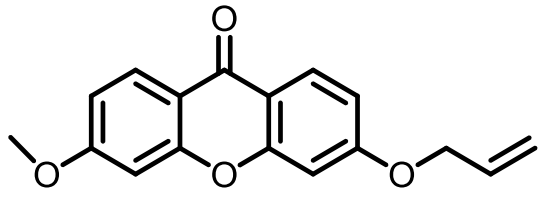

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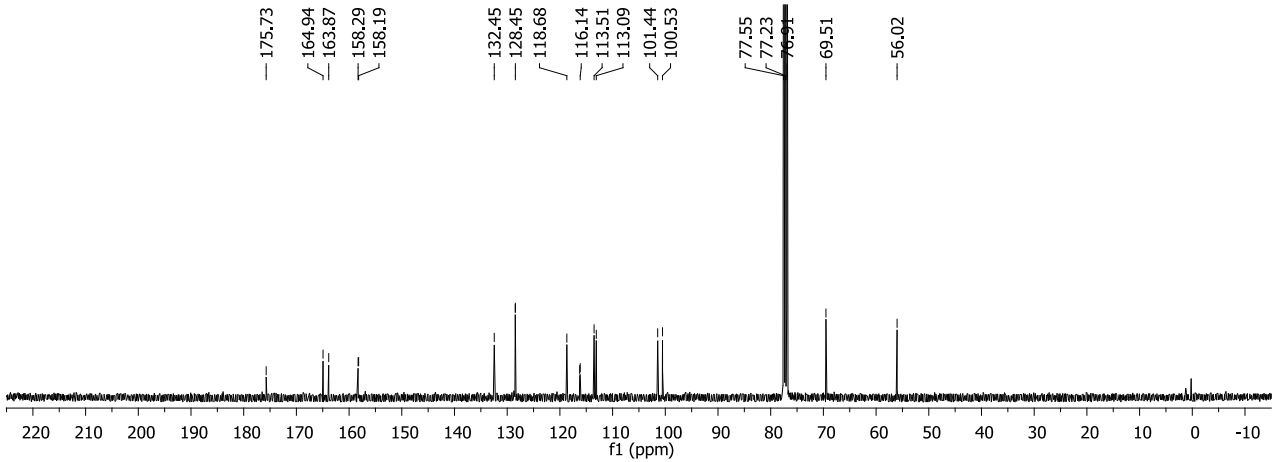

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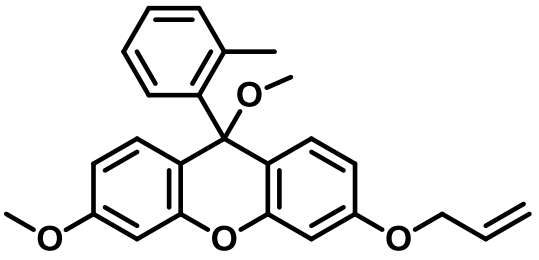

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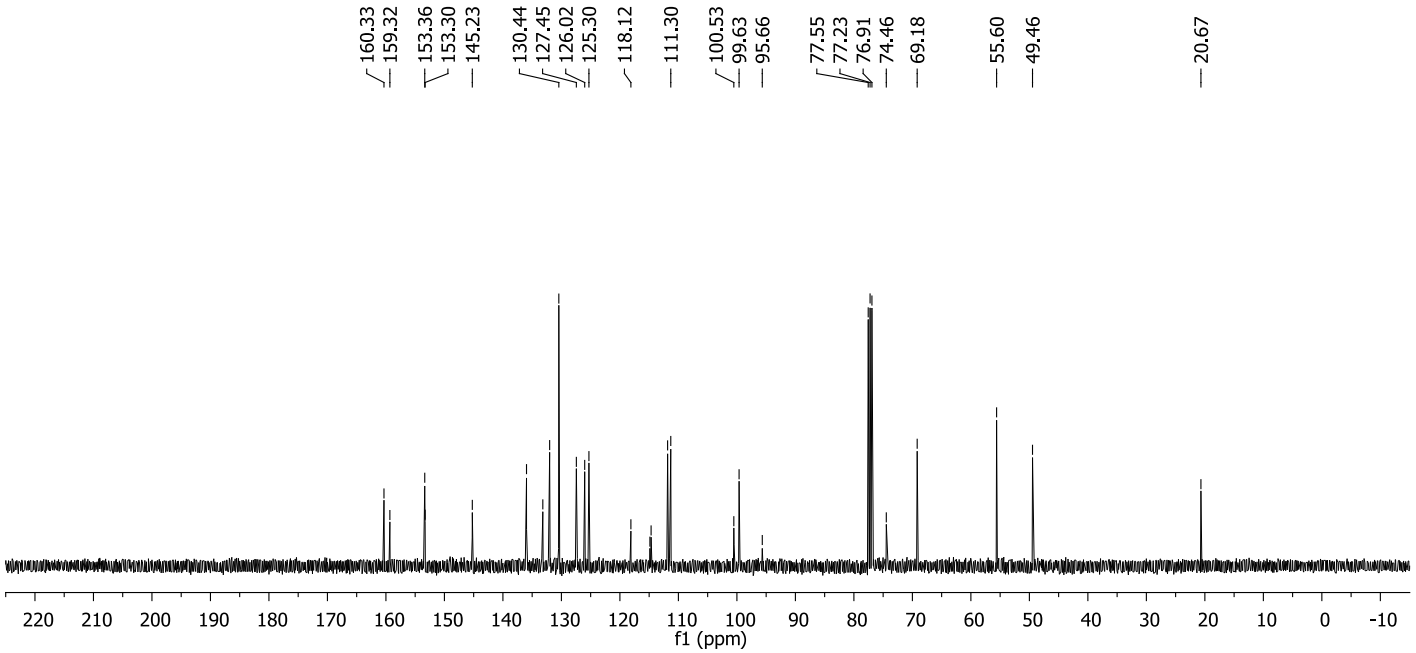

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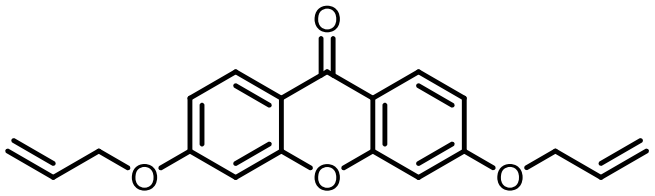

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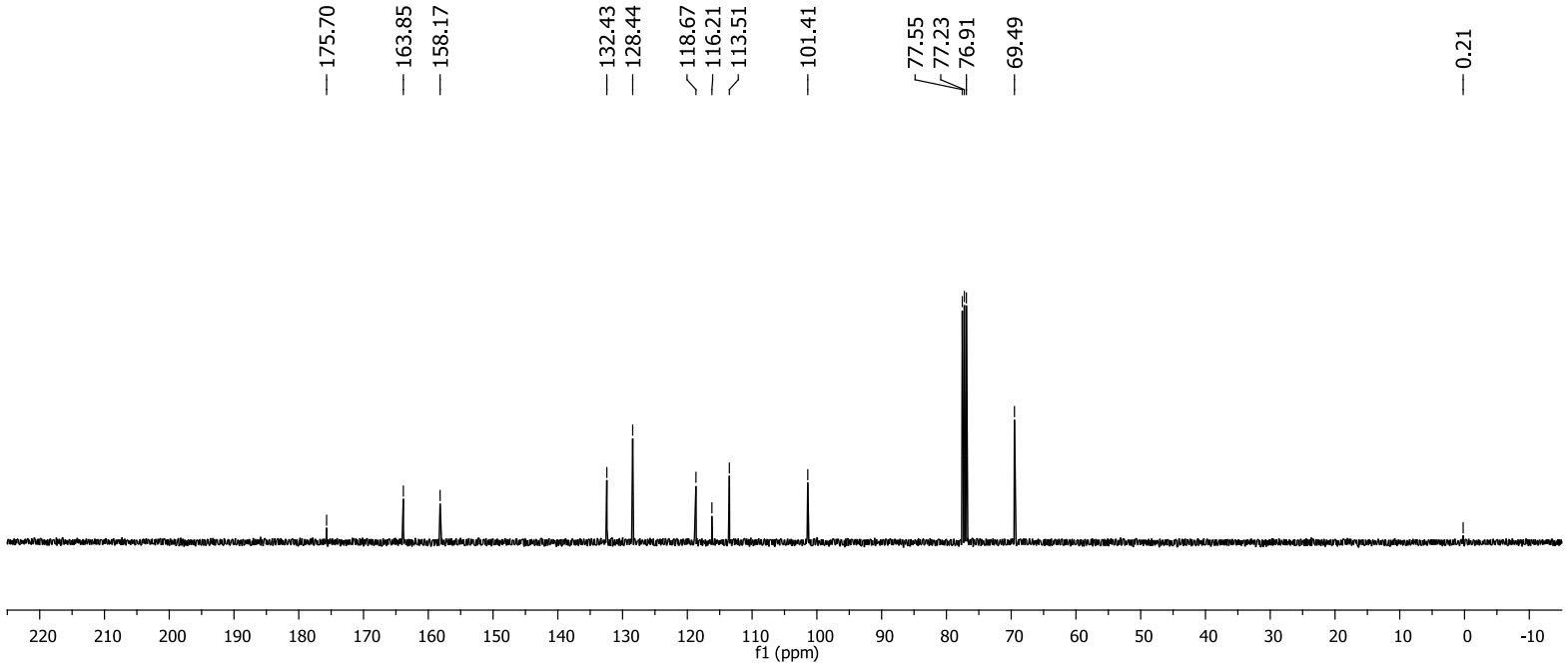

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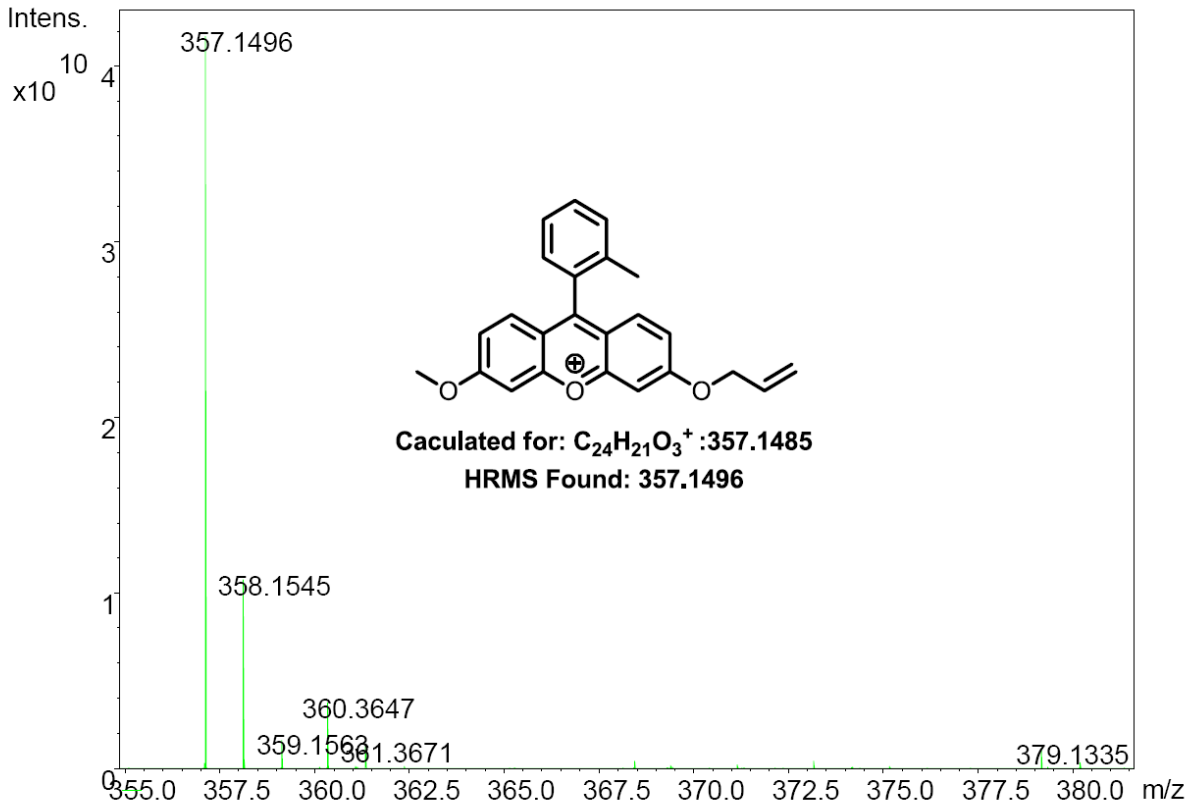
Figure S1. HRMS of cation 2c in Methanol : TFA solution



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