Supporting Information for

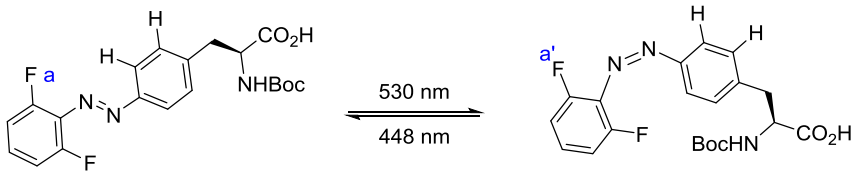
Synthesis and Site-Specific Incorporation of Red-Shifted Azobenzene Amino Acids into Proteins

Alford A. John, Carlo P. Ramil, Yulin Tian, Gang Cheng, and Qing Lin*

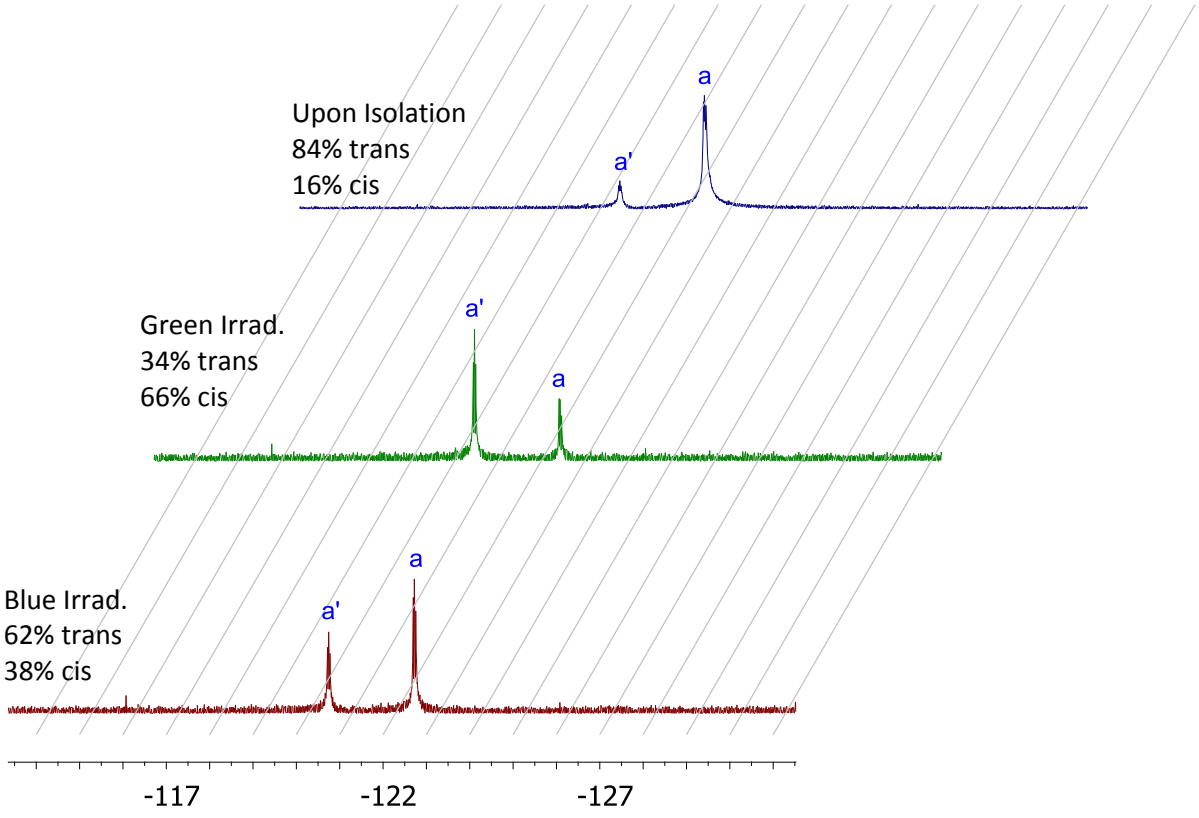
Department of Chemistry, State University of New York at Buffalo, Buffalo, New York 14260-3000, United States, qinglin@buffalo.edu

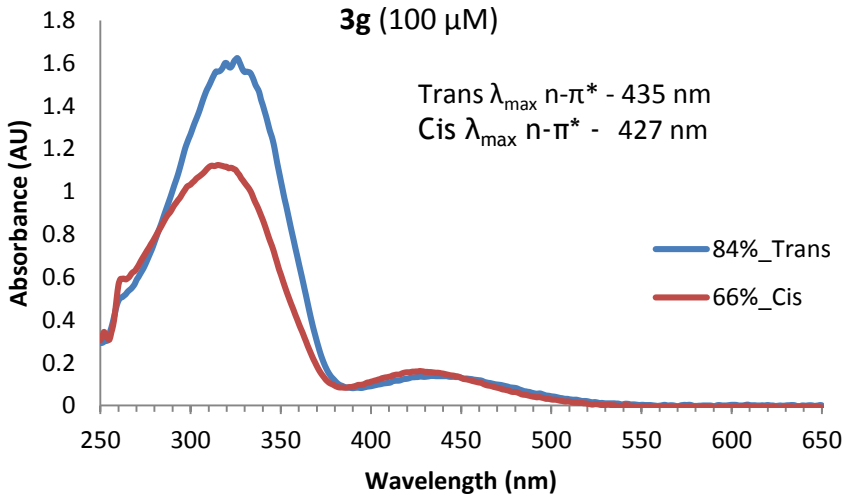
Supplemental Data

Table 2, compound **3g:**



trans-**3g** *cis*-**3g**





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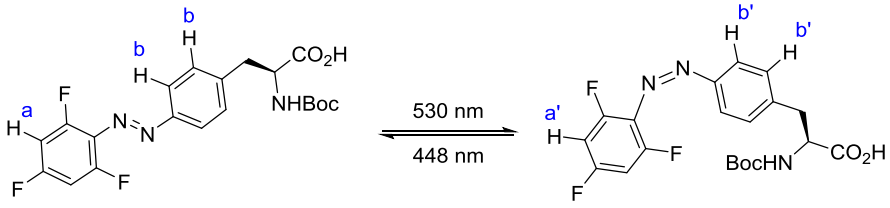
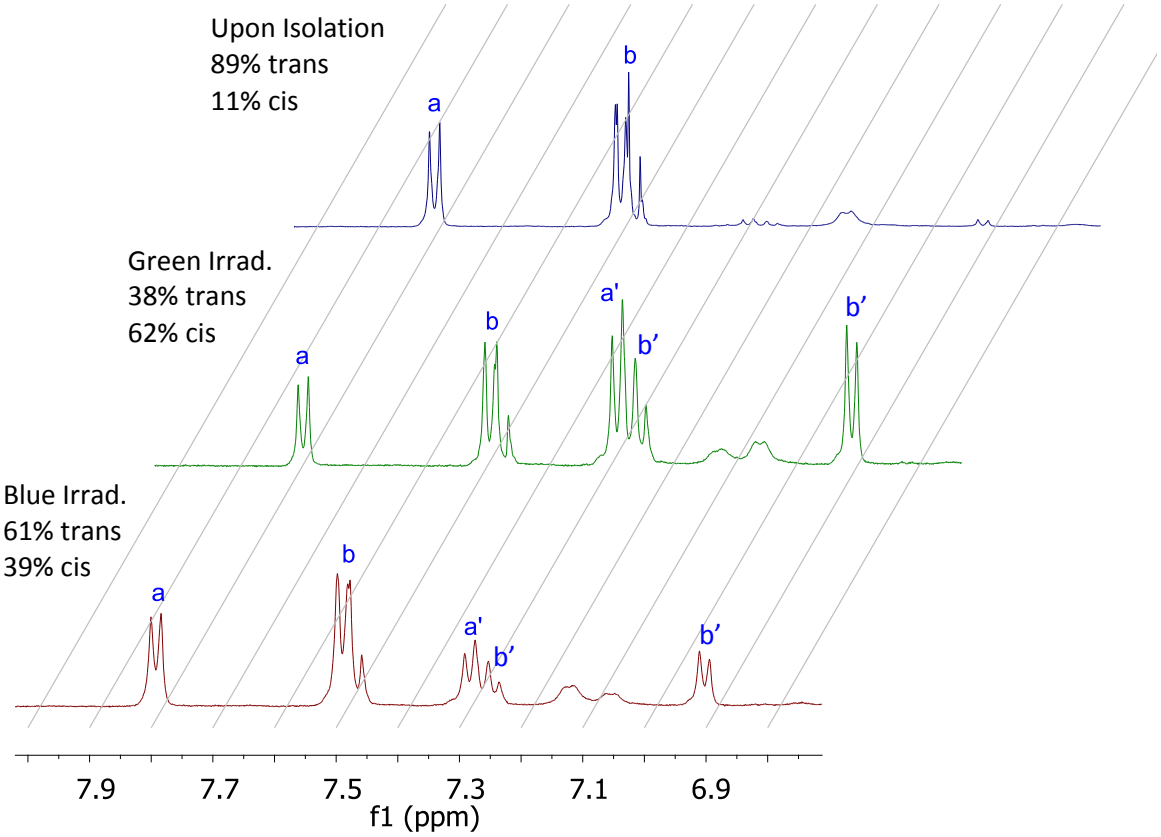
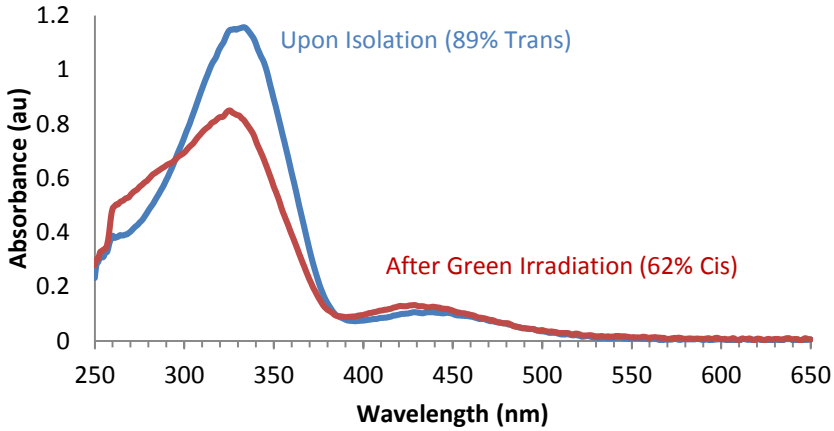


trans-**3h** *cis*-**3h**





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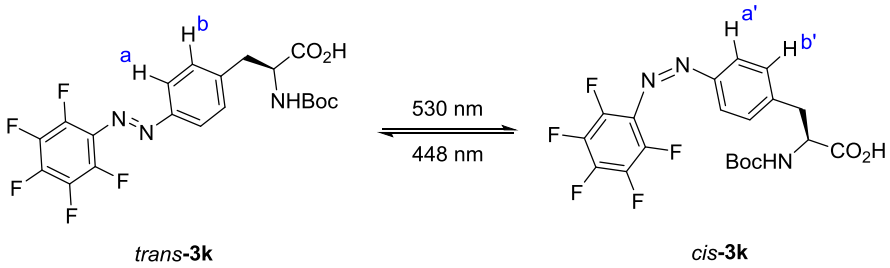


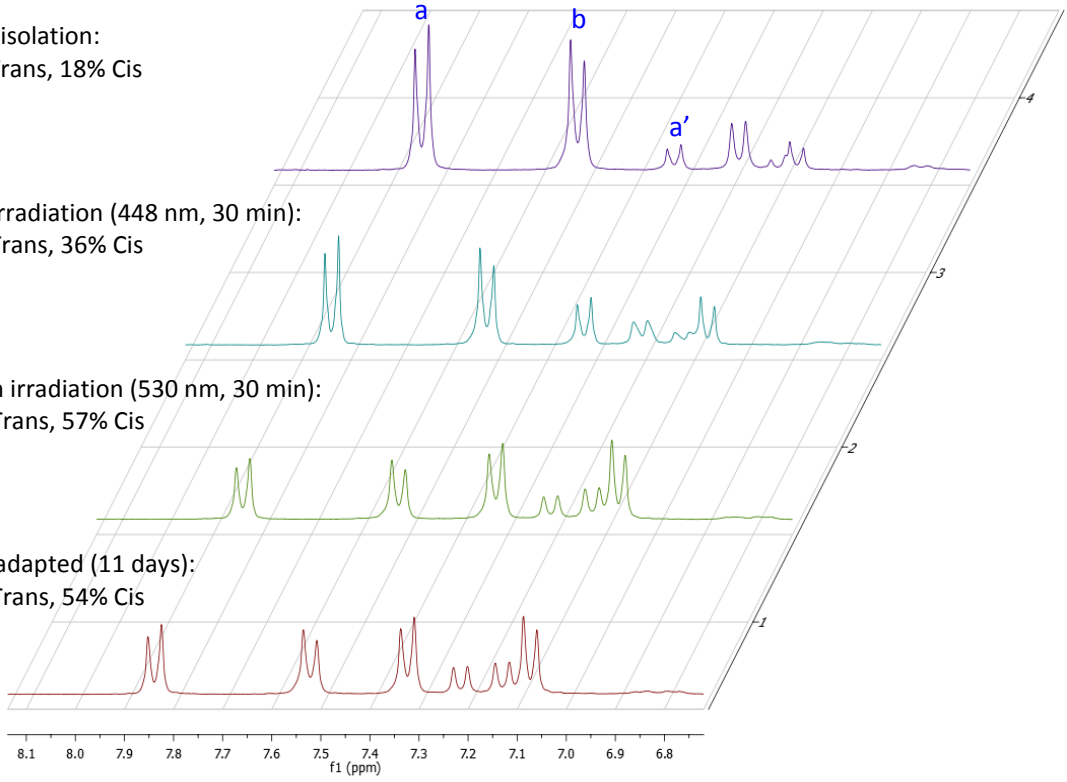
trans-**3k** *cis*-**3k**

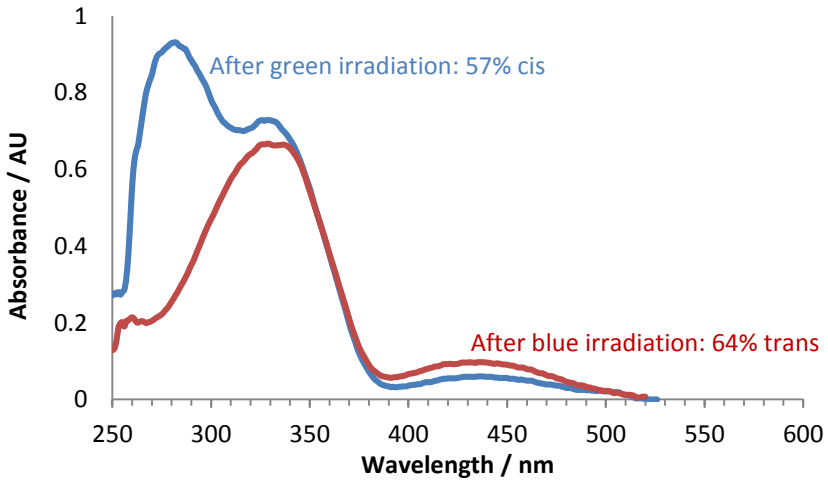
Upon isolation:
82% Trans, 18% Cis

Blue irradiation (448 nm, 30 min):
64% Trans, 36% Cis

Green irradiation (530 nm, 30 min):
43% Trans, 57% Cis

Dark adapted (11 days):
46% Trans, 54% Cis





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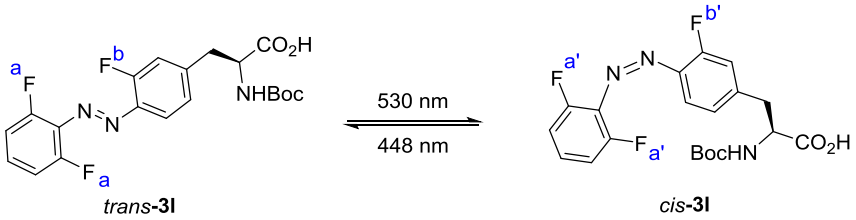

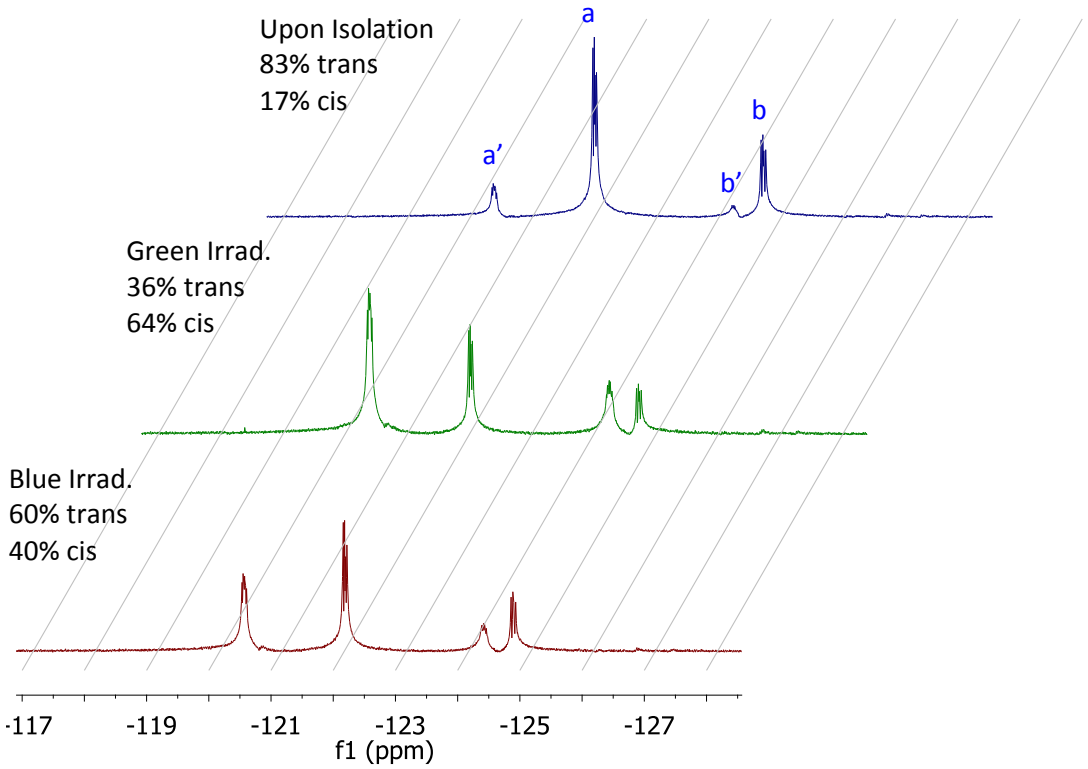

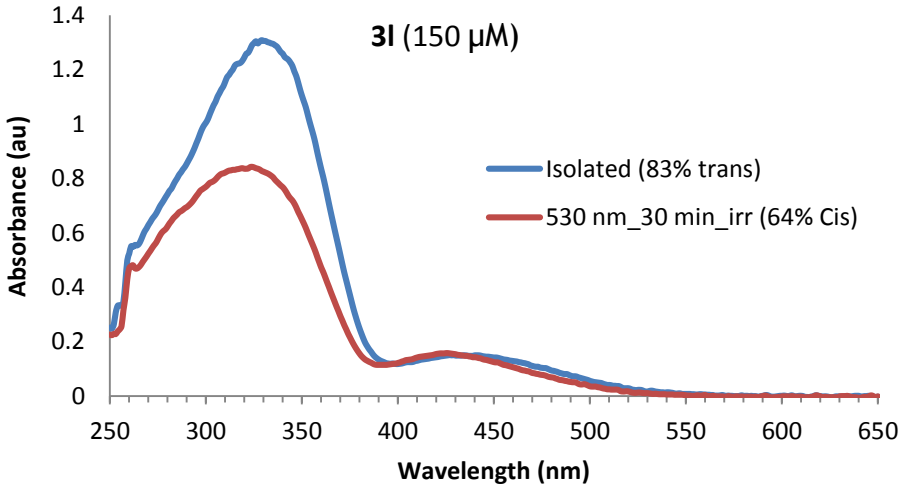

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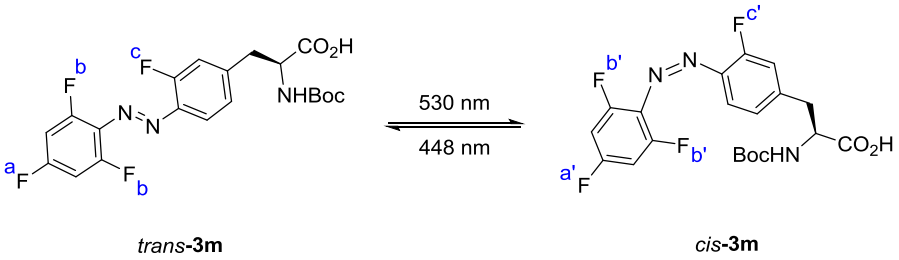
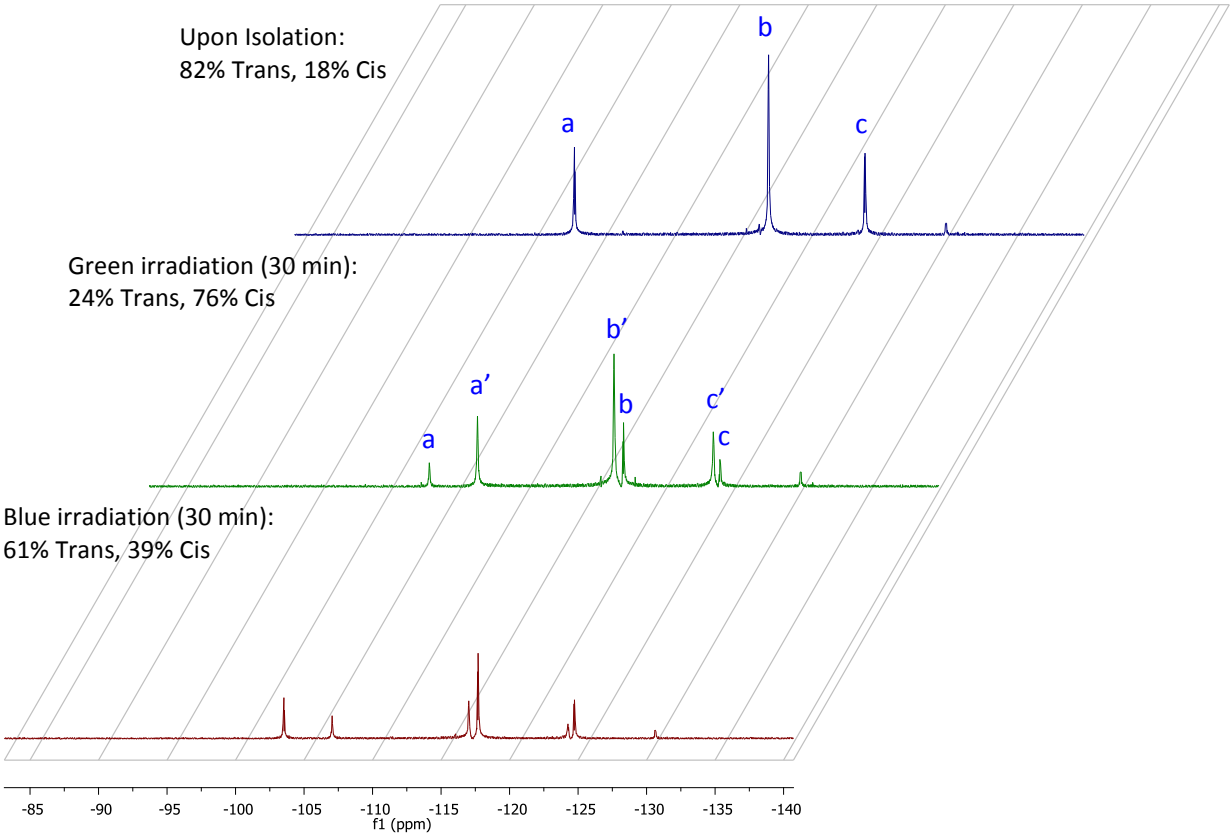
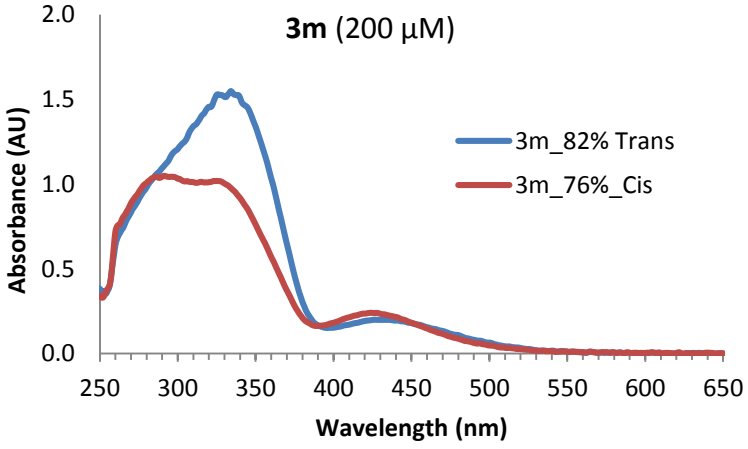


trans-**3m** *cis*-**3m**





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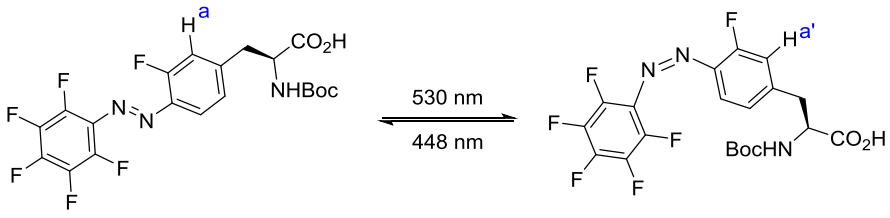
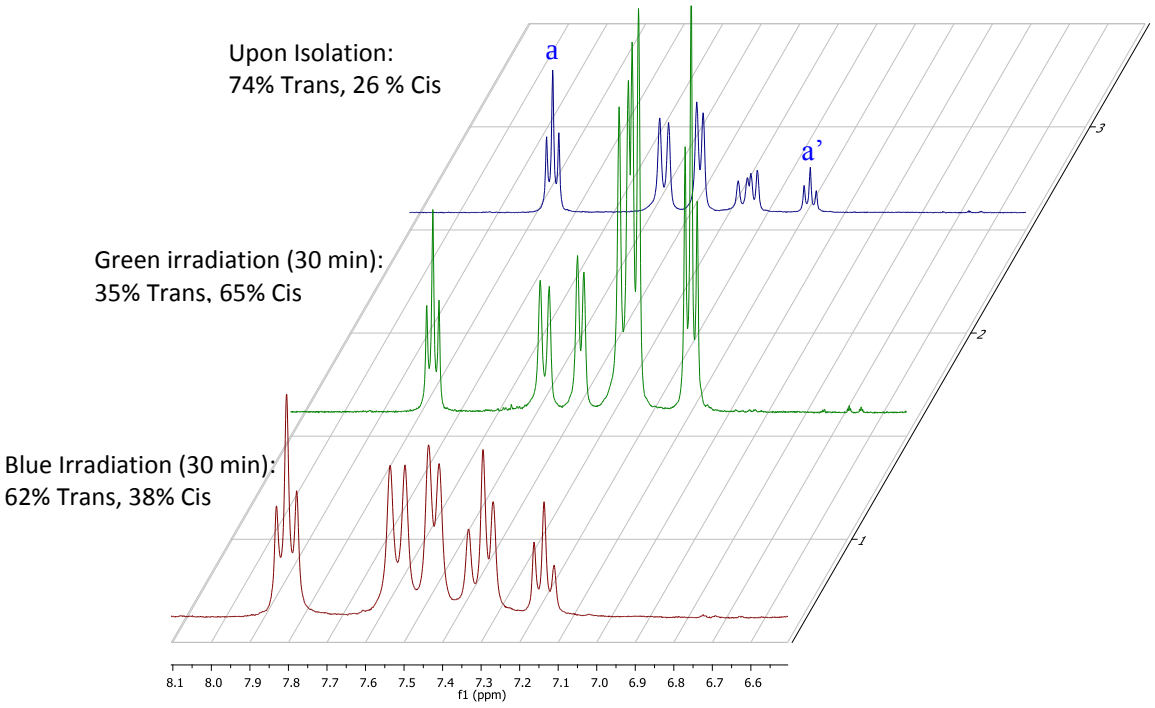
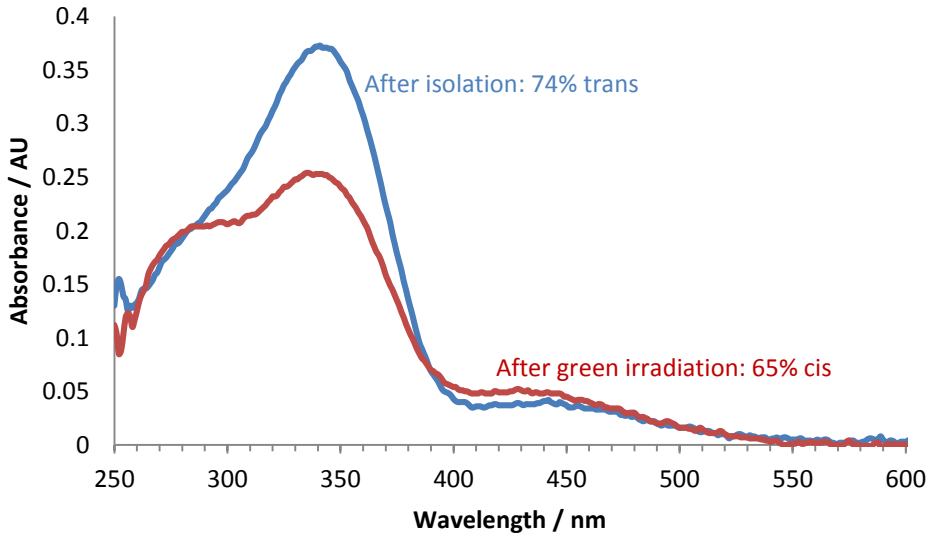


trans-**3n** *cis*-**3n**





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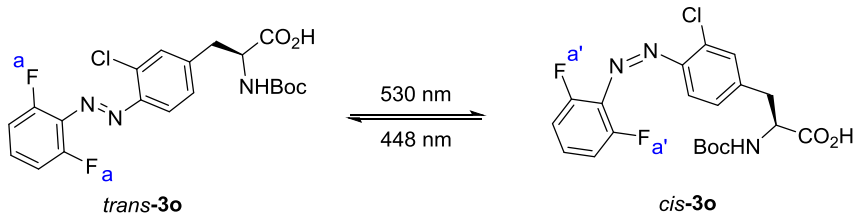
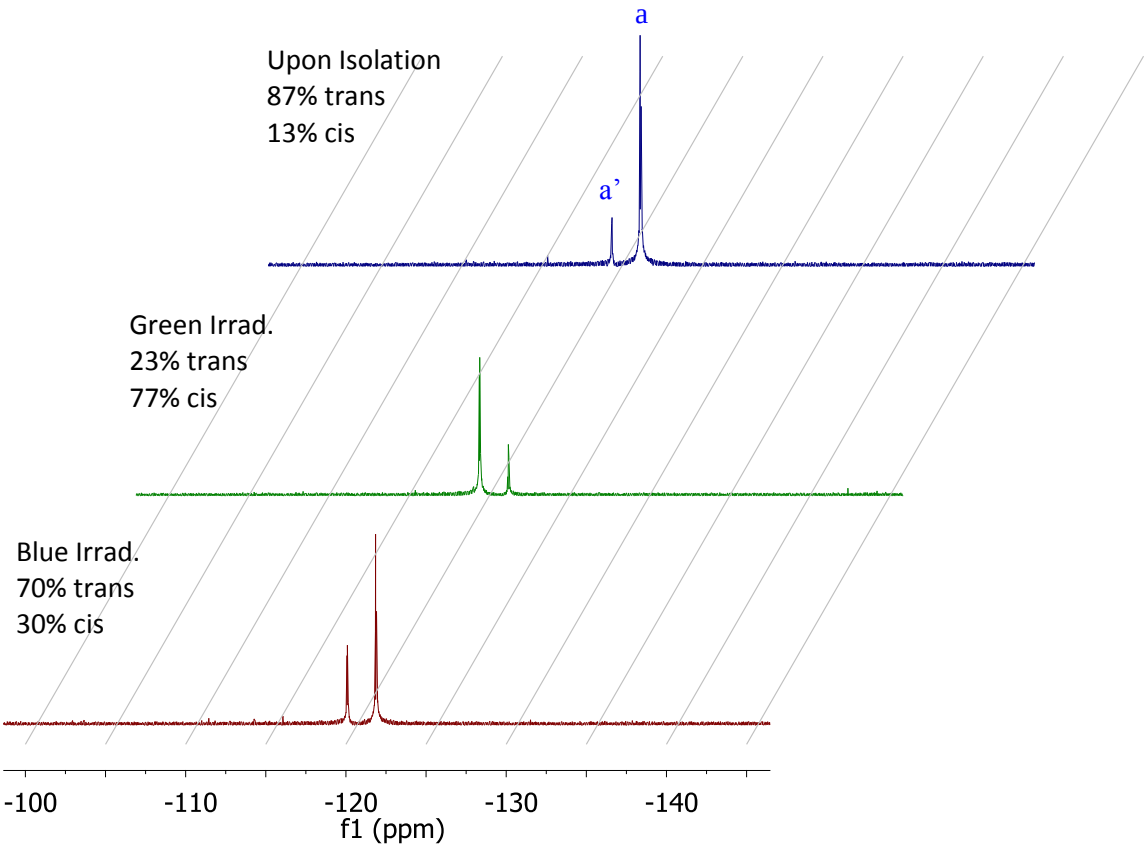
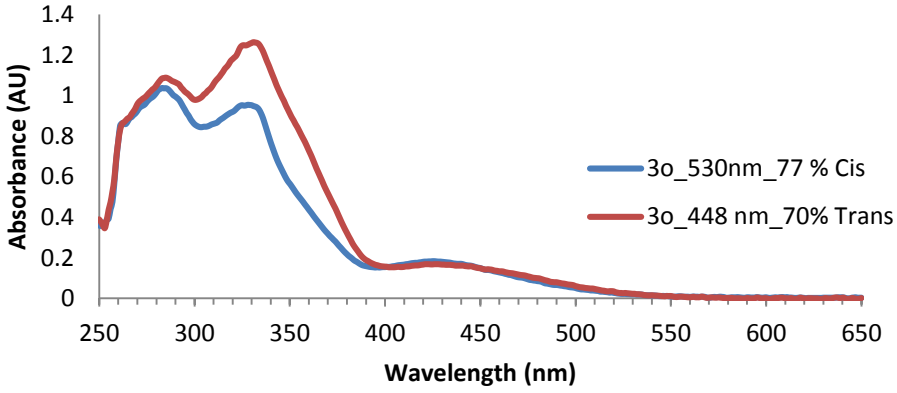


trans-**3o** *cis*-**3o**



3o (100 µM)



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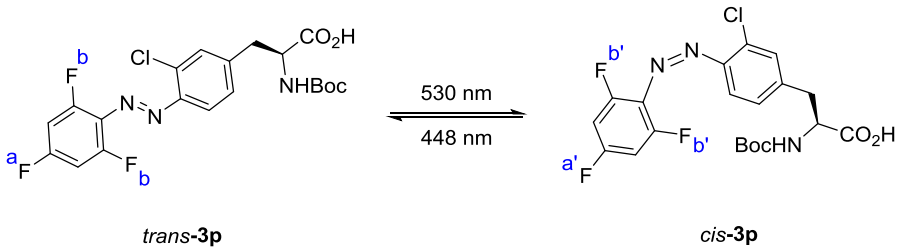

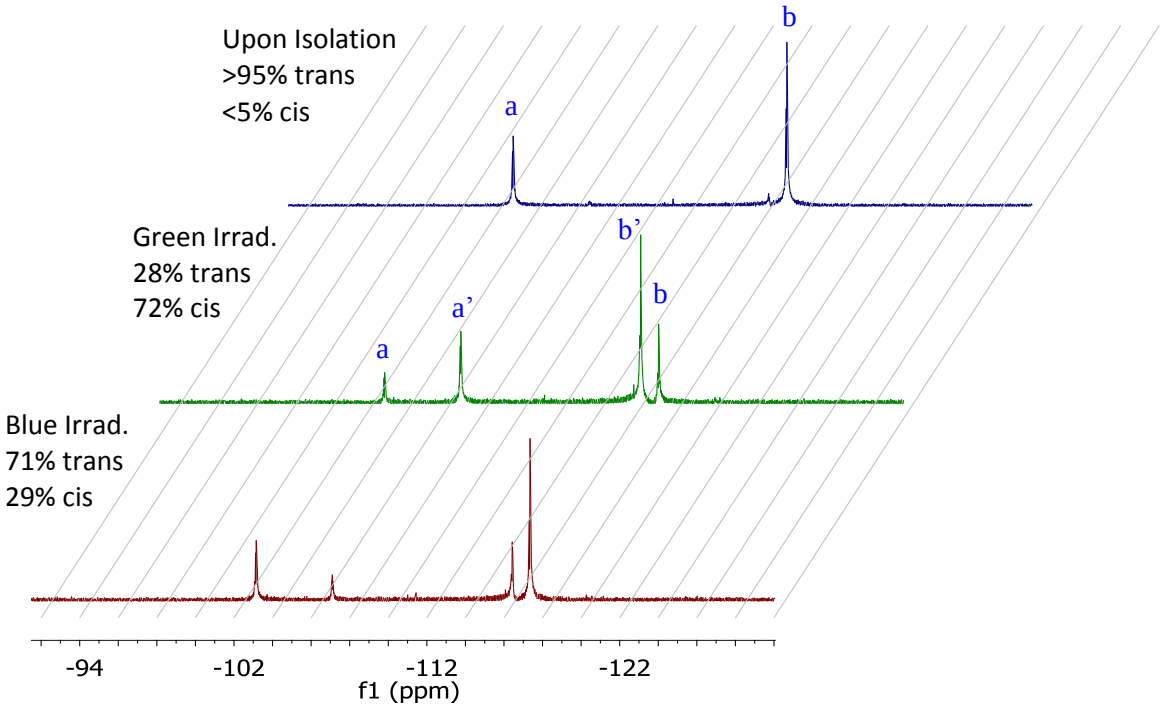

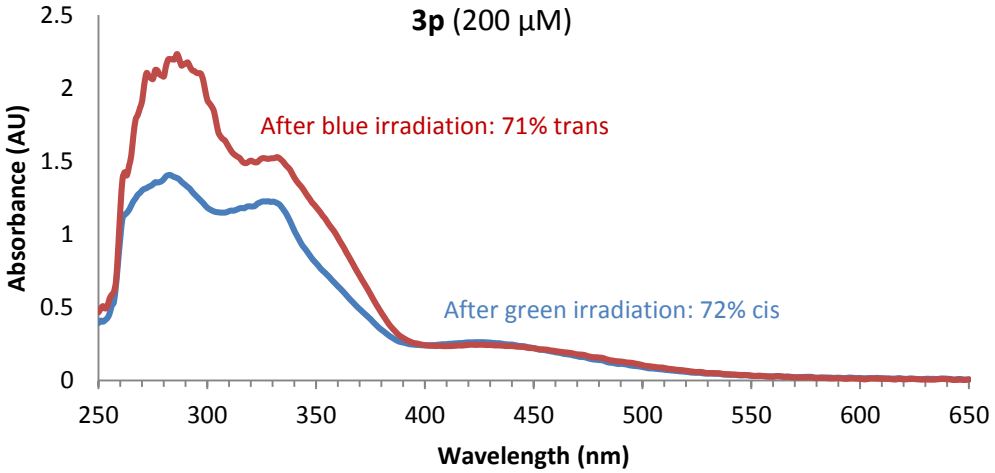

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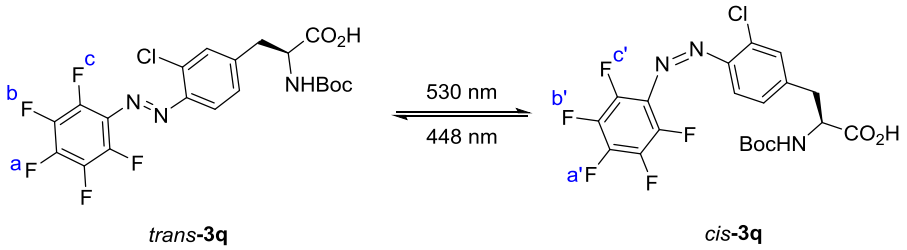
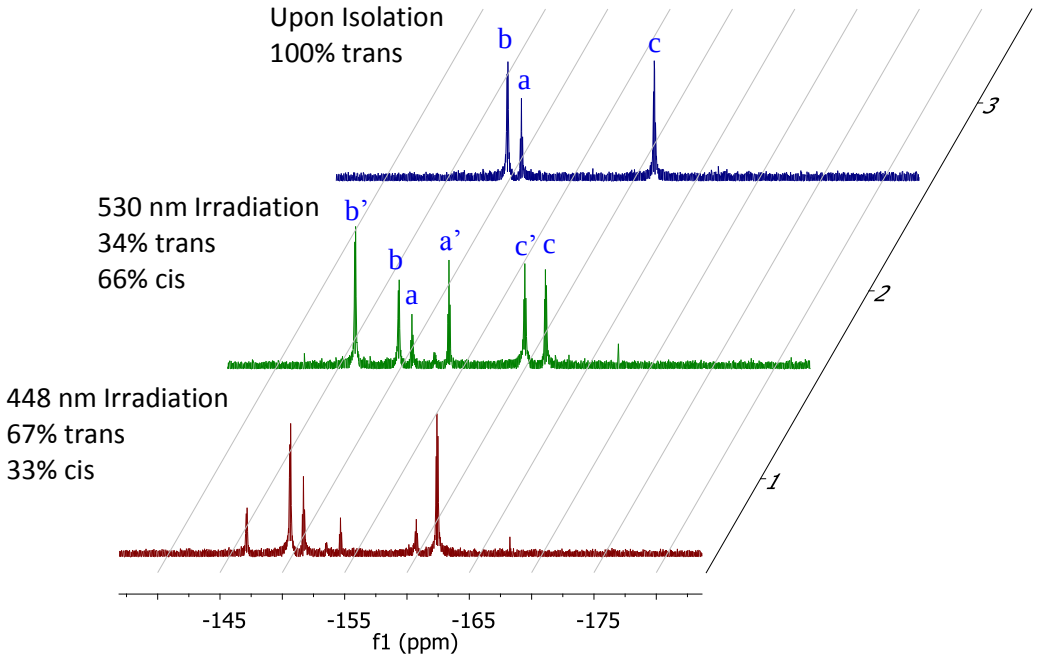




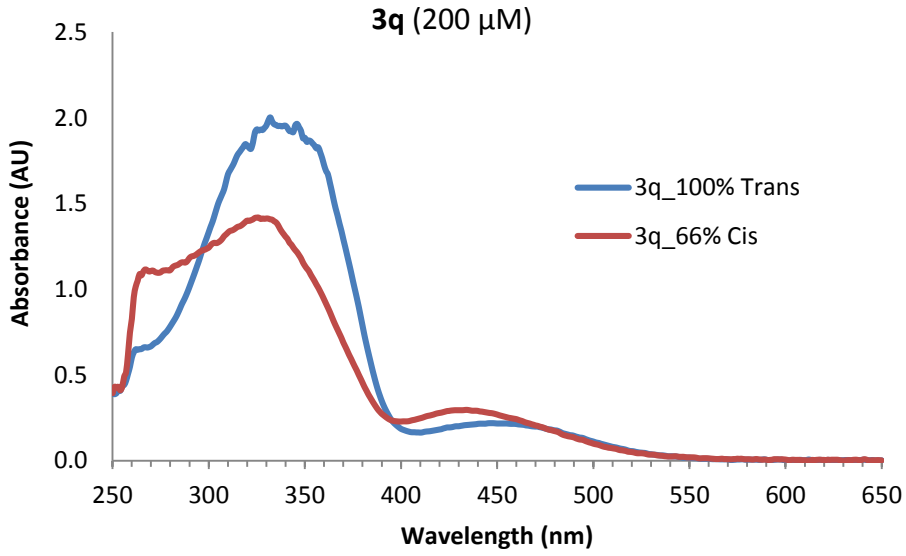
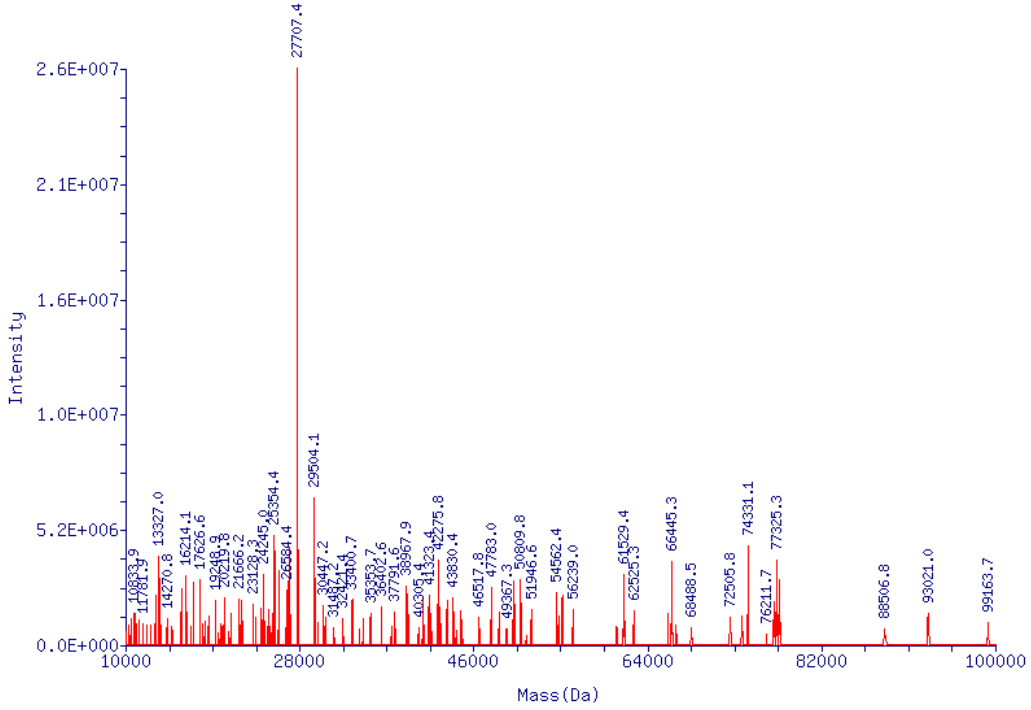


Table 3, (-) Control: sfGFP-S2Gln with first Met removed: MS-ESI calcd for sfGFP-S2Gln, 27708.6, found 27707.4.



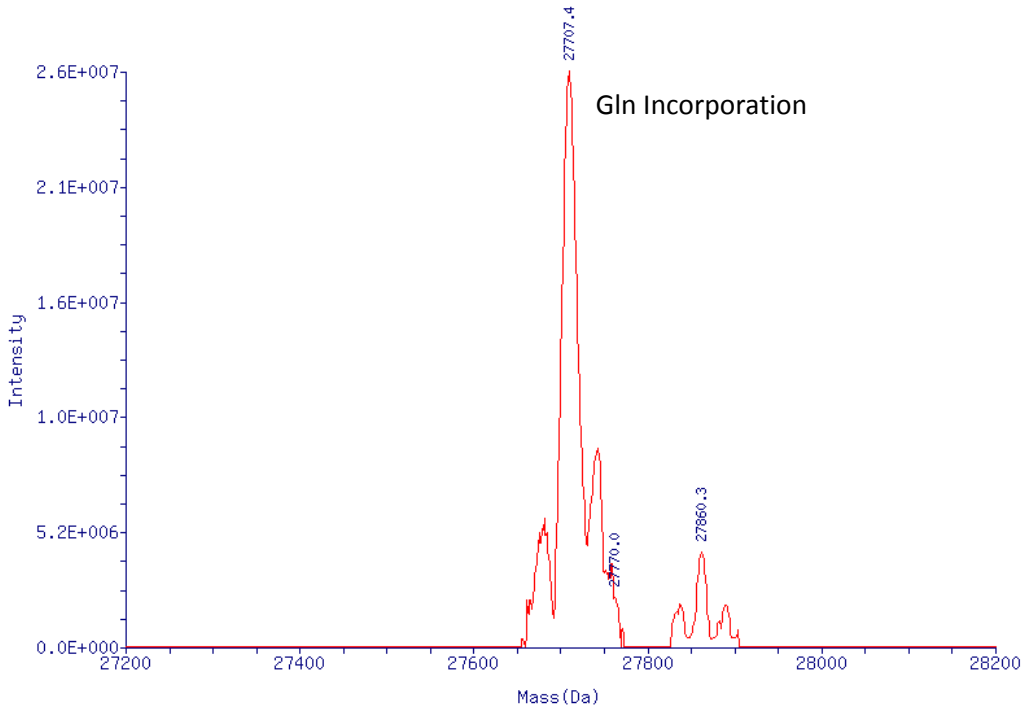
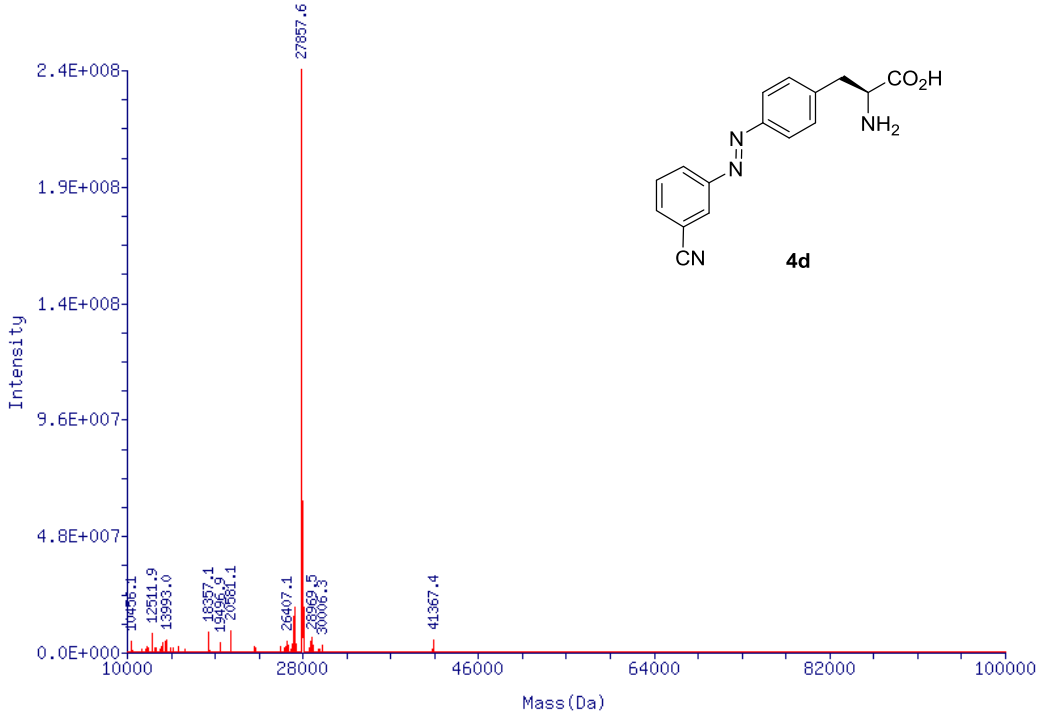


Table 3, compound **4d**: sfGFP-S2→**4d** with first Met removed: MS-ESI calcd 27856.3, found 27857.6.



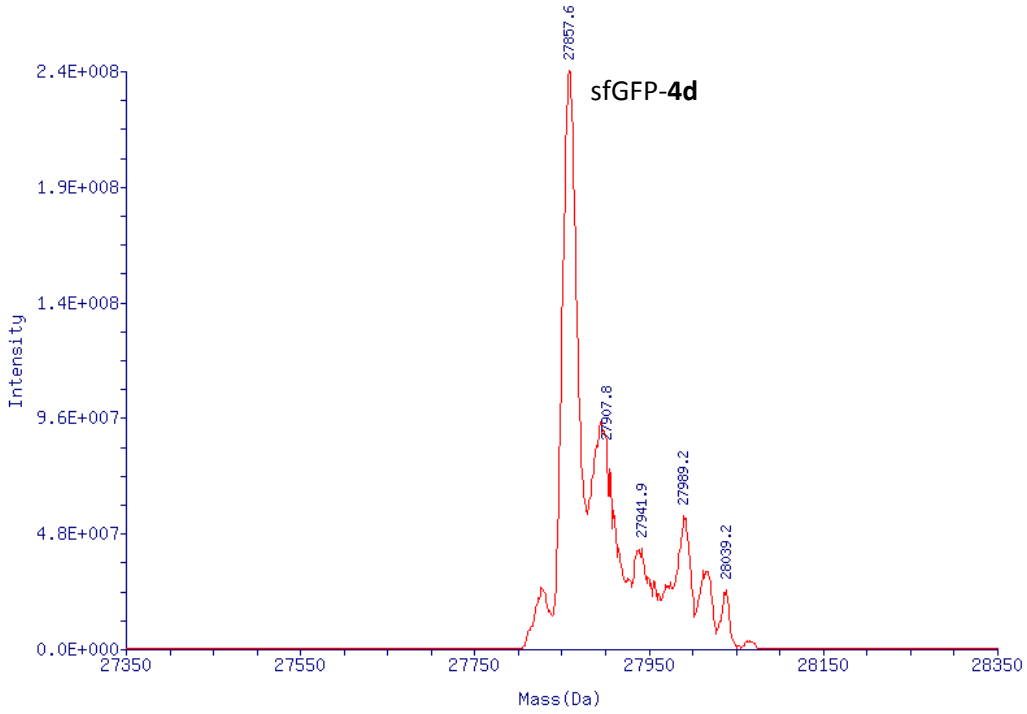
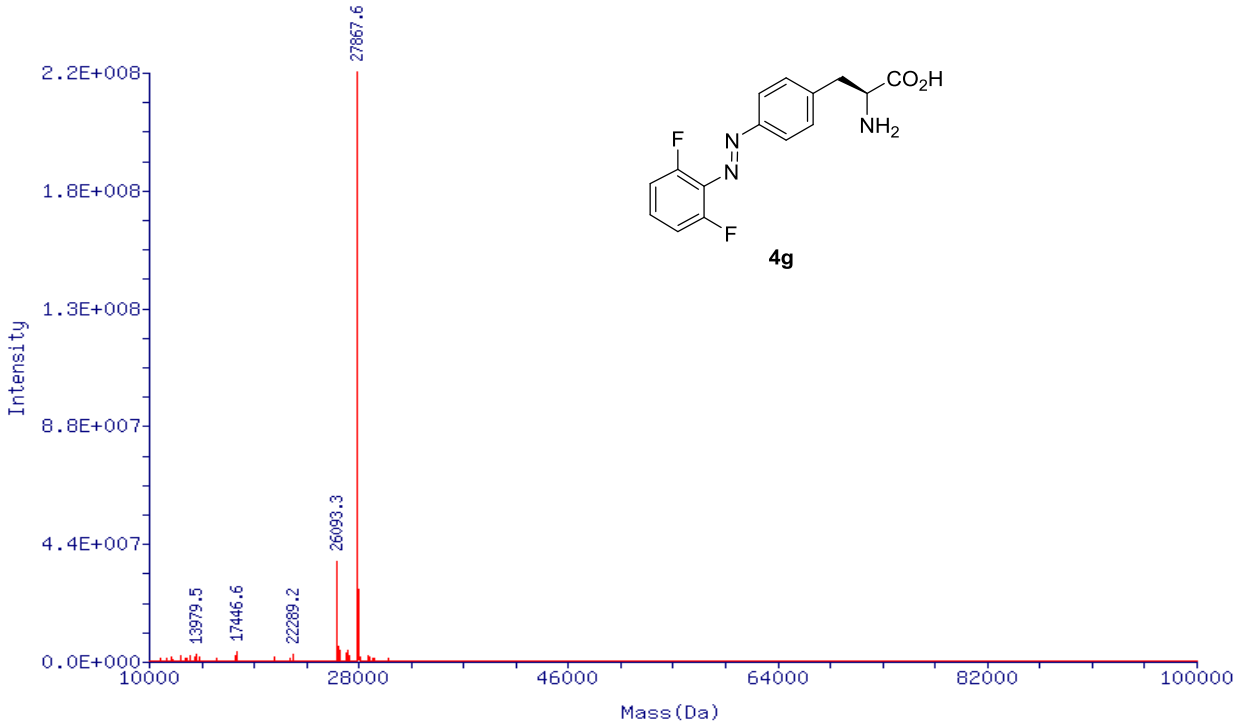


Table 3, compound **4g**: sfGFP-S2→**4g** with first Met removed: MS-ESI calcd 27868.1, found 27867.6.



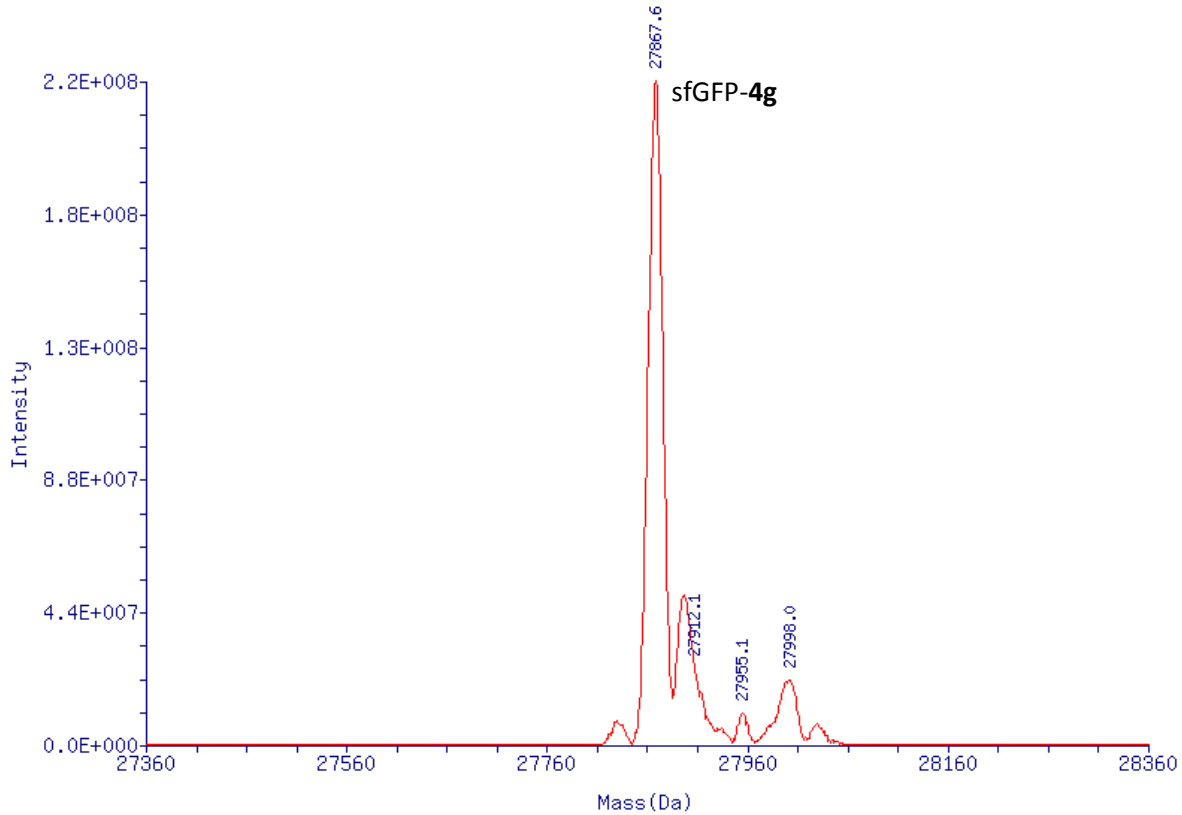
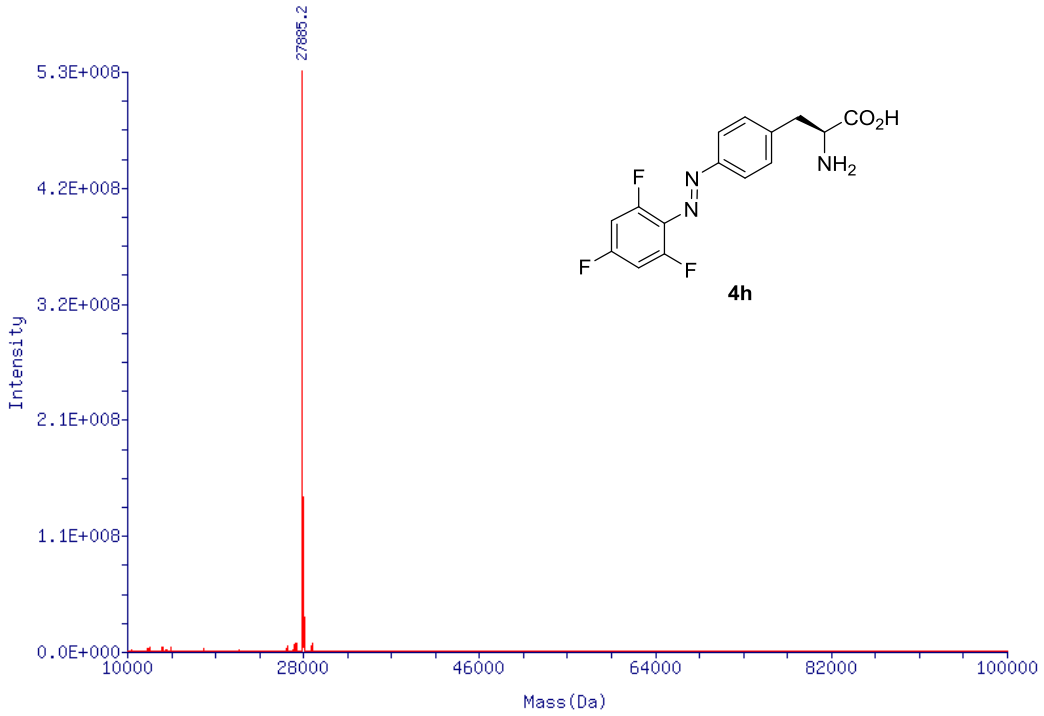


Table 3, compound **4h**: sfGFP-S2→**4h** with first Met removed: MS-ESI calcd 27886.1, found 27885.2.



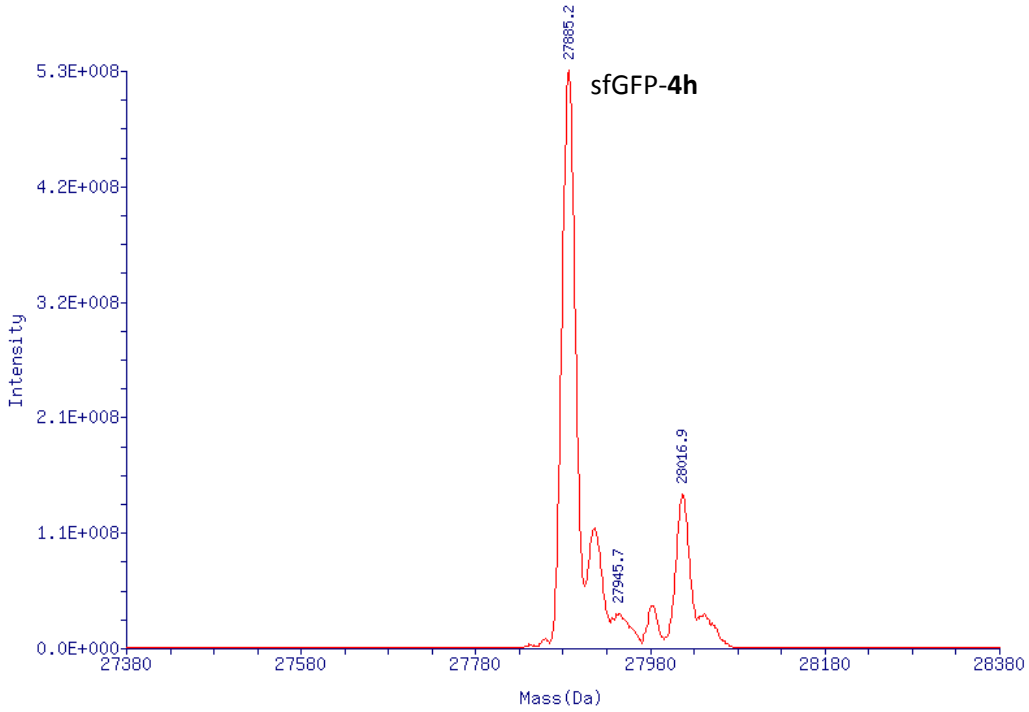
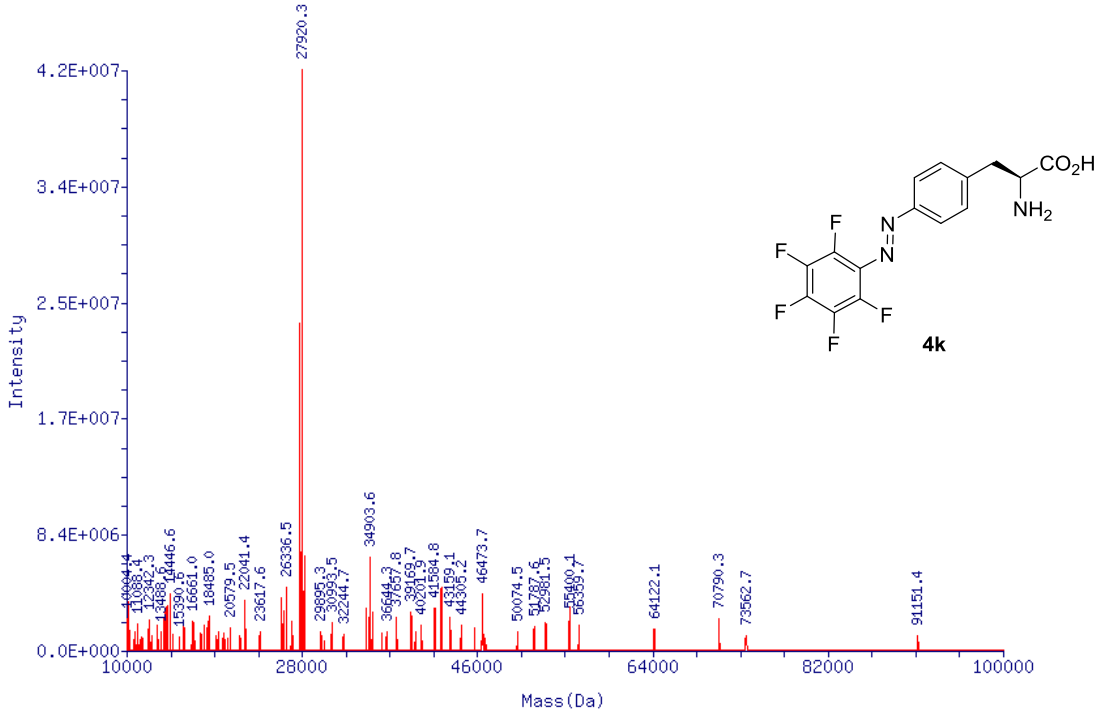


Table 3, compound **4k**: sfGFP-S2→**4k** with first Met removed: MS-ESI calcd 27921.3, found 27920.3.



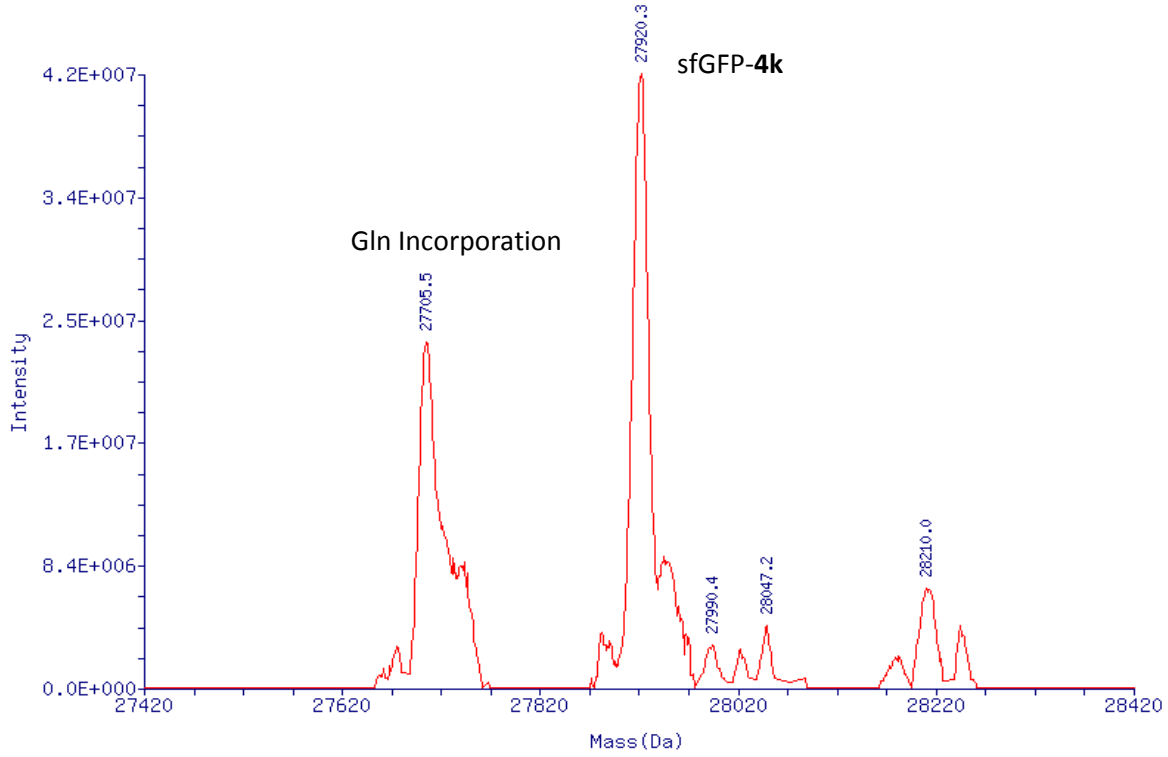
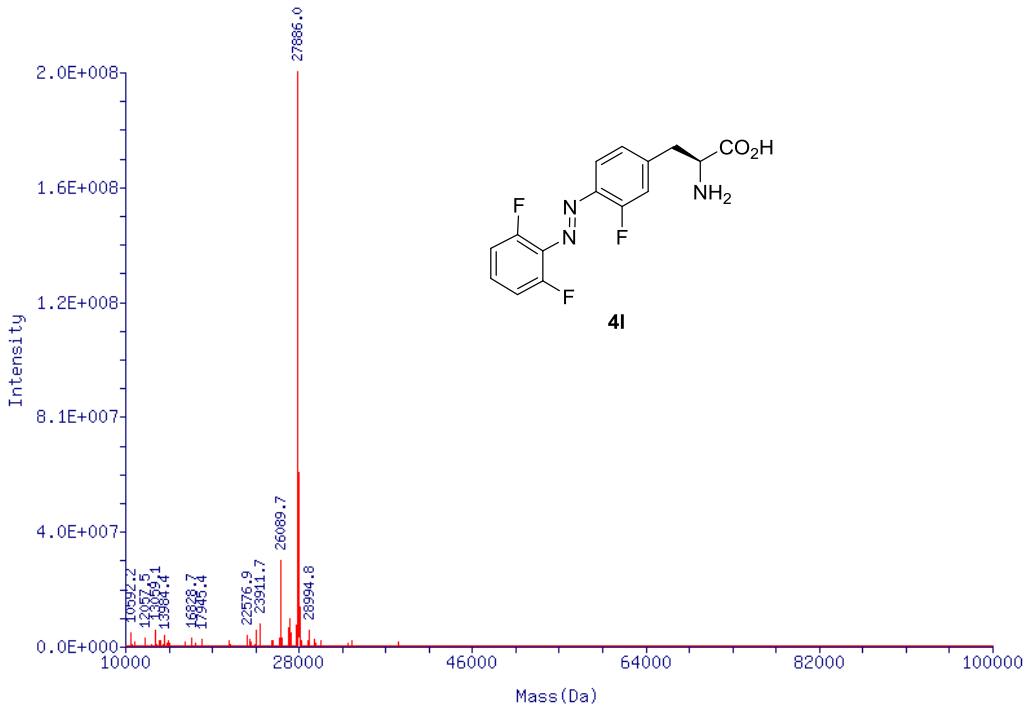


Table 3, compound **4l**: sfGFP-S2→**4l** with first Met removed: MS-ESI calcd 27886.1, found 27886.0.



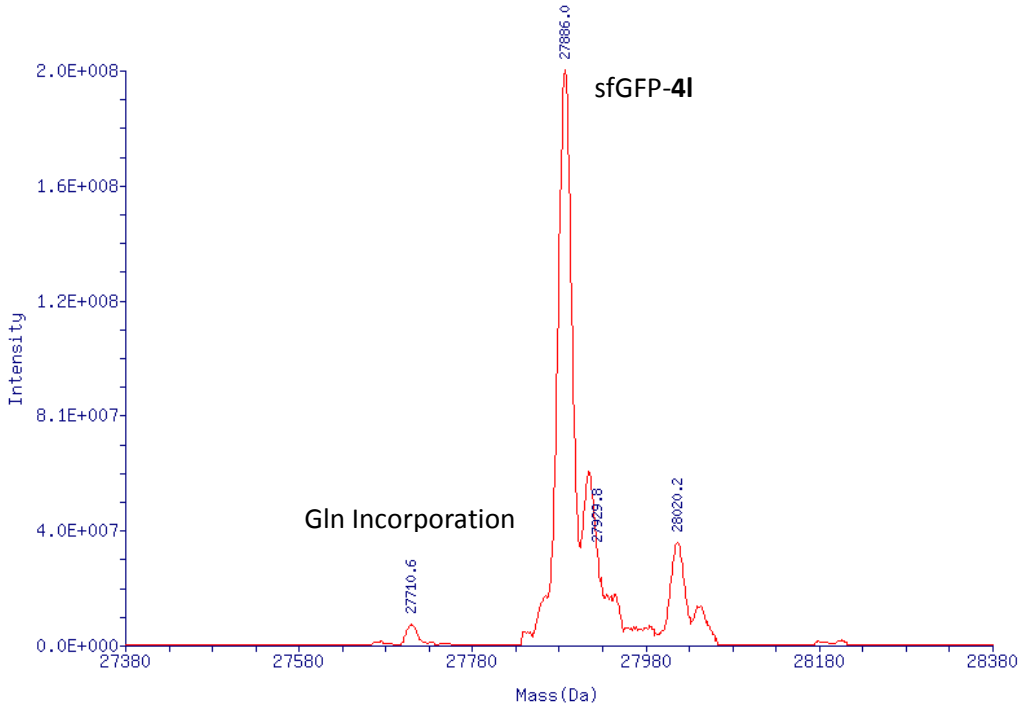
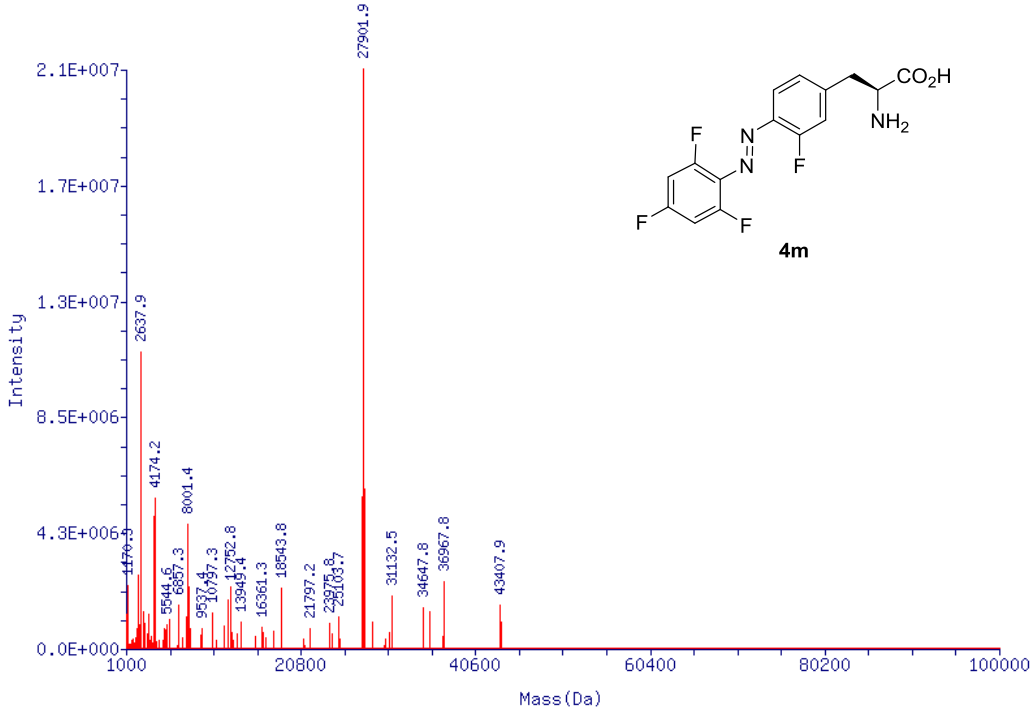


Table 3, compound **4m**: sfGFP-S2→**4m** with first Met removed: MS-ESI calcd 27903.3, found 27901.9.



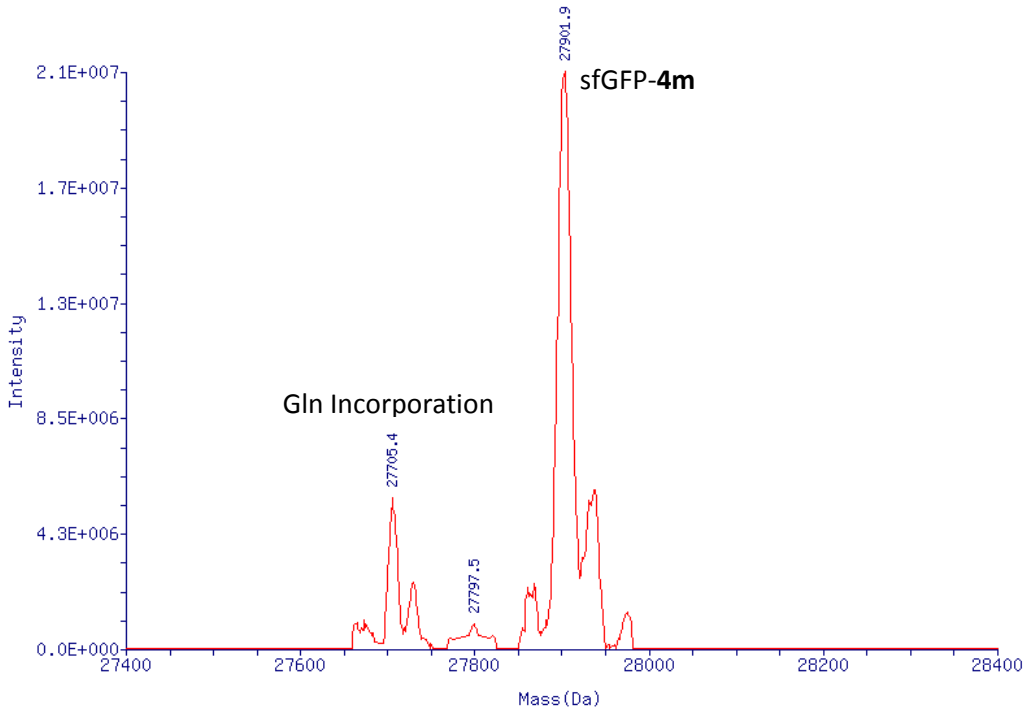
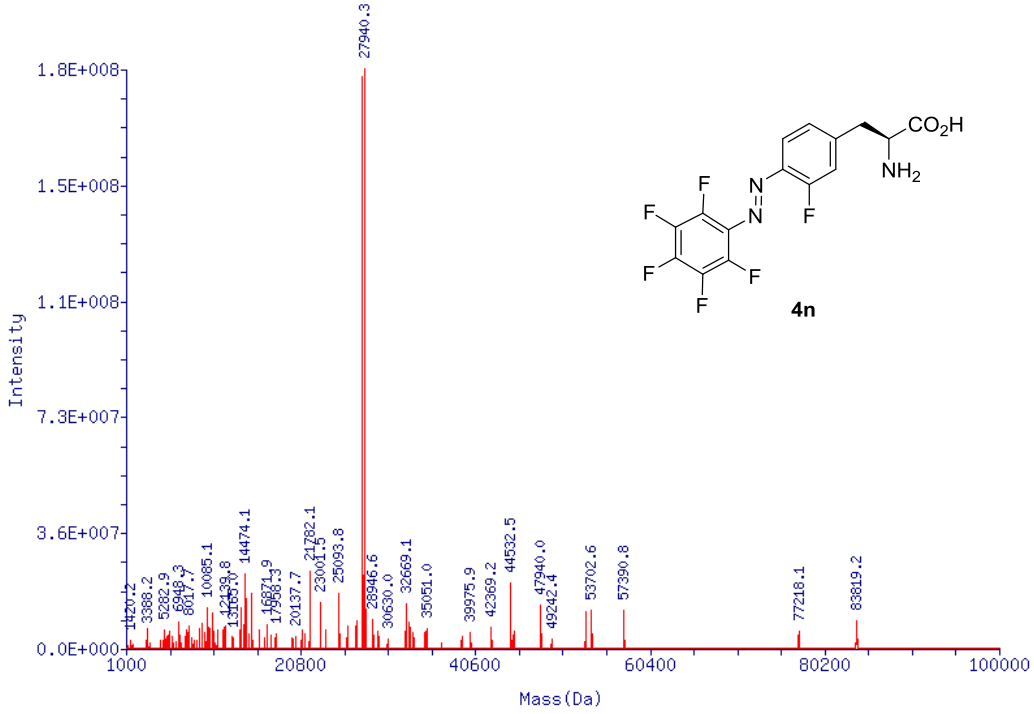


Table 3, compound **4n**: sfGFP-S2→**4n** with first Met removed: MS-ESI calcd 27941.1, found 27940.3.



Gln Incorporation sfGFP-**4n**

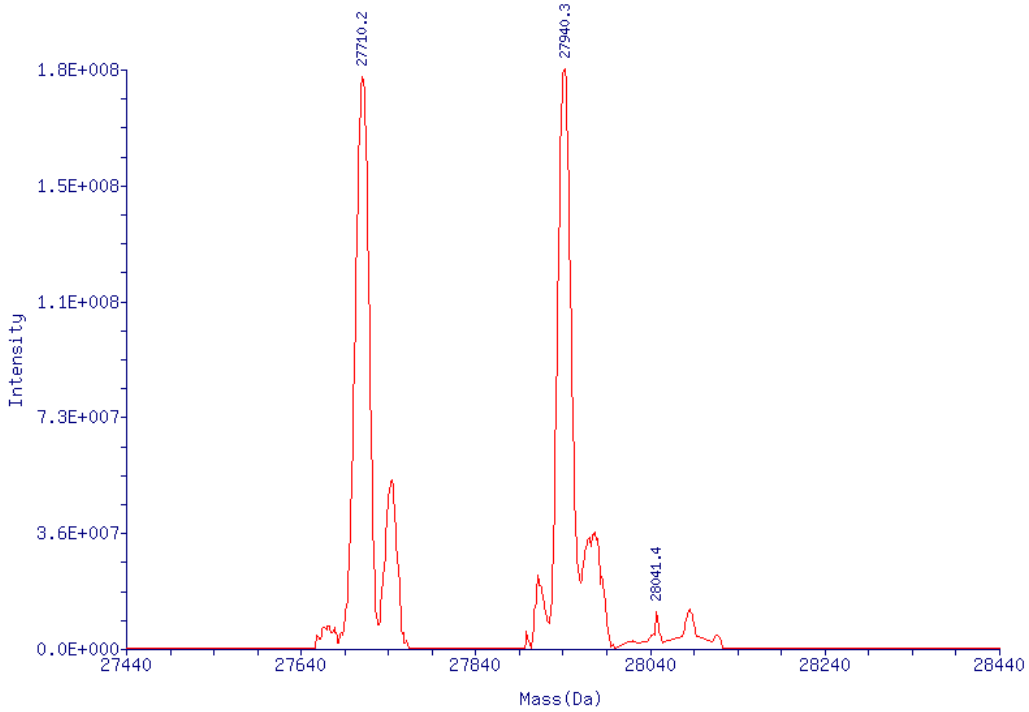
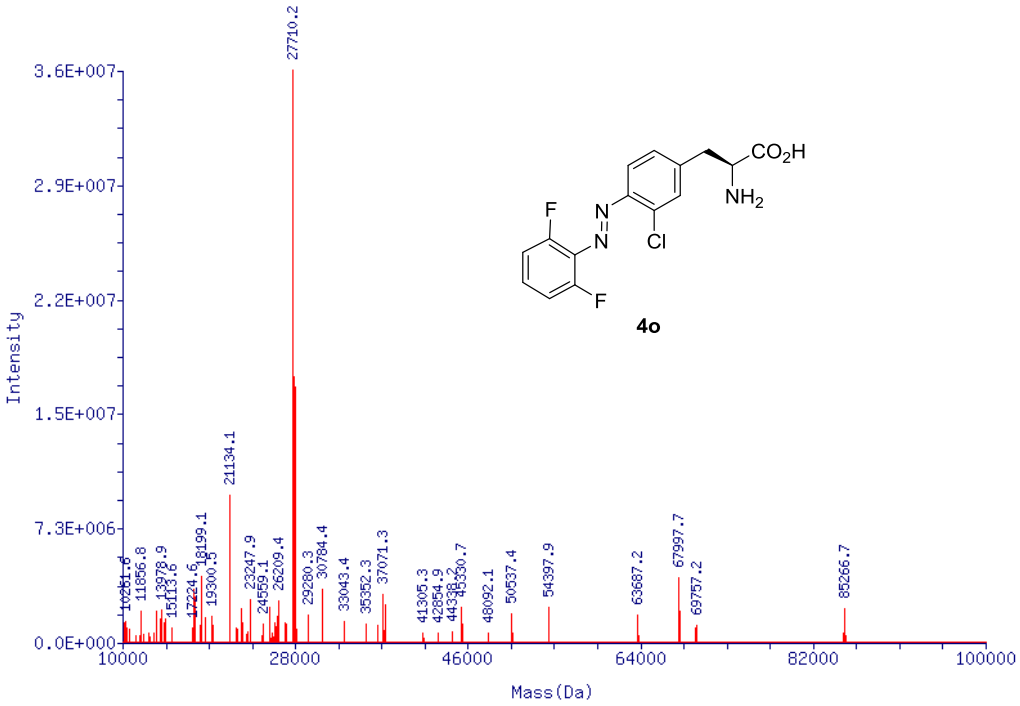
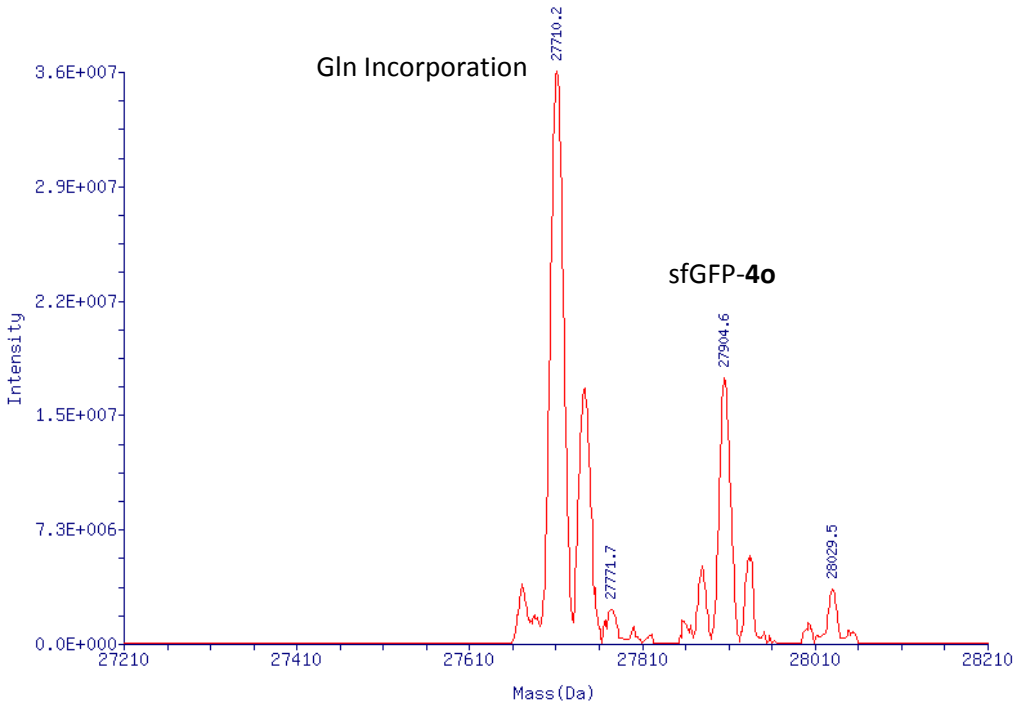


Table 3, compound **4o**: sfGFP-S2→**4o** with first Met removed: MS-ESI calcd 27902.1, found 27904.6.





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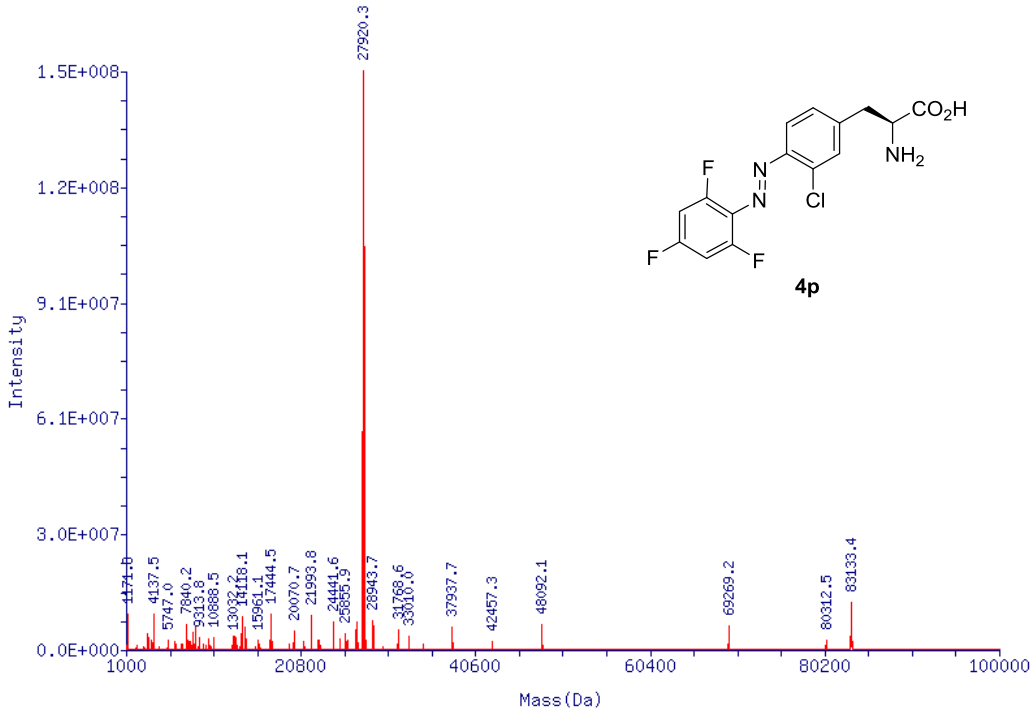


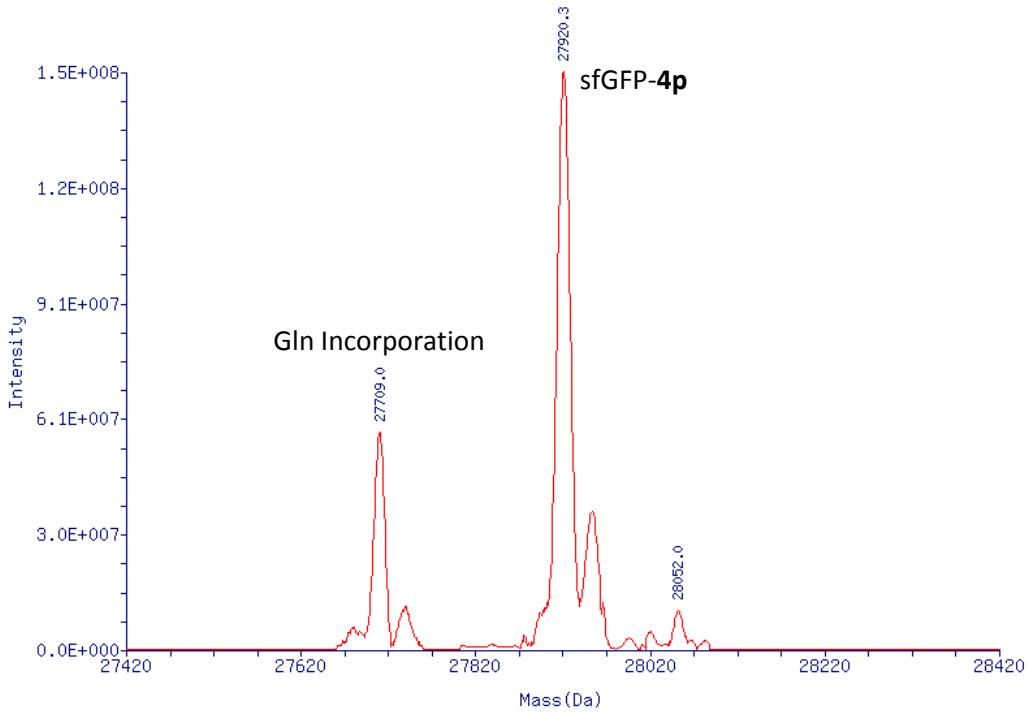
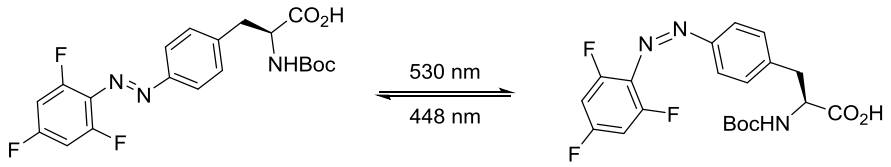


Table S1. Measuring trans:cis ratios for **3h** with varying photoirradiation time.



trans-**3h** *cis*-**3h**

LED light source (nm)	*Irradiation Time (min)*	*Trans : Cis*[a]
530	5	37 : 63
448	5	64 : 36
530	10	37 : 63
448	10	63 : 37
530	15	36 : 64
448	15	63 : 37
530	30	38 : 62
448	30	61 : 39

[a]The trans:cis ratio was determined by ^{19}F NMR.

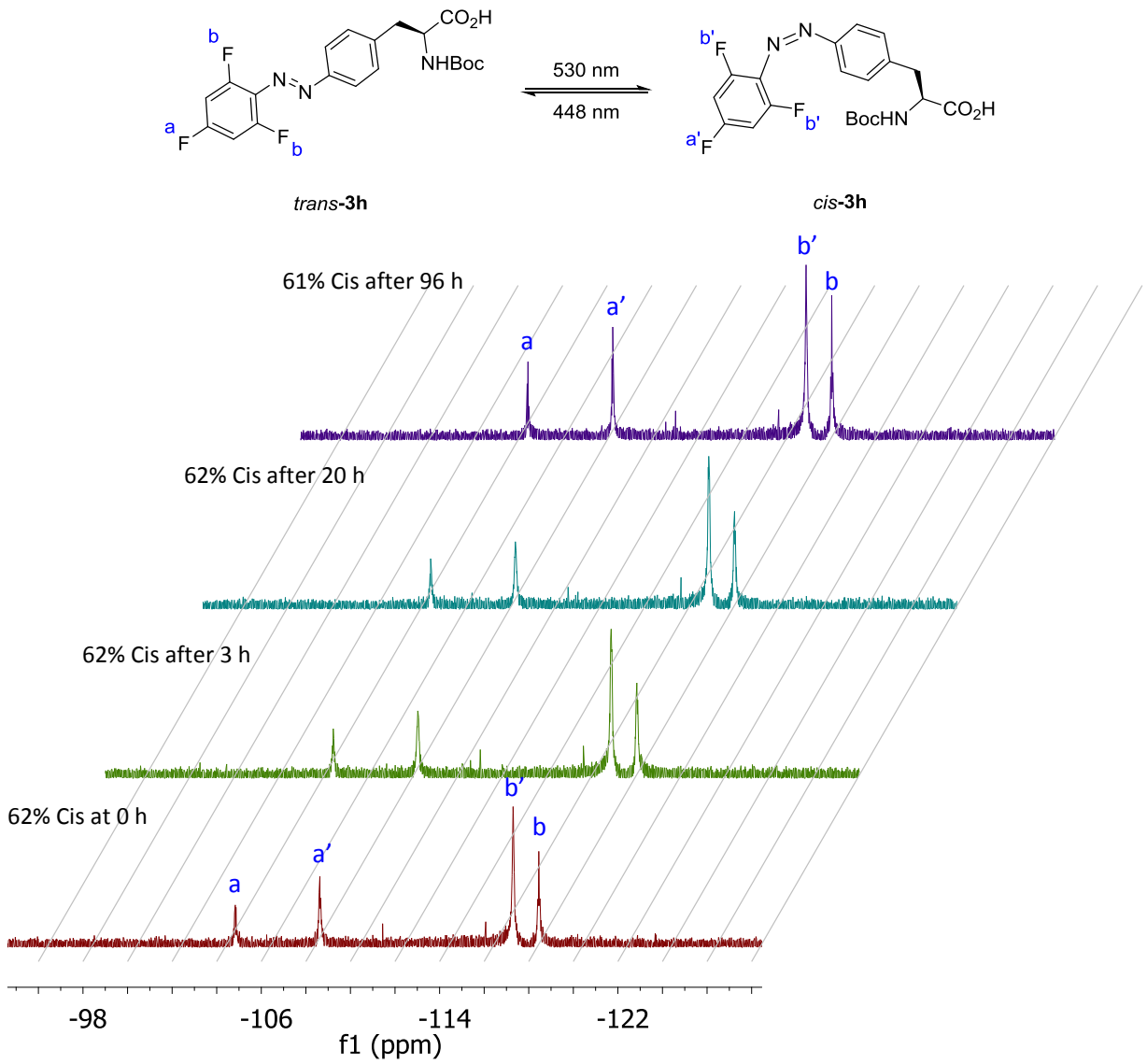
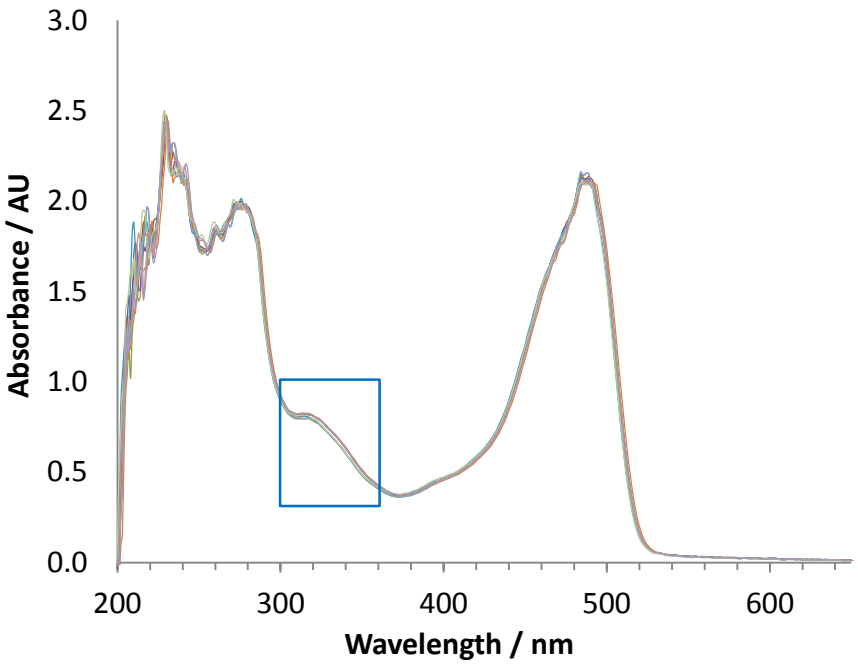


Figure S1. Measurement of *cis*-pss at various time points by ^{19}F NMR after photo-irradiating compound **3h** with a 530 nm LED light for 30 min followed by dark adaptation at room temperature.

a)



b)

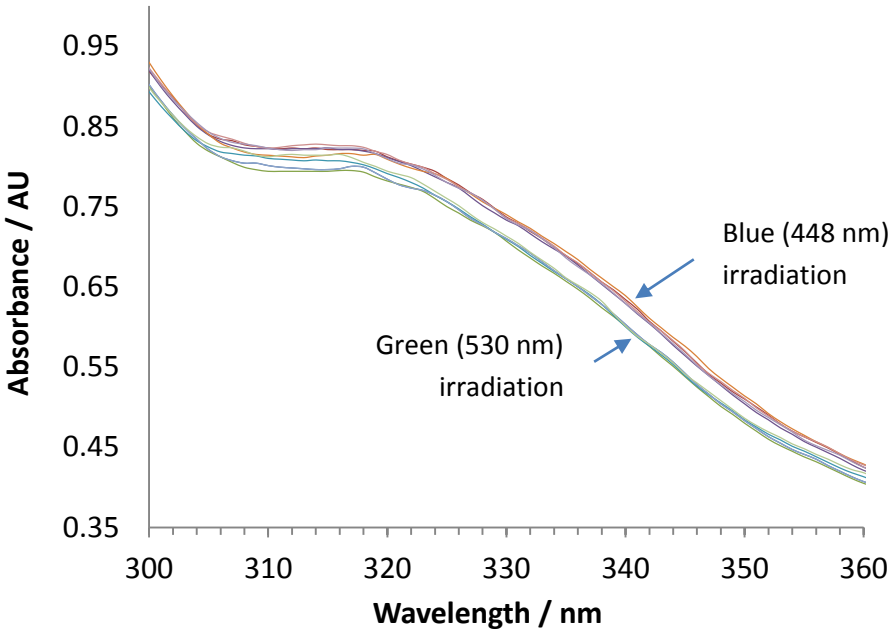
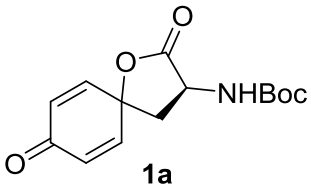


Figure S2. (a) Overlaid UV-vis spectra of sfGFP-**4h** with 5-min alternating rounds of blue/green LED light irradiation. The protein was dissolved in PBS buffer to obtain a concentration of 57 μM. (b) A zoom-in view of the 300-360 nm region (boxed in a) associated with the π→π* transition of the trifluoroazobenzene **4h**.

General Information

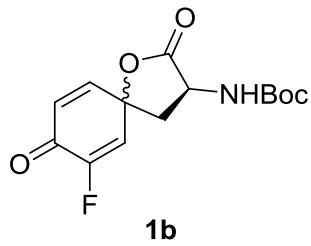
Solvents and chemicals were purchased from commercial sources and used directly without further purification. Flash chromatography was performed with SiliCycle P60 silica gel (40-63 μm, 60Å). ^{1}H NMR spectra were recorded with Inova-300, -400 or -500 MHz spectrometers and chemical shifts were reported in ppm using either TMS or deuterated solvents as internal standards (TMS, 0.00; $CDCl_3$, 7.26; CD_3OD, 3.31; DMSO-d_6, 2.50). Multiplicity was reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, brs = broad. ^{13}C NMR spectra were recorded at 75.4 MHz, and chemical shifts were reported in ppm using the deuterated solvents as internal standards ($CDCl_3$, 77.0; DMSO-d_6, 39.5; CD_3OD, 49.05). Absorption spectra were recorded using 1-cm quartz cuvette on a Thermo Scientific NanoDrop 2000c spectrometer. LED lights were purchased from Luxeonstar: Royal-Blue LED light (447.5 nm), LUXEON Rebel ES LED on a SinkPAD-II 20 mm Star Base, 1030 mW @ 700 mA; and Green LED light (530 nm), LUXEON Rebel LED on a SinkPAD-II 20 mm Star Base, 125 lm @ 700 mA.

Experimental Procedures and Characterization Data



1a

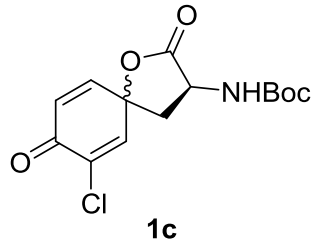
***tert*-Butyl (2,8-dioxo-1-oxaspiro[4.5]deca-6,9-dien-3-yl)carbamate (1a):** Phenyliodonium diacetate (PIDA) (126 mg, 0.39 mmol) was added to a round-bottom flask containing 10 mL acetonitrile, and the mixture was stirred at room temperature. A solution of Boc-tyrosine (0.36 mmol) in 10 mL acetonitrile was added to the above round-bottom flask dropwise over 2 h. The mixture was allowed to stir for 4 h before diluting with 20 mL ethyl acetate and 20 mL brine. The reaction mixture was extracted with ethyl acetate (20 mL x 2), and the organic layers were combined, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The crude mixture was purified using the Yamazen Smart Flash chromatography purification system (gradient elution - 25-55% ethyl acetate in hexanes over 30 min). The titled compound was obtained as a white solid (37 mg, 37% yield): ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.57 (d, J = 8.1 Hz, 1H), 7.41-7.37 (m, 1H), 7.01-6.97 (m, 1H), 6.29-6.20 (m, 2H), 4.80-4.71 (m, 1H), 2.56-2.53 (m, 1H), 2.41-2.33 (m, 1H), 1.40 (s, 9H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 184.6, 174.6, 155.4, 148.1, 147.4, 129.2, 128.0, 79.4, 75.6, 49.7, 40.8, 36.6, 28.5; HRMS (ESI) calcd for $C_{14}H_{17}NO_5$ 278.1028 [M-H$^+$], found 278.1044.



1b

***tert*-Butyl (7-fluoro-2,8-dioxo-1-oxaspiro[4.5]deca-6,9-dien-3-yl) carba mate (1b):** The compound was synthesized from 3-fluoro-*N*-Boc-L-tyrosine (190 mg, 0.603 mmol) using the same procedure as **1a**. After flash chromatography, the desired product was obtained as a white diastereomeric mixture (32 mg, 17% yield). **Diastereomer 1**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.97 ((s, 1H), 6.35-6.30 (m, 1H), 5.29 (d, J = 6.3 Hz, 1H), 4.55-4.46 (m, 1H) 2.85-2.78 (m, 1H), 2.59-2.50 (m, 1H), 1.46 (s, 9H); **Diastereomer 2:** ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.88-6.84 (m, 1H), 6.50 (s, 1H), 6.32-6.27 (m, 1H), 6.35-6.30 (m, 1H), 5.21 (s, 1H), 4.49 (s, 1H), 2.83-2.74 (m, 1H), 2.62-2.53 (m,

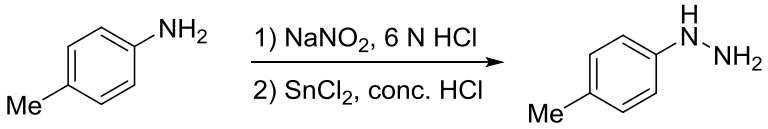
1H), 1.45 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.1, 156.0, 154.7, 151.1, 147.9, 127.4, 122.0, 121.8, 79.9, 49.5, 36.7, 27.3; HRMS (ESI) calcd for $C_{14}H_{16}FNO_5$ 314.3 [M + H_2O - H^+]$^-$, found 314.1.



1c

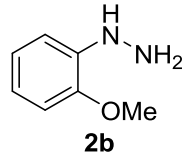
***tert*-Butyl (7-chloro-2,8-dioxo-1-oxaspiro[4.5]deca-6,9-dien-3-yl) carbamate:** The compound was synthesized from 3-chloro-*N*-Boc-L-tyrosine (127 mg, 0.402 mmol) using the same procedure as **1a**. After flash chromatography, the desired product was obtained as a white diastereomeric mixture (25 mg, 20% yield). **Diastereomer 1:** ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.05 (d, J = 2.7 Hz, 1H), 6.95 (s, 1H), 6.40 (d, J = 10.2, 1H), 5.30 (s, 1H), 4.56-4.48 (m, 1H), 2.81-2.74 (m, 1H), 2.58-2.50 (m, 1H), 1.45 (s, 9H); **Diastereomer 2:** ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.05 (d, J = 2.7 Hz, 1H), 7.10 (s, 1H), 6.90-6.86 (m, 1H), 5.27 (s, 1H), 4.54-4.46 (m, 1H), 2.80-2.73 (m, 1H), 2.59-2.51 (m, 1H), 1.46 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.5, 155.4, 150.6, 129.9, 129.2, 119.9, 116.4, 80.4, 54.5, 36.8, 31.0, 28.3; HRMS (ESI) calcd for $C_{14}H_{16}ClNO_5$ 368.0871 [M + MeOH + Na^+], found 368.1083.

General procedure for synthesis of phenylhydrazine derivatives



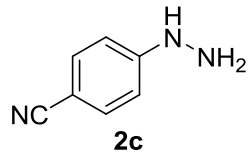
2a

***p*-Tolylhydrazine (2a):** 4-Methylaniline (428 mg, 4 mmol) was added to a round bottom flask containing 10 mL 6 N HCl. The solution was vigorously stirred at 0 ºC for 10 min. Sodium nitrite (280 mg, 4 mmol) dissolved in 2 mL water was added dropwise to the aniline solution, and the resulting mixture was stirred at 0 ºC for an additional 1 h. To a beaker containing $SnCl_2$ (1.9 g, 10 mmol) was added 10 mL of concentrated HCl, and the solution was sonicated and cooled to 0 ºC. The $SnCl_2$ solution was then added dropwise to the diazonium salt solution, and the mixture was stirred for additional 90 min at room temperature. Then, sodium hydroxide (7.2 g, 0.18 mol) dissolved in 20 mL water was added to quench the reaction. The solution was extracted with 20 mL of diethyl ether three times, and the organic layers were combined and concentrated under reduced pressure to give pale-orange crude product (347 mg, 71% yield). The crude product was further purified by recrystallization in hot ethanol. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.11-7.00 (m, 2H), 6.77-6.71 (m, 2H), 3.85 (s, 3H), 2.27 (s, 3H).



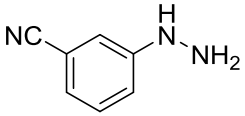
2b

2-Methoxyphenyl)hydrazine (2b): The hydrazine derivative was synthesized from *o*-anisidine (123 mg, 1 mmol) using the same procedure as procedures as **2a**.The desired product (69 mg, 50% yield) was used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.01-6.89 (m, 2H), 6.79 (dd, J = 5.2, 2.6 Hz, 2H), 3.85 (d, J = 2.6 Hz, 3H), 3.67 (s, 3H).



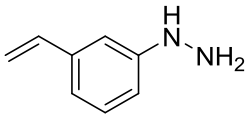
2c

4-Hydrazinylbenzonitrile (2c): The hydrazine derivative was prepared from 4-cyanoaniline (236 mg, 2.0 mmol) using the same procedure as **2a**; 51% yield: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.56-7.39 (m, 2H), 6.90-6.76 (m, 2H), 5.61 (s, 1H), 3.65 (s, 2H).



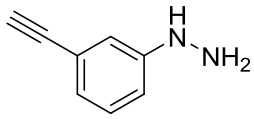
2d

3-Hydrazinylbenzonitrile (2d): The hydrazine derivative was prepared from 3-aminobenzonitrile (118 mg, 1.0 mmol) using the same procedure as **2a**; 80% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.23 (dd, J = 17.4, 9.4 Hz, 2H), 7.08 (s, 1H), 7.01 (dd, J = 8.4, 2.3 Hz, 1H), 6.96-6.89 (m, 1H), 4.11 (s, 2H).



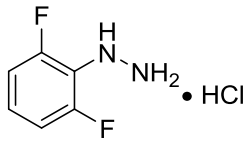
2e

3-Vinylphenyl)hydrazine (2e): The hydrazine derivative was synthesized from 3-aminostyrene (300 mg, 2.5 mmol) using the same procedure as **2a**. The titled compound was obtained as a yellow oil (246 mg, 73%), which was used without further purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.06 (t, J = 7.8 Hz, 1H), 6.88 (s, 1H), 6.69 - 6.58 (m, 4H), 5.70 (dd, J = 17.6, 1.1 Hz, 1H), 5.17 (dd, J = 10.8, 1.0 Hz, 1H), 3.93 (s, 2H).



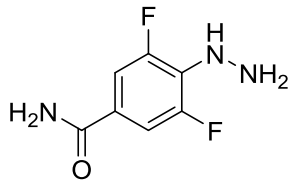
2f

(3-Ethynylphenyl)hydrazine (2f): The hydrazine derivative was prepared from 3-ethynylaniline (200 mg, 1.7mmol) using the same procedure as **2a**. The titled compound was obtained as a red oil (250 mg, 91% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.21-7.14 (m, 1H), 6.97-6.94 (m, 2H), 6.83-6.77 (m, 1H), 5.20 (s, 1H), 3.57 (s, 2H), 3.03 (s, 1H).



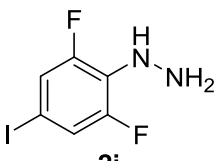
2g

(2,6-Difluorophenyl)hydrazine hydrochloride (2g): The hydrazine derivative was prepared from 2,6-difluoroaniline (2.1 g, 16 mmol) using the same procedure as **2a**. The HCl salt form of the product was obtained after acidification (2.3 g, 79% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.22 (s, 3H), 7.88 (s, 1H), 7.36 – 6.98 (m, 3H).



2i

3,5-Difluoro-4-hydrazinylbenzamide (2i): The hydrazine derivative was prepared from 4-amino-3,5-difluorobenzamide (200 mg, 1.2 mmol) using the procedure as **2a**. Purification was carried out using silica gel flash chromatography with methylene chloride/methanol (8:1) as eluent to give the titled compound as a brown solid (164 mg, 76% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.83 (s, 1H), 7.57-7.12 (m, 3H), 6.68 (s, 1H), 4.48 (s, 2H).

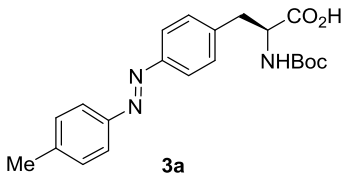


2j

(2,6-Difluoro-4-iodophenyl)hydrazine (2j): The hydrazine derivative was synthesized from 2,6-difluoro-4-iodoaniline (322 mg, 1.3 mmol) using the same procedure as **2a**. The titled compound was obtained as a dark brown solid (305 mg, 87 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.19 (s, 2H), 5.14 (s, 1H), 3.87 (s, 2H).

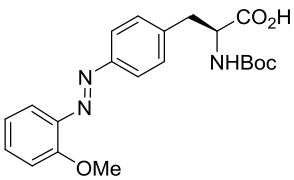
General procedure for synthesis of azobenzene amino acids

The azobenzene amino acid was synthesized by incubating 90 mM spirolactone with 100 mM phenylhydrazine in 2 mL acetonitrile in the presence of 3 mol % of cerium(IV) ammonium nitrate at room temperature. The reaction was monitored by TLC and the solution was concentrated under reduced pressure after the disappearance of the spirolactones starting materials. The crude product were purified by flash chromatography using ethyl acetate/hexanes (2:3) first and then acetic acid/ethyl acetate/hexanes (1:9:10) as eluents to yield the desired azobenzene amino acids.



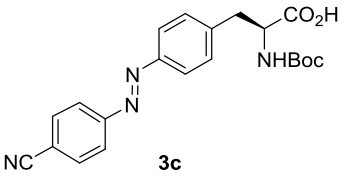
3a

(*S*,*E*)-2-((*tert*-Butoxycarbonyl)amino)-3-(4-(*p*-tolyldiazenyl) phenyl)propanoic acid (3a): The corresponding hydrazine (**2a**, 24 mg, 0.2 mmol) and spirolactone **1a** (50 mg, 0.18 mmol) were combined, incubated, and purified according to the general procedure. An orange product was obtained (51 mg, 73% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.80-7.77 (m, 4H), 7.46-7.38 (m, 4H), 7.11-7.09 (m, 1H), 4.18-4.10 (m, 1H), 3.15-3.09 (m, 1H), 2.96-2.92 (m, 1H), 2.40 (s, 3H), 1.31 (s, 9H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 174.0, 150.4, 141.9, 130.6, 130.3, 127.7, 122.8, 122.6, 115.3, 104.5, 78.4, 55.7, 49.0, 28.5, 21.5; HRMS (ESI) calcd for $C_{21}H_{25}N_3O_4$ 384.1924 [M+H$^+$], found 384.1928.



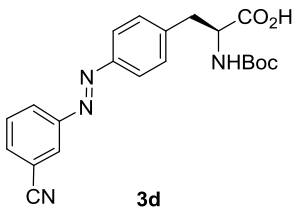
3b

(*S*,*E*)-2-((*tert*-Butoxycarbonyl)amino)-3-(4-((2-methoxyphenyl) diazenyl)phenyl)propanoic acid (3b): The corresponding hydrazine (**2b**, 27 mg, 0.2 mmol) and spirolactone **1a** (50 mg, 0.18 mmol) were combined, incubated, and purified according to the general procedure. An orange product was obtained (53 mg, 72% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.71 (d, J = 7.9 Hz, 2H), 7.52-7.47 (m, 2H), 7.39 (d, J = 7.9 Hz, 2H), 7.27-7.25 (m, 2.8 Hz, 1H), 7.07 – 6.99 (m, 1H), 6.39 (d, J = 6.2 Hz, 1H), 4.08 – 3.90 (m, 4H), 3.25 – 3.12 (m, 1H), 3.04 – 2.89 (m, 1H), 1.37 – 1.22 (m, 9H)., ^{13}CNMR (75 MHz, DMSO-d_6) δ 157.0, 155.3, 151.4, 143.2, 142.0, 139.0, 133.1, 130.8, 122.6, 121.0, 116.7, 113.9, 78.0, 56.5, 28.6; HRMS (ESI) calcd for $C_{21}H_{25}N_3O_5$ 400.1873 [M+H$^+$], found 400.1876.



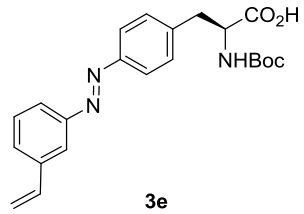
3c

(*S*,*E*)-2-((*tert*-Butoxycarbonyl)amino)-3-(4-((4-cyanophenyl) diazenyl)phenyl)propanoic acid (3c): The corresponding hydrazine (**2c**, 27 mg, 0.2 mmol) and spirolactone **1a** (50 mg, 0.18 mmol) were combined, incubated, and purified according to the general procedure. An orange product was obtained (51 mg, 72% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.09 (d, J = 5.1 Hz, 2H), 8.00 (d, J = 4.8 Hz, 2H), 7.87 (d, J = 4.8 Hz, 2H), 7.51 (d, J = 5.1 Hz, 2H), 7.13 (d, J = 5.1 Hz, 1H), 4.18 (s, 1H), 3.18-3.14 (m, 1H), 2.99-2.94 (m, 1H), 1.32 (s, 9H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 173.8, 155.8, 154.4, 150.9, 144.0, 134.2, 130.8, 123.5, 123.3, 118.9, 113.5, 78.5, 55.4, 37.0, 28.6; HRMS (ESI) calcd for $C_{21}H_{22}N_4O_4$ 395.1720 [M + H$^+$], found 395.1702.



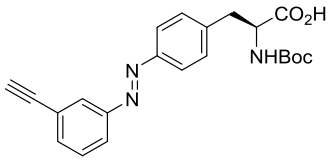
3d

(*S*,*E*)-2-((*tert*-Butoxycarbonyl)amino)-3-(4-((3-cyanophenyl) diazenyl)phenyl)propanoic acid (3d): The corresponding hydrazine (**2d**, 27 mg, 0.2 mmol) and spirolactone **1a** (50 mg, 0.18 mmol) were combined, incubated, and purified according to the general procedure. An orange product was obtained (60 mg, 85% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.26 (s, 1H), 8.16 (d, J = 5.1 Hz, 1H), 8.01 (d, J = 4.2 Hz, 1H), 7.85-7.78 (m, *3*H), 7.48 (d, J = 4.8 Hz, 2H), 7.08 (d, J = 4.8 Hz, 1H), 4.16-4.14 (m, 1H), 3.16-3.12 (m, 1H), 2.97-2.92 (m, 1H), 1.31 (s, 9H); ^{13}C NMR (75 MHz, DMSO-d$_6$) δ 173.9, 155.8, 152.3, 143.7, 134.8, 131.3, 130.8, 127.3, 126.4, 123.2, 118.5, 113.0, 78.5, 55.4, 37.0, 28.6; HRMS (ESI) calcd for $C_{21}H_{22}N_4O_4$ 393.1562 [M-H$^+$], found 393.1581.



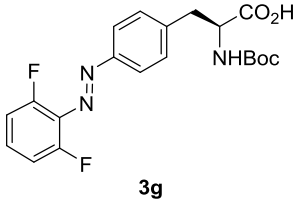
3e

(*S*,*E*)-2-((*tert*-Butoxycarbonyl)amino)-3-(4-((3-vinylphenyl) diazenyl)phenyl)propanoic acid (3e):): The corresponding hydrazine (**2e**, 38 mg, 0.29 mmol) and spirolactone **1a** (73 mg, 0.26 mmol) were combined, incubated, and purified according to the general procedure. An orange product was obtained (70 mg, 68% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.92 (s, 1H), 7.82-7.44) (m, 7H), 7.12 (d, J = 8.1 Hz, 1), 6.87-6.81 (m, 1H), 5.96 (d, J = 17.4 Hz, 1H), 5.36 (d, J = 11.1 Hz, 1H), 4.19 (s, 1H), 3.15-3.09 (m, 1H), 2.97-2.92 (m, 1H), 1.29 (s, 9H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 173.8, 155.9, 152.8, 151.1, 142.7, 138.9, 136.4, 130.7, 130.1, 129.2, 122.9, 122.1, 120.7, 116.2, 78.5, 55.4, 36.8, 28.6; HRMS (ESI) calcd for $C_{22}H_{25}N_3O_4$ 396.1924 [M+H$^+$], found 396.1931.



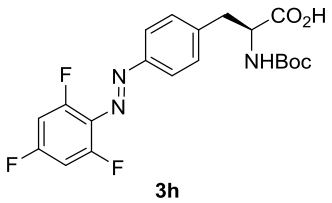
3f

(*S*,*E*)-2-((*tert*-Butoxycarbonyl)amino)-3-(4-((3-ethynylphenyl) diazenyl)phenyl)propanoic acid (3f): The corresponding hydrazine (**2f**, 26 mg, 0.2 mmol) and spirolactone **1a** (50 mg, 0.18 mmol) were combined, incubated, and purified according to the general procedure. A red product was obtained (59 mg, 83% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.90-7.88 (m, 2H), 7.78 (d, J = 8.0 Hz, 2H), 7.66 – 7.55 (m, 2H), 7.40 (d, J = 8.0 Hz, 2H), 6.29 (d, J = 6.5 Hz, 1H), 4.32 (s, 1H), 4.04 – 3.95 (m, 1H), 3.24-3.17 (m, 1H), 3.03-2.96 (m, 1H), 1.30 (s, 9H)$_4$^{13}C NMR (75 MHz, DMSO-d_6) δ 155.3, 152.3, 150.7, 144.3, 134.4, 130.9, 130.4, 125.4, 123.7, 123.3, 122.7, 83.0, 82.2, 77.9, 56.3, 37.8, 28.6; HRMS (ESI) calcd for $C_{22}H_{23}N_3O_4$ 394.1768 [M+H$^+$], found 394.1768.



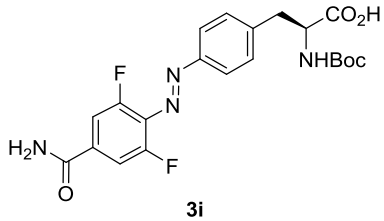
3g

(*S*,*E*)-2-((*tert*-Butoxycarbonyl)amino)-3-(4-((2,6-difluorophenyl) diazenyl)phenyl)propanoic acid (3g): The corresponding hydrazine (**2g**, 29 mg, 0.2 mmol) and spirolactone **1a** (50 mg, 0.18 mmol) were combined, incubated, and purified according to the general procedure. A red product was obtained (69 mg, 95% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.79 (d, J = 8.0 Hz, 2H), 7.48 (d, J = 8.0 Hz, 2H), 7.01 (d, J = 8.0 Hz, 1H), 4.18-4.12 (m, 1H), 3.18-3.13 (m,1H), 3.01-2.92 (m, 1H), 1.32 (s, 9H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 173.8, 155.8, 151.6, 144.1, 130.8, 122.8, 113.6. 113.4, 110.0, 104.5, 79.4, 55.5, 37.0, 28.6; HRMS (ESI) calcd for $C_{20}H_{21}F_2N_3O_4$ 406.1579 [M+H$^+$], found 406.1571.



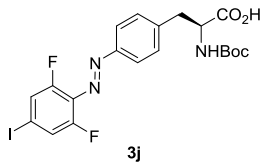
3h

(*S*,*E*)-2-((*tert*-Butoxycarbonyl)amino)-3-(4-((2,4,6-trifluoro phenyl)diazenyl)phenyl)propanoic acid (3h): The corresponding hydrazine (**2h**, 32 mg, 0.2 mmol) and spirolactone **1a** (50 mg, 0.18 mmol) were combined, incubated, and purified according to the general procedure. A red product was obtained (69 mg, 91% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.4 (s, 1H), 7.79-7.77 (m, 2H), 7.50-7.45 (m, 4H), 4.20-4.13 (m,1H), 3.24-3.11 (m, 1H), 2.98-2.90 (m, 1H), 1.31 (s, 9H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 173.7, 172.4, 155.8, 151.6, 143.9, 130.8, 122.8, 113.4, 102.5, 78.5, 55.4, 40.8, 40.5, 40.2, 39.9, 39.7, 39.4, 39.1, 28.5, 21.5; MS (ESI) calcd for $C_{20}H_{20}F_3N_3O_4$ 422.1327 [M-H$^+$], found 422.1349.



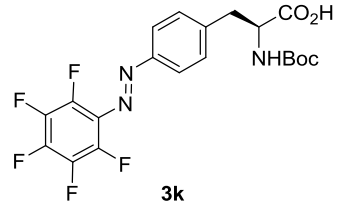
3i

(*S*,*E*)-2-((*tert*-Butoxycarbonyl)amino)-3-(4-((4-carbamoyl-2,6-difluorophenyl)diazenyl) phenyl)propanoic acid (3i): The corresponding hydrazine (**2i**, 37 mg, 0.2 mmol) and spirolactone **1a** (50 mg, 0.18 mmol) were combined, incubated, and purified according to the general procedure. An orange product was obtained (78 mg, 94% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.30 (s, 1H), 7.79 (dd, J = 8.7, 3.7 Hz, 4H), 7.45 (d, J = 8.0 Hz, 2H), 6.66 (d, J = 6.7 Hz, 1H), 4.09 (s, 1H), 3.26-3.08 (m, 1H), 3.07-2.88 (m, 1H), 1.52-0.92 (m, 9H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 165.1, 156.4, 156.4, 155.5, 153.0, 153.0, 151.6, 145.1, 136.9, 131.0, 122.9, 118.4, 112.7, 112.4, 112.4, 78.3, 78.2, 56.1, 28.6; HRMS (ESI) calcd for $C_{21}H_{22}F_2IN_4O_5$ 449.1637 [M+H$^+$], found 449.1634.



3j

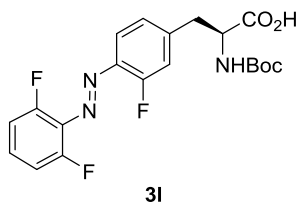
(*S*,*E*)-2-((*tert*-Butoxycarbonyl)amino)-3-(4-((2,6-difluoro-4-iodo phenyl)diazenyl)phenyl)propanoic acid (3j): The corresponding hydrazine (**2j**, 54 mg, 0.2 mmol) and spirolactone **1a** (50 mg, 0.18 mmol) were combined, incubated, and purified according to the general procedure. An orange product was obtained (78 mg, 82% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.87 – 7.73 (m, 4H), 7.48 (d, J = 8.3 Hz, 2H), 7.10 (d, J = 8.4 Hz, 1H), 4.20 - 4.12 (m, 1H), 3.17 - 3.11 (m, 1H), 2.98 - 2.90 (m, 1H), 1.31 (s, 9H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 173.7, 172.4, 156.6, 155.8, 153.1, 153.0, 151.7, 144.2, 130.8, 122.9, 122.5, 78.5, 55.3, 28.6, 21.5; HRMS (ESI) calcd for $C_{20}H_{20}F_2IN_3O_4$ 532.0546 [M+H$^+$], found 532.0554.



3k

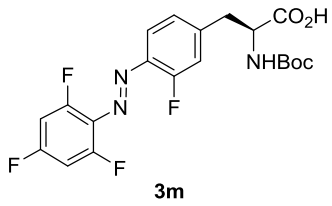
(*S*,*E*)-2-((*tert*-Butoxycarbonyl)amino)-3-(4-((perfluorophenyl) diazenyl)phenyl)propanoic acid (**3k**): The corresponding hydrazine (**2k**, 20 mg, 0.1 mmol) and spirolactone **1a** (30 mg, 0.11 mmol) were combined, incubated, and purified according to the general procedure. An orange product was obtained (41 mg, 89% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.68 (s, 1H), 7.84 (d, J = 8.1 Hz, 2H), 7.52 (d, J = 8.7 Hz, 2H), 7.20 (d, J = 8.4 Hz, 1H), 4.21-4.14 (m, 1H), 3.18-3.12 (m, 1H), 3.02-2.91 (m, 1H), 1.31-1.29 (m, 9H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 173.7, 155.9, 151.5, 145.0, 141.5, 130.9,

130.5, 123.1, 119.9, 78.5, 55.2, 36.9, 28.5; HRMS (ESI) calcd for $C_{20}H_{18}F_5N_3O_4$ 458.1138 [M-H^+], found 458.1159.



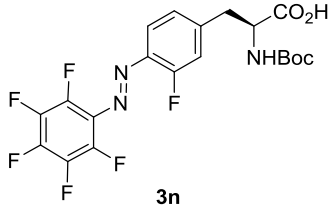
3l

(*S*,*E*)-2-((*tert*-Butoxycarbonyl)amino)-3-(3-fluoro-4-((2,6-difluoro phenyl)diazenyl)phenyl)propanoic acid (3l): The corresponding hydrazine (**2g**, 15 mg, 0.11 mmol) and spirolactone **1b** (29 mg, 0.10 mmol) were combined, incubated, and purified according to the general procedure. An orange product was obtained (30 mg, 72% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.61-7.51 (m, 2H), 7.37-7.19 (m, 4H), 4.12 (s,1H), 3.20-3.14 (m, 1H), 2.97-2.81 (m, 1H), 1.30 (s, 9H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.5, 157.9, 155.6, 153.6, 147.3, 139.3, 132.6, 126.5, 116.8, 113.7, 113.4, 113.3, 78.5, 28.6, 21.5; HRMS (ESI) calcd for $C_{20}H_{20}F_3N_3O_4$ 424.1485 [M+H^+], found 424.1489.



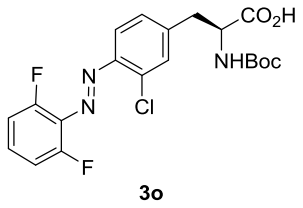
3m

(*S*,*E*)-2-((*tert*-Butoxycarbonyl)amino)-3-(4-((2,4,6-trifluoro phenyl)diazenyl)phenyl)propanoic acid (3m): The corresponding hydrazine (**2h**, 29 mg, 0.18 mmol) and spirolactone **1b** (49 mg, 0.16 mmol) were combined, incubated, and purified according to the general procedure. An orange product was obtained (51 mg, 72% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.60-7.38 (m, 3H), 7.25-7.10 (m, 2H), 4.20-4.12 (m,1H), 3.19-3.13 (m, 1H), 2.97-2.89 (m, 1H), 1.31 (s, 9H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.4, 155.8, 147.4, 146.6, 135.6, 132.1, 129.6, 116.9, 78.6, 54.9, 36.6, 28.5; HRMS (ESI) calcd for $C_{20}H_{20}F_4N_3O_4$ 442.1391 [M+H^+], found 442.1386.



3n

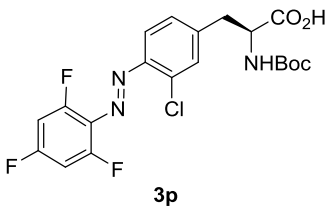
(*S*,*E*)-2-((*tert*-Butoxycarbonyl)amino)-3-(3-fluoro-4-((perfluoro phenyl)diazenyl)phenyl)propanoic acid (3n): The corresponding hydrazine (**2k**, 13 mg, 0.07 mmol) and spirolactone **1b** (16 mg, 0.05 mmol) were combined, incubated, and purified according to the general procedure. A red-orange product was obtained (14 mg, 60% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.83 (s, 1H), 7.63 (t, J = 7.8 Hz, 1H), 7.44 (d, J = 11.9 Hz, 1H), 7.28 (d, J = 8.2 Hz, 1H), 7.17 (d, J = 8.5 Hz, 1H), 4.19 (s, 1H), 3.18 (d, J = 13.1 Hz, 1H), 3.04 – 2.88 (m, 1H), 1.43 – 1.14 (m, 9H); ^{13}C NMR (75 MHz, CD_3COCD_3) δ 172.4, 161.9, 158.5, 147.2, 147.1, 139.4, 126.2, 126.1, 118.4, 118.1, 116.5, 92.6, 78.5, 54.4, 37.4, 27.6 ; HRMS (ESI) calcd for $C_{20}H_{17}F_6N_3O_4$ 476.1044 [M-H^+], found 476.1064.



3o

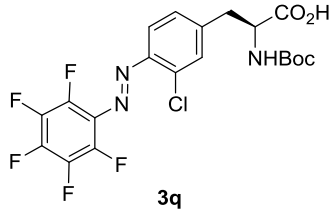
(*S*,*E*)-2-((*tert*-Butoxycarbonyl)amino)-3-(3-chloro-4-((2,6-difluoro phenyl)diazenyl)phenyl)propanoic acid (3o): The corresponding hydrazine (**2g**, 36 mg , 0.25 mmol) and spirolactone **1c** (31 mg, 0.10 mmol) were combined, incubated, and purified according to the general procedure. An orange product was obtained (30 mg, 68% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.75 – 7.24 (m, 4H), 7.22 – 7.00 (m, 2H), 6.85 (s, 1H), 3.96 (s, 1H), 3.17 - 3.11 (m, 1H), 3.05 – 2.83 (m, 1H), 1.29 (s, 9H; ^{13}C NMR (75 MHz, DMSO-d_6) δ 170.6, 158.6, 157.0, 153.6, 148.2, 142.4, 135.0, 133.0, 132.3, 129.9, 117.4,

113.5, 53.5, 46.0, 35.8, 34.6; HRMS (ESI) calcd for $C_{20}H_{20}F_2ClN_3O_4$ 440.1189 [M+H$^+$], found 440.1176.



3p

(*S*,*E*)-2-((*tert*-Butoxycarbonyl)amino)-3-(3-chloro-4-((2,4,6-trifluorophenyl)diazenyl)phenyl) propanoic acid (3p): The corresponding hydrazine (**2h**, 65 mg, 0.40 mmol) and spirolactone **1c** (50 mg, 0.16 mmol) were combined, incubated, and purified according to the general procedure. An orange product was obtained (49 mg, 67% yield). ^{1}H (300 MHz, DMSO-d_6) δ 7.63-7.44 (m, 4H), 7.37 (d, J = 8.4 Hz, 1H), 7.05 (d, J = 7.9 Hz, 1H), 4.14 (s, 1H), 3.18 - 3.12 (m , 1H), 2.96 - 2.88 (m, 1H), 1.32 (s, 9H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.5, 155.6, 154.6, 147.5, 146.0, 134.7, 131.9, 129.6, 116.7, 102.6, 102.3, 78.3, 55.5, 28.5, 21.6; MS (ESI) calcd for $C_{20}H_{19}ClF_3N_3O_4$ 458.1095 [M+H$^+$], found 458.1108.

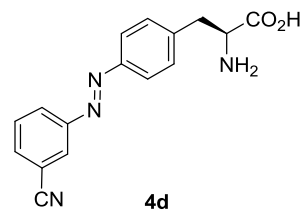


3q

(*S*,*E*)-2-((*tert*-Butoxycarbonyl)amino)-3-(3-chloro-4-((perfluorophenyl)diazenyl)phenyl)propanoic acid (3q): The corresponding hydrazine (**2k**, 13 mg, 0.06 mmol) and spirolactone **1c** (18 mg, 0.06 mmol) were combined, incubated, and purified according to the general procedure. An orange product was obtained (19 mg, 63% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.64 (s, 1H), 7.61 (d, J = 8.2 Hz, 1H), 7.40 (d, J = 8.2 Hz, 1H), 7.00 (s, 1H), 4.14 (s, 1H), 3.20 - 3.16 (m, 2H), 2.97 - 2.93 (m, 1H), 1.32 (d, J = 9.7 Hz, 9H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.4, 155.8, 147.4, 146.6, 146.6, 135.6, 132.1, 129.6, 116.9, 78.6, 54.8, 36.5, 28.5; HRMS (ESI) calcd for $C_{20}H_{17}F_5$ ClN_3O_4 494.0907 [M+H$^+$], found 494.0902.

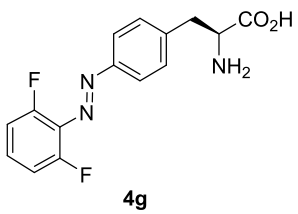
General procedure for Boc-deprotection

The Boc-protected azobenzene amino acid (50 mg) was dissolved in 1 mL of methylene chloride/trifluoroacetic acid (1:1). The resulting solution was stirred for 20 min, and then concentrated under reduced pressure. The solid was dried by lyophilization for 24 h. The dried product was characterized and used without further purification.



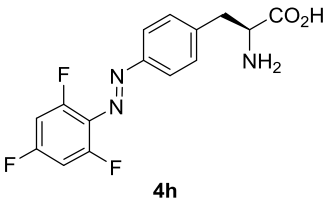
4d

(*S*,*E*)-2-Amino-3-(4-((3-cyanophenyl)diazenyl)phenyl)propanoic acid (4d): ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.45 (s, 3H), 8.45 (s, 3H), 8.25 (s, 1H), 8.16 (d, J = 8.0 Hz, 1H), 8.01 (d, J = 8.0, 1H), 7.89 (d, J = 8.0, 2H), 7.81-7.77 (m, 1H), 7.52 (d, J = 8.0, 2H), 4.11 (s, 1H), 3.24-2.99 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.7, 152.3, 151.3, 140.2, 135.0, 131.4, 131.2, 127.4, 126.4, 123.5, 118.5, 113.0, 104.5, 53.4, 36.1; HRMS (ESI) calcd for $C_{16}H_{14}N_4O_2$ 295.1196 [M+H$^+$], found 295.1200.



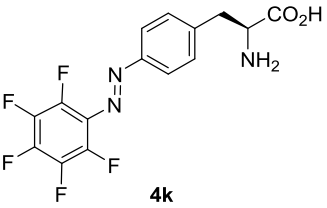
4g

(S,E)-2-amino-3-(4-((2,6-difluorophenyl)diazenyl)phenyl)propanoic acid (4g): ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.43 (s, 3H), 7.84 (d, J = 8.4 Hz, 2H), 7.60 – 7.48 (m, 3H), 7.37 – 7.27 (m, 2H), 4.28 (s, 1H), 3.37 – 3.13 (m, 2H) ; ^{13}C NMR (125 MHz, DMSO-d_6) δ 170.7, 156.2, 154.2, 152.2, 140.5, 131.2, 123.2, 113.5, 113.4, 53.5, 40.4, 40.3, 40.1, 39.9, 39.8, 39.6, 39.4, 36.1; HRMS (ESI) calcd for $C_{15}H_{13}F_2N_3O_2$ 306.1079 [M+H$^+$], found 306.1065.



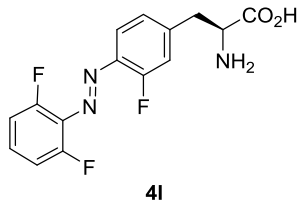
4h

(*S*,*E*)-2-Amino-3-(4-((2,4,6-trifluorophenyl)diazenyl)phenyl) propanoic acid (4h): ^{1}H NMR (300 MHz, CD_3OD) δ 7.94-7.86 (m, 2H), 7.54-7.49 (m, 2H), 7.15-7.03 (m, 2H), 4.32 (dd, J = 7.8, 5.6 Hz, 1H), 3.42 (dd, J = 14.5, 5.6 Hz, 1H), 3.29-3.21 (m, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 170.5, 148.2, 142.1, 135.0, 132.3, 129.9, 117.5, 113.73 113.4, 53.3, 34.6; HRMS (ESI) calcd for $C_{15}H_{12}F_3N_3O_2$ 324.0961 [M+H$^+$], found 324.0959.



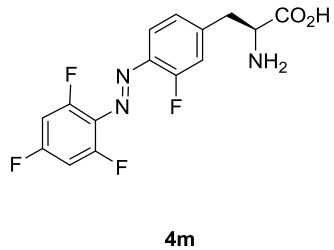
4k

(S,*E*)-2-Amino-3-(4-((perfluorophenyl)diazenyl)phenyl) propanoic acid (4k): ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.52 (s, 2H), 7.86 (d, J = 8.4 Hz, 2H), 7.55 (d, J = 8.4 Hz, 2H), 4.23 (t, J = 6.4 Hz, 1H), 3.25 (d, J = 6.4 Hz, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.7, 152.0, 141.6, 131.4, 123.5, 116.0, 104.2, 53.4, 36.1; HRMS (ESI) calcd for $C_{15}H_{10}F_5N_3O_2$ 360.0772 [M+H$^+$], found 360.0775.



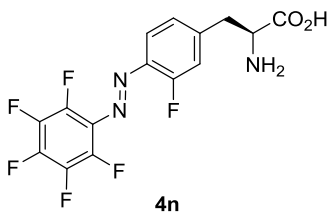
4l

(*S*,*E*)-2-Amino-3-(4-((2,6-difluorophenyl)diazenyl)-3-fluorophenyl) propanoic acid (4l): ^{1}HNMR (400 MHz, DMSO-d_6) δ 8.37 (s, 2H), 7.73-7.49 (m, 2H), 7.38-7.15 (m, 3H), 7.03-6.78 (m, 1H), 4.27 (d, J = 6.0 Hz, 1H), 3.26-3.14 (m, 1H), 3.10-2.82 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.6, 143.4, 126.9, 126.1, 121.4, 119.1, 118.9, 117.5, 116.0, 113.7, 113.5, 53.8, 53.2; HRMS (ESI) calcd for $C_{15}H_{12}F_3N_3O_2$ 324.0961 [M+H$^+$], found 324.0953.



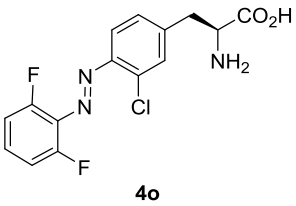
4m

(*S*,*E*)-2-Amino-3-(3-fluoro-4-((2,4,6-trifluorophenyl)diazenyl) phenyl)propanoic acid (4m):^{1}H NMR (300 MHz, DMSO-d_6) δ 8.38 (s, 3H), 7.64-7.60 (m, 1H), 7.51-7.44 (m, 3H), 7.29-7.26 (m, 1H), 4.32 (m, 1H), 3.36-3.23 (m, 2H);^{13}C NMR (75 MHz, DMSO-d_6) δ 170.5, 158.4, 143.1, 140.0, 126.8, 119.0, 118.8, 117.6, 102.9, 102.7, 102.4, 49.0, 35.9; HRMS (ESI) calcd for $C_{15}H_{11}F_4N_3O_2$ 342.0866 [M+H$^+$], found 342.0862.



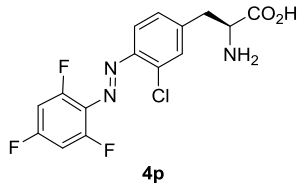
4n

(*S*,*E*)-2-Amino-3-(3-fluoro-4-(perfluorophenyl)diazenyl)phenyl) propanoic acid (4n): ^{1}H NMR (300 MHz, CD_3OD) δ 7.71 (t, J = 8.0 Hz, 1H), 7.38-7.34 (m, 1H), 7.25 (dd, J = 8.4, 1.3 Hz, 3H), 4.37-4.33 (m, 1H), 3.43-3.36 (m, 1H), 3.31-3.27 (m, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 169.6, 162.1, 1585.6, 142.8, 142.7, 140.2, 140.1, 125.7, 125.6, 118.3, 118.0, 117.1, 53.2, 35.6; HRMS (ESI) calcd for $C_{15}H_9F_6N_3O_2$ 378.0678 [M+H$^+$], found 378.0678.



4o

(*S*,*E*)-2-Amino-3-(3-chloro-4-((2,6-difluorophenyl)diazenyl)phenyl) propanoic acid (4o): ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.47 (s, 3H), 7.81 (d, J = 8.3 Hz, 2H), 7.51 (d, J = 8.3 Hz, 2H), 7.41 (t, J = 9.3 Hz, 2H), 4.27 (t, J = 6.1 Hz, 1H), 3.23 (d, J = 6.4 Hz, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 170.5, 148.2, 142.1, 135.0, 133.1, 132.3, 131.3, 129.9, 117.5, 113.7, 113.7, 113.4, 53.3, 34.7; HRMS (ESI) calcd for $C_{15}H_{12}F_2ClN_3O_2$ 340.0665 [M+H$^+$], found 340.0681.



4p

(*S*,*E*)-2-Amino-3-(3-chloro-4-((2,4,6-trifluorophenyl)diazenyl) phenyl)propanoic acid (4p): ^{1}H NMR (300 MHz, CD_3OD) δ 7.65 (t, J =9.0 Hz, 2H), 7.36 (d, J = 9.0 Hz 1H), 7.11 (t, J = 9.0 Hz, 2H), 4.36 (dd, J = 9.0, 6.0 Hz, 1H), 3.38-3.36 (m, 1H), 3.30-3.24 (m, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 170.6, 148.2, 142.1, 134.9, 132.3, 129.9, 117.4, 100.5, 53.2, 35.6; MS (ESI) calcd for $C_{15}H_{11}ClF_3N_3O_2$ 358.0571 [M + H$^+$], found 358.0592.

Construction of the Aba-specific *p*EVOL-*Mm*PylRS plasmid

The pyrrolysyl-tRNA synthetase variant suitable for charging azobenzene amino acids was previously reported by Hoppmann et al.[1] We used *p*CMV6-*Mm*PylRS-U6-tRNA plasmid[2] as the template and performed three rounds of site-directed mutagenesis to generate mutations: A302T, L309S, N346V, C348G using the following primers pairs:

*Mm*PylRS-A302T-PO-F: 5'- cccatgcttacccaaccctttacaactactcccgcaag -3'
*Mm*PylRS-A302T-PO-R: 5'- aagggttgggtaagcatgggtctcaggcagaagttg -3'
*Mm*PylRS_309_F: 5'- gctccaaacctttacaactactcccgcaagcttgacagggccctg -3'
*Mm*PylRS_309_R: 5'- cagggccctgtcaagcttgcggagtagttgtaaaggtttggagc -3'
*Mm*PylRS_346_348_F: 5'- cctcgaagagtttaccatgctggtgttcggccagatgggatcgggatgcac -3'
*Mm*PylRS_346_348_R: 5'- gtgcatcccgatcccatctggccgaacaccagcatggtaaactcttcgagg -3'

The *Mm*PylRS mutant gene was amplified by PCR using the following primers to introduce the *SpeI* and *SalI* restriction sites at the 5'- and 3'- ends, respectively:

*Mm*PylRS-SpeI-F: 5'- taagcaactagtatggataaaaaaccactaaac -3'
*Mm*PylRS-SalI-R: 5'- tgcttagtcgacttacaggttggtagaaatccc -3'

The gene fragment was cloned into the *p*GEM-T (Promega) vector using TA cloning following manufacturer's instructions to give *p*GEM-*Mm*PylRS and subsequently subcloned into the *p*EVOL vector (GENEWIZ, South Plainfield, NJ) to obtain the Aba-specific *p*EVOL-*Mm*PylRS plasmid.

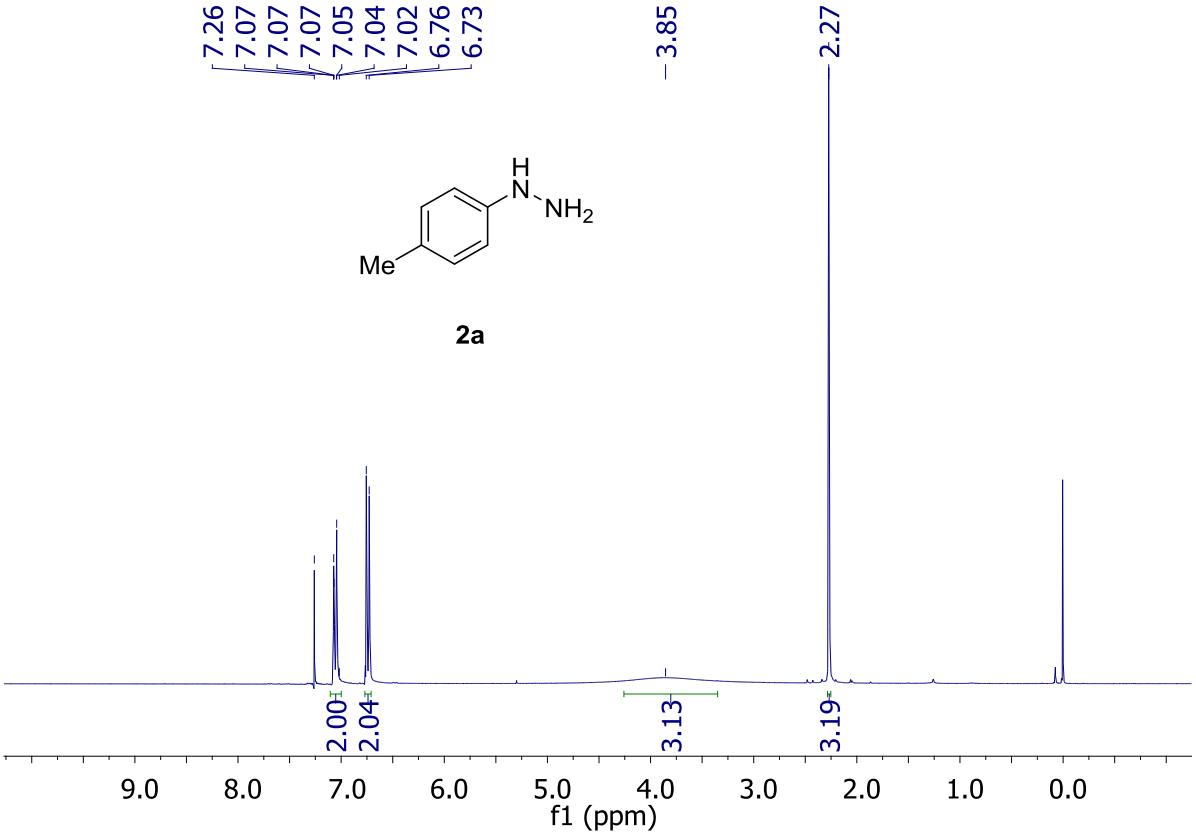
Expression and purification of the azobenzene-alanine (Aba)-containing sfGFP proteins

BL21(DE3) cells were co-transformed with Aba-specific pEVOL-*Mm*PylRS and pET-sfGFP-S2TAG plasmids. The cells were recovered in 950 μL Terrific Broth following transformation and incubated at 37 °C for 1 hour before plating on LB agar plate containing chloramphenicol (Cam, 34 μg/mL) and ampicillin (Amp, 100 μg/mL). A 2-mL overnight culture from a single colony was used to inoculate 10 mL LB medium supplemented with Cam and Amp. Cells were grown at 37 °C in a shaker-incubator (280 rpm), and protein expression was induced by adding 1 mM IPTG, 0.2% arabinose and 1 mM of the desired azobenzene amino acid when OD_{600} reached 0.5. The control experiment contained only arabinose and IPTG. After 7-hour induction, cells were harvested, resuspended in a lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0): BugBuster Protein Extraction Reagent (3:1), vortexed for 60 sec, and then rotated at room temperature for 15 min. The lysate was centrifuged (40 min, 17,136 g, 4 °C). The supernatant was incubated with 0.1 mL Ni-NTA beads (Thermo HisPur™) at 4 °C overnight. The bound protein was eluted off the beads using an elution buffer (50 mM Na_2HPO4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The supernatant was concentrated using an Amicon Ultra-15 Centrifugal Filter (10k MWCO, Millipore). The protein purity was analyzed by LC/ESI-MS. The calculated mass corresponded to the mass of sfGFP-S2Aba with the first methionine removed, and the observed mass was derived from the deconvolution of the protein charge ladder using ProMass as shown previously.[3]

References

[1] Hoppmann, C.; Lacey, V. K.; Louie, G. V.; Wei, J.; Noel, J. P.; Wang, L. *Angew. Chem. Int. Ed.* **2014**, *53*, 3932–3936.
[2] Li, N.; Ramil, C. P.; Lim, R. K.; Lin, Q. *ACS Chem. Biol.* **2015**, *10*, 379–384.
[3] Yu, Z.; Lin, Q. *J. Am. Chem. Soc.* **2014**, *136*, 4153−4156.

^{1}H and ^{13}C NMR Spectra



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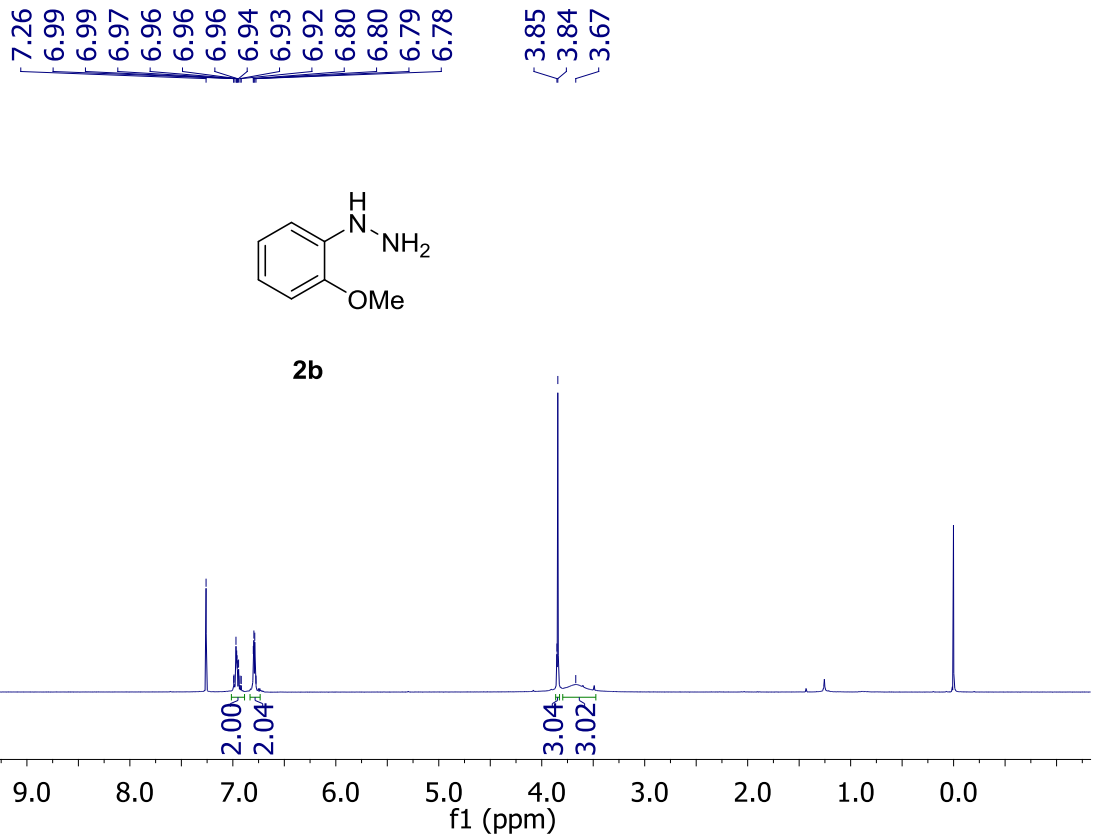
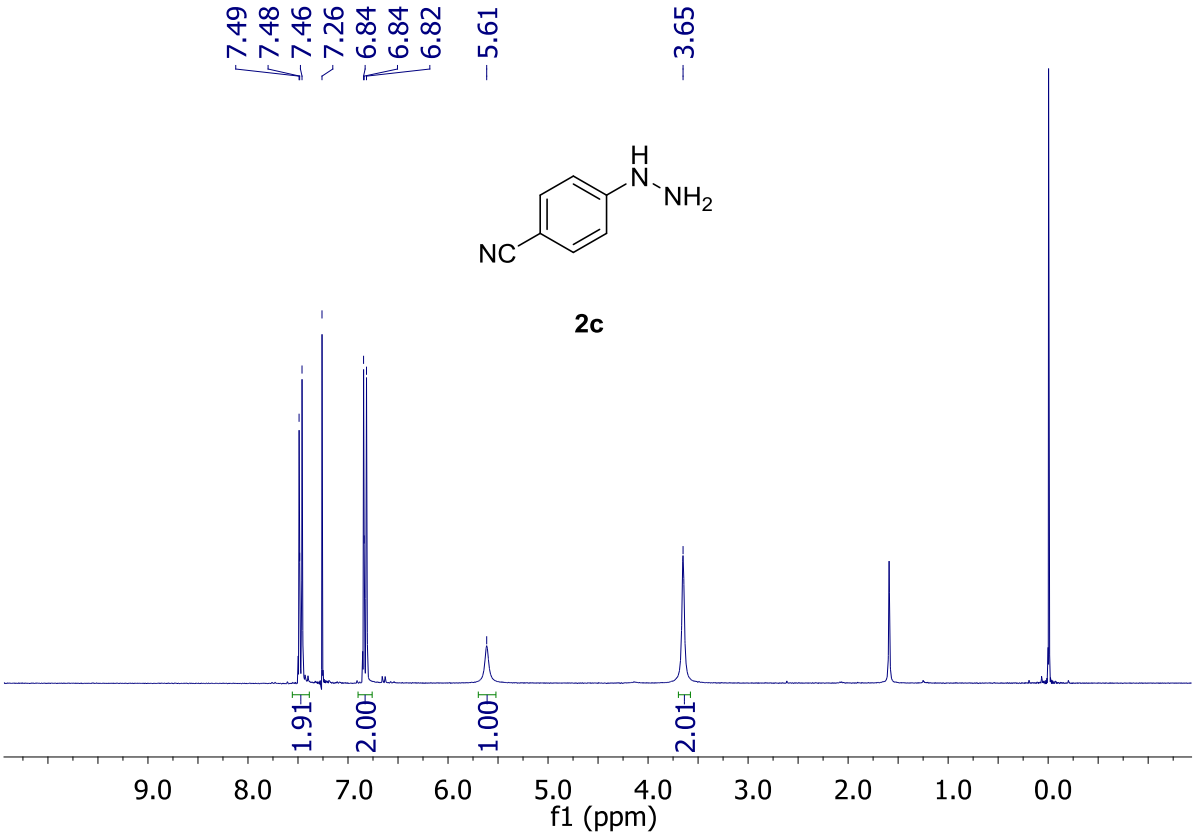


2b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5.61
3.65
H
N
NH2
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2.00
1.00
2.01
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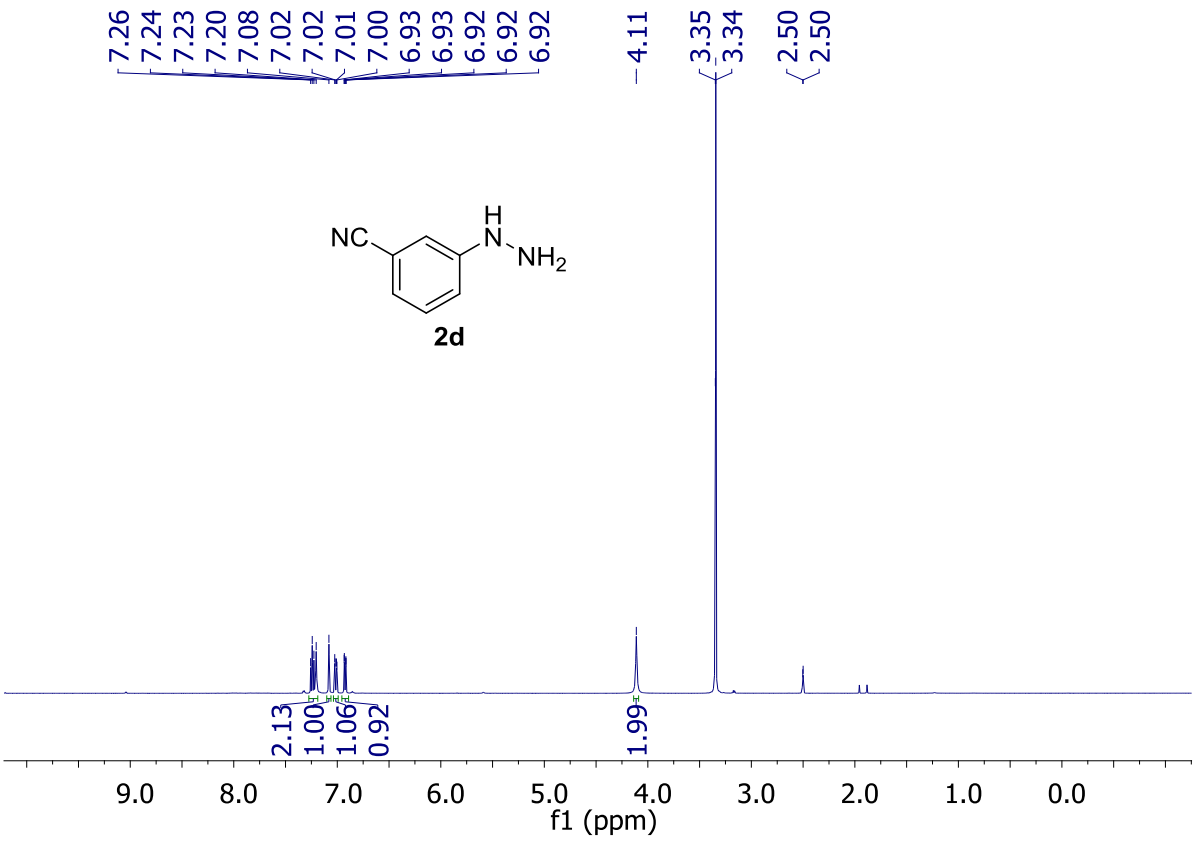
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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7.23
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7.01
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6.93
6.93
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6.92
4.11
3.35
3.34
2.50
2.50
NC
H
N
NH2
2d
2.13
1.00
1.06
0.92
1.99
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)


7.08
7.06
7.04
6.88
6.69
6.69
6.67
6.67
6.65
6.63
6.61
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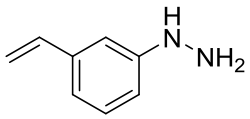
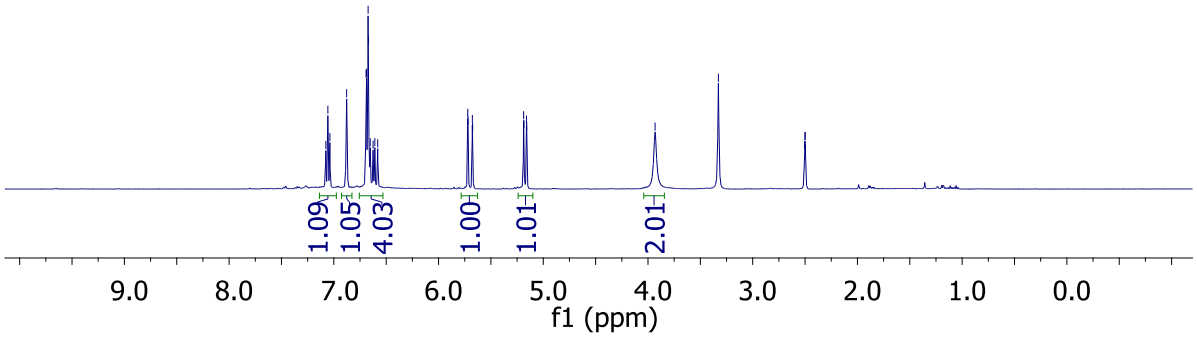
5.16
3.33
2.50
2.50
2.50
2.50


H
N
NH2

2e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8.0
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6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)


7.26
7.26
7.20
7.18
7.17
7.15
6.97
6.96
6.96
6.94
6.94
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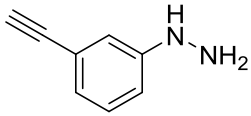
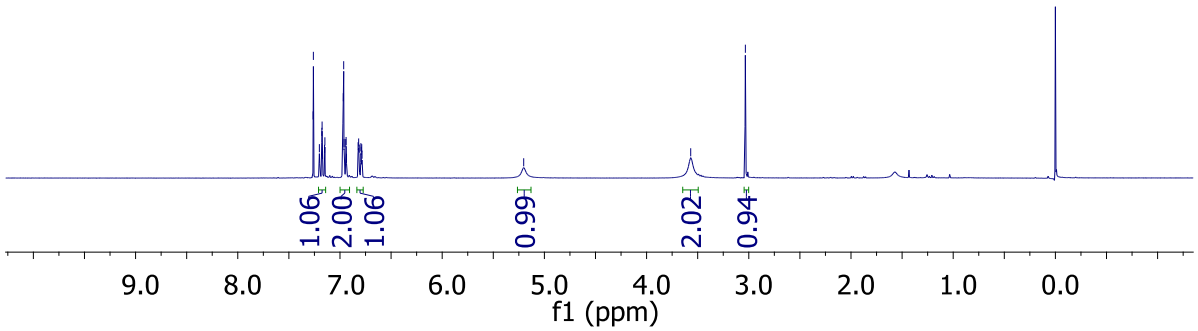
6.82
6.80
6.79
5.20
3.57
3.03


H
N
NH2

2f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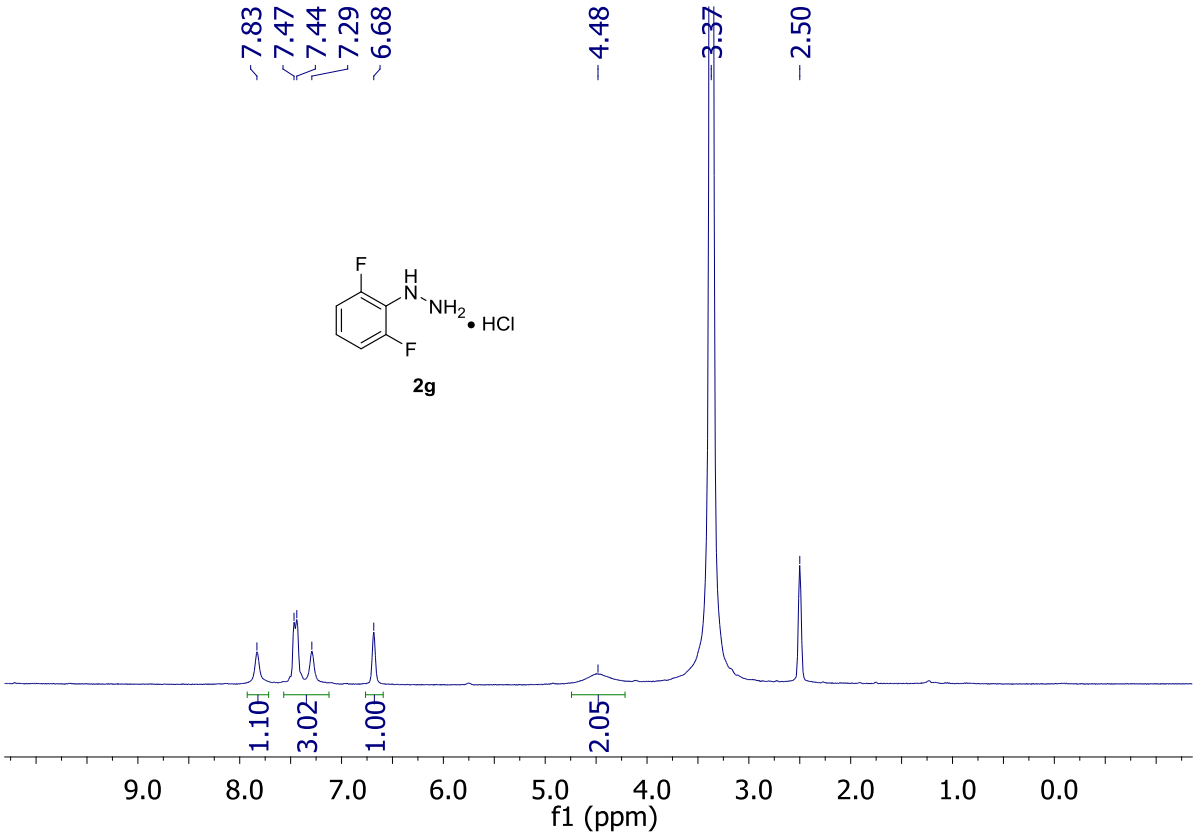
2.0
1.0
0.0
f1 (ppm)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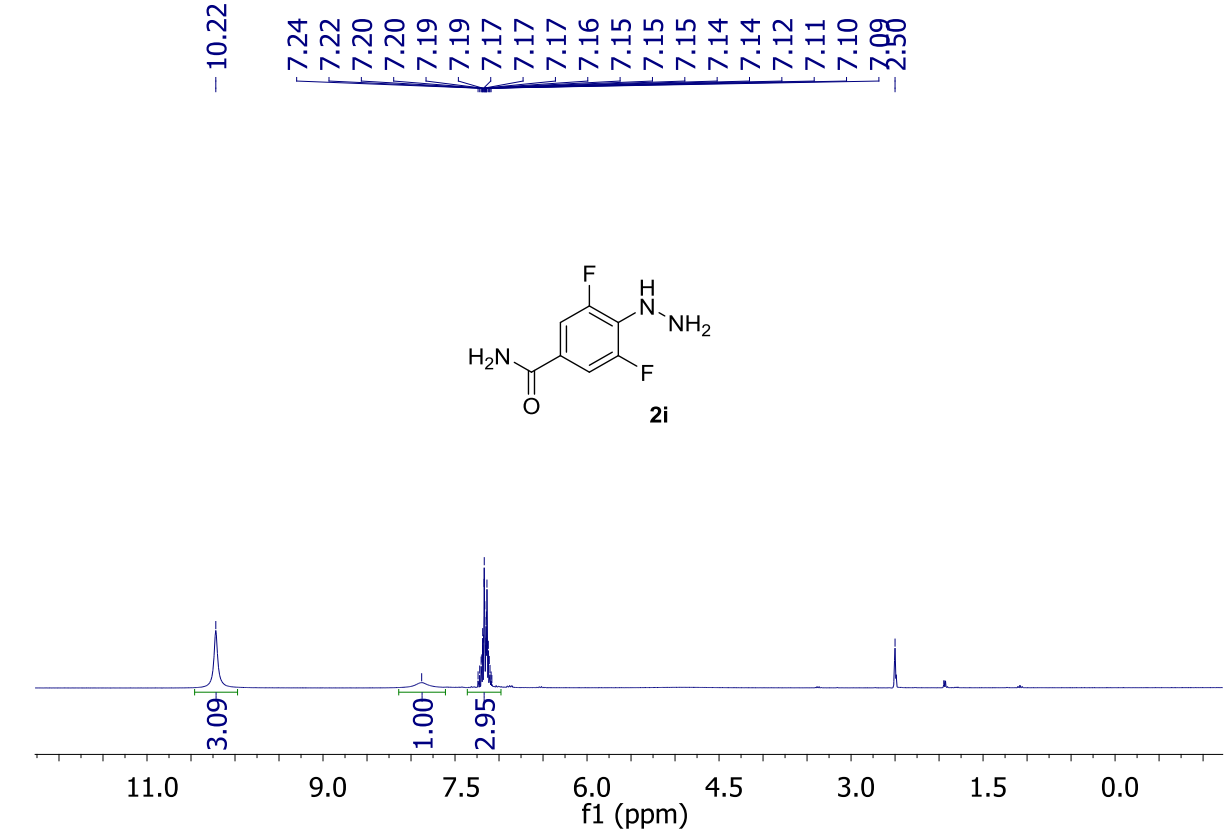
f1 (ppm)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7.19
7.17
7.17
7.17
7.16
7.15
7.15
7.15
7.14
7.14
7.12
7.11
7.10
7.09
2.50
F
H
N
NH2
H2N
O
F
2i
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11.0
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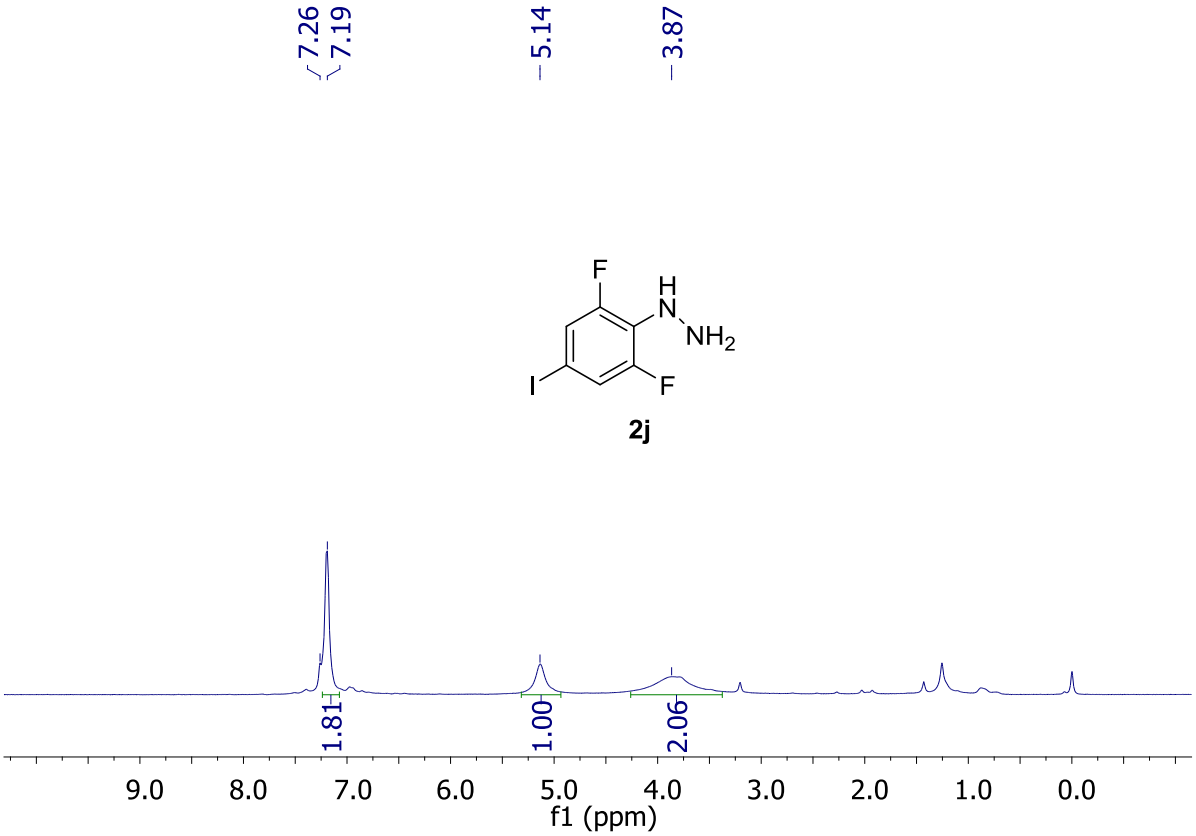
7.5
6.0
4.5
3.0
1.5
0.0
f1 (ppm)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F
H
N
NH2
I
F
2j
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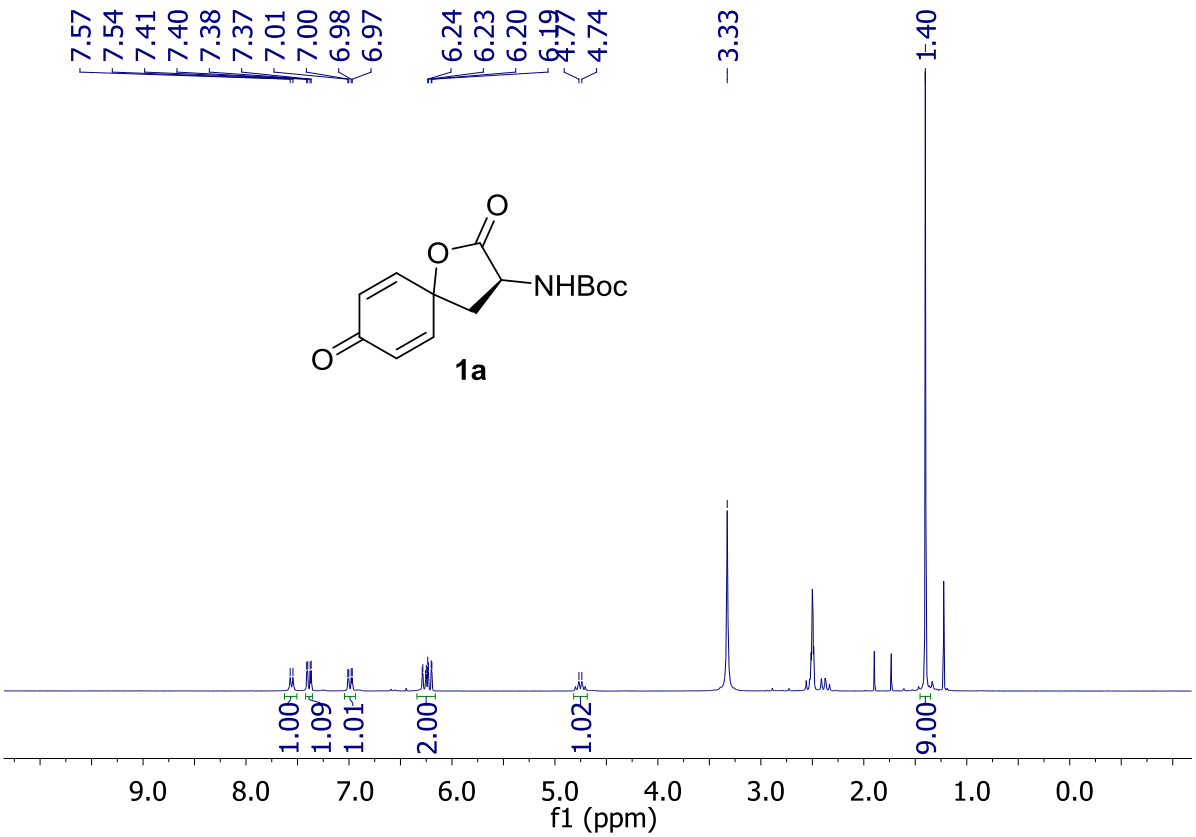
f1 (ppm)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6.20
6.19
4.77
4.74
3.33
1.40
O
O
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O
1a
1.00
1.09
1.01
2.00
1.02
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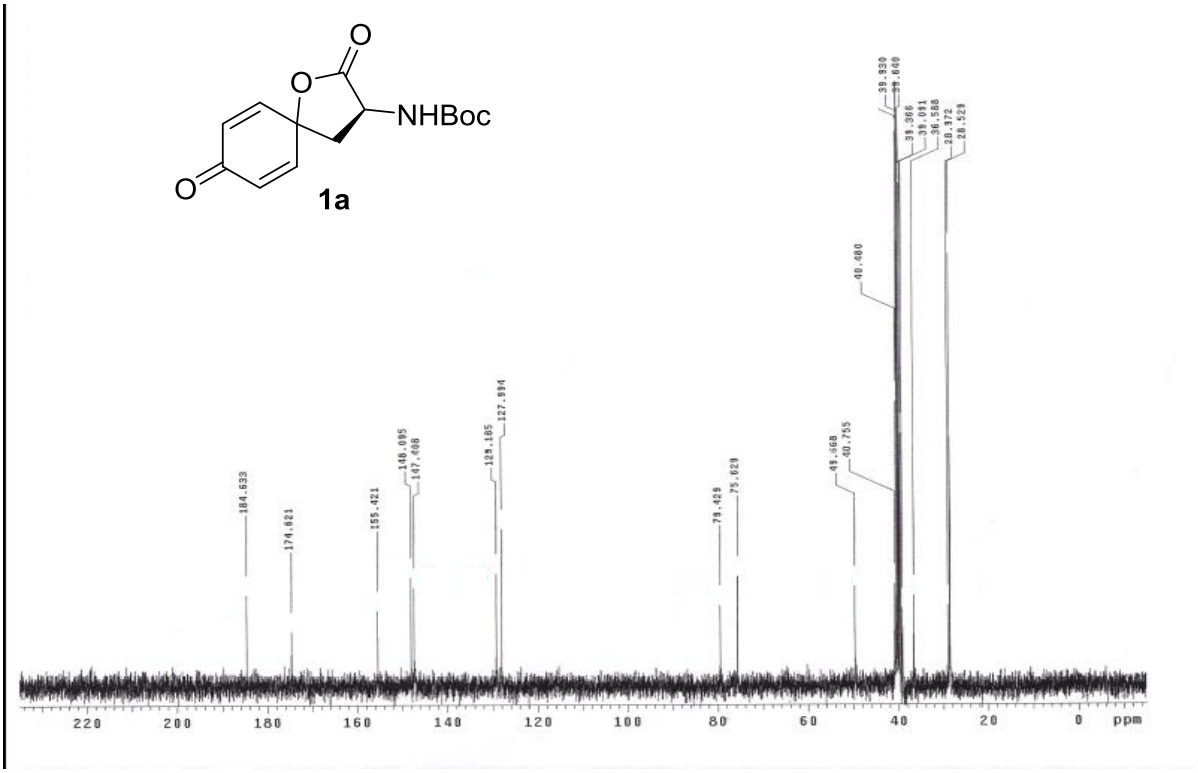
f1 (ppm)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49.755
40.480
39.930
39.640
39.366
39.091
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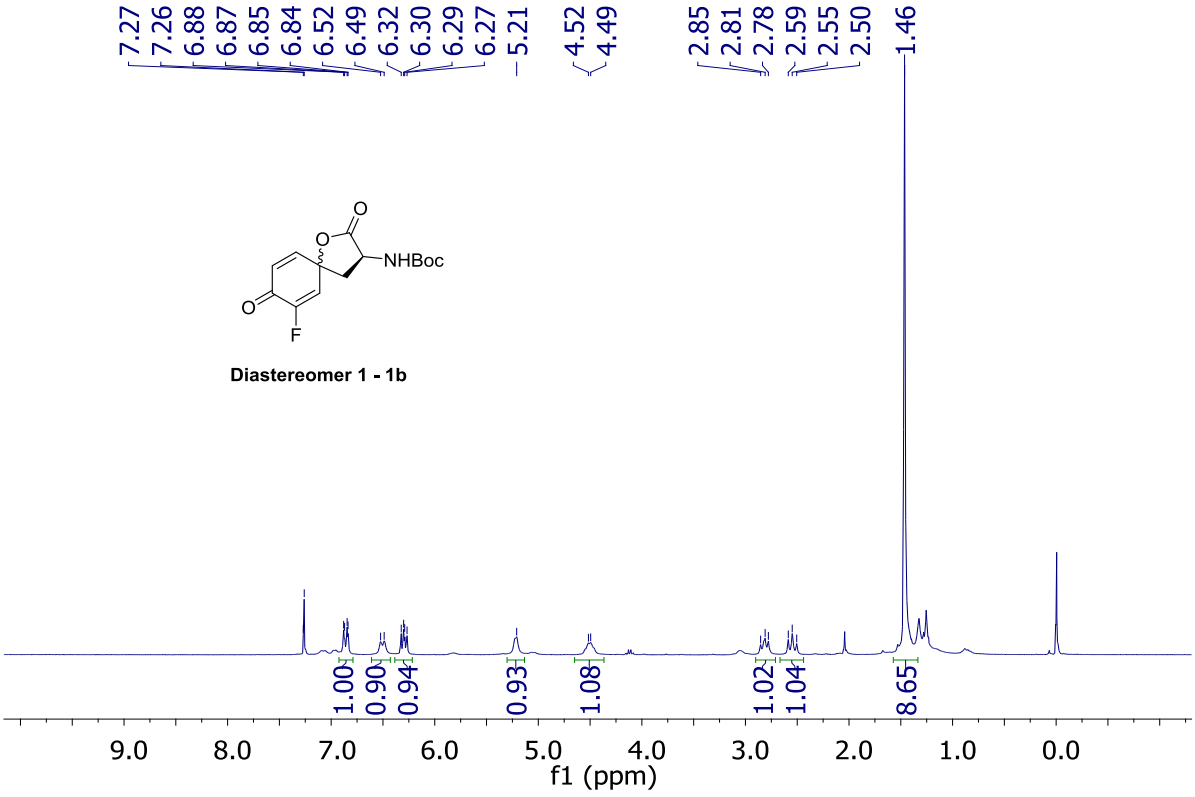
60
40
20
0
ppm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4.52
4.49
2.85
2.81
2.78
2.59
2.55
2.50
1.46
O
O
NHBoc
O
F
Diastereomer 1 - 1b
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0.94
0.93
1.08
1.02
1.04
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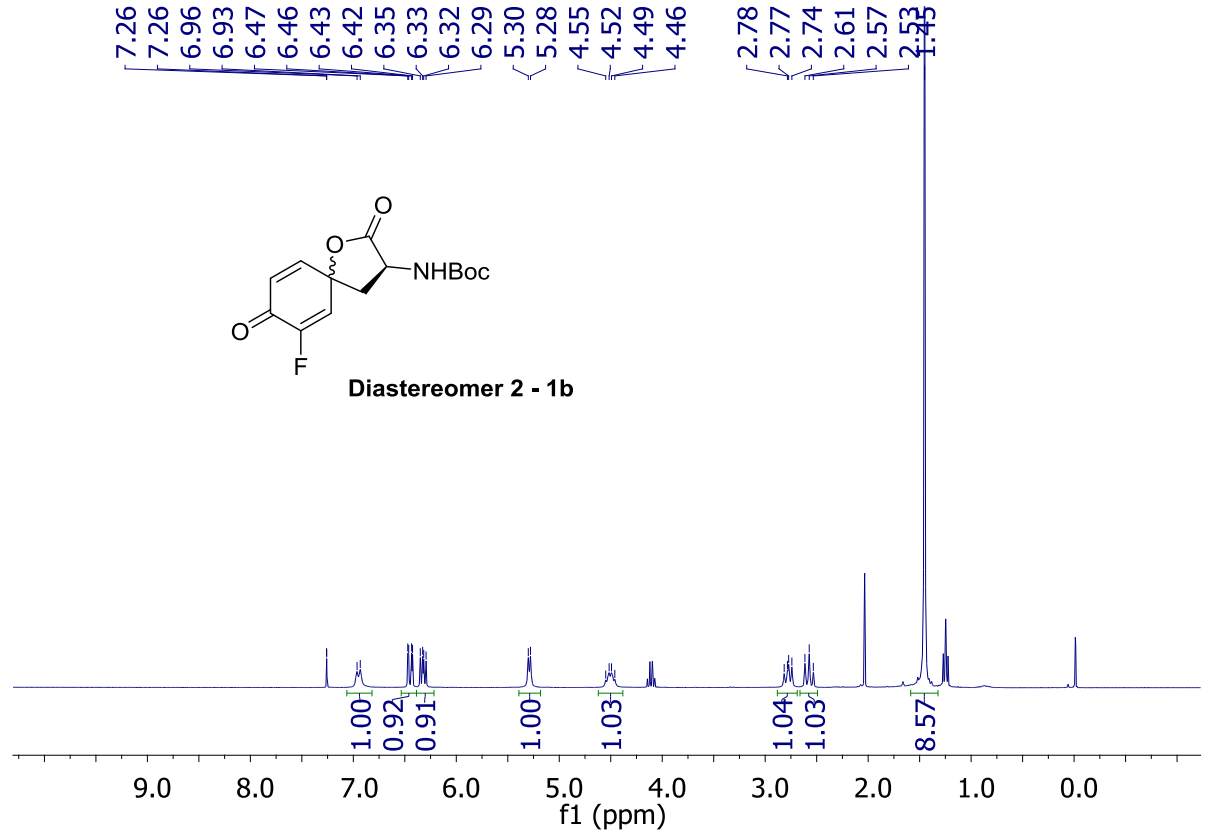
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2.77
2.74
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2.53
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Diastereomer 2 - 1b
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0.91
1.00
1.03
1.04
1.03
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7.0
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5.0
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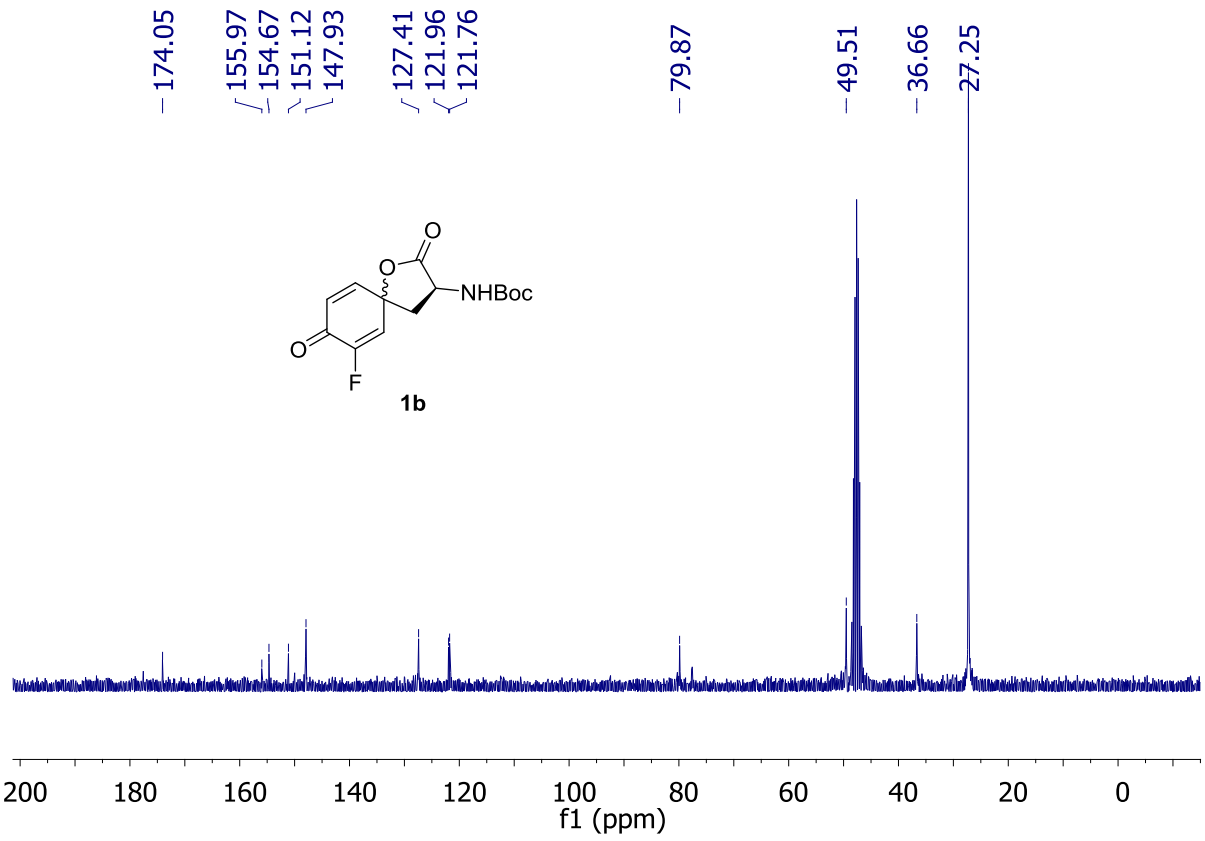
f1 (ppm)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20
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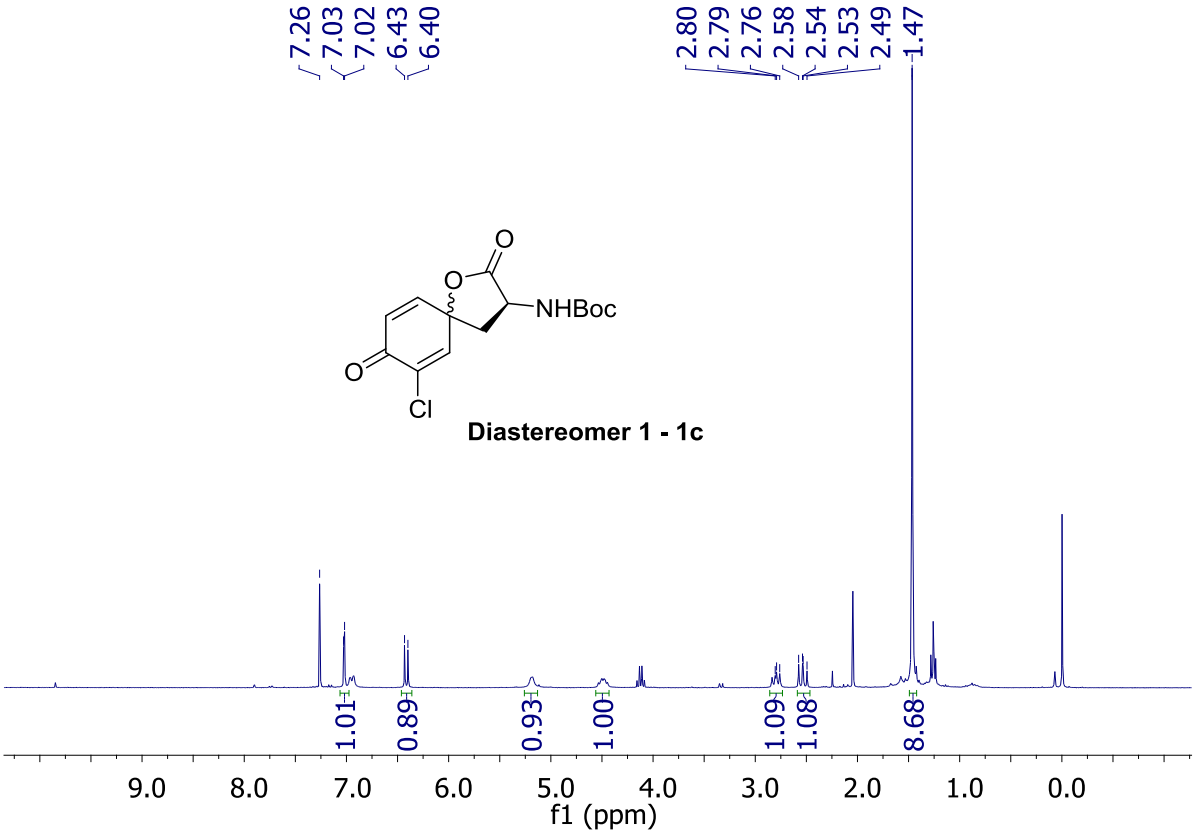
f1 (ppm)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2.76
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2.53
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1.47
O
O
NHBoc
O
Cl
Diastereomer 1 - 1c
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0.89
0.93
1.00
1.09
1.08
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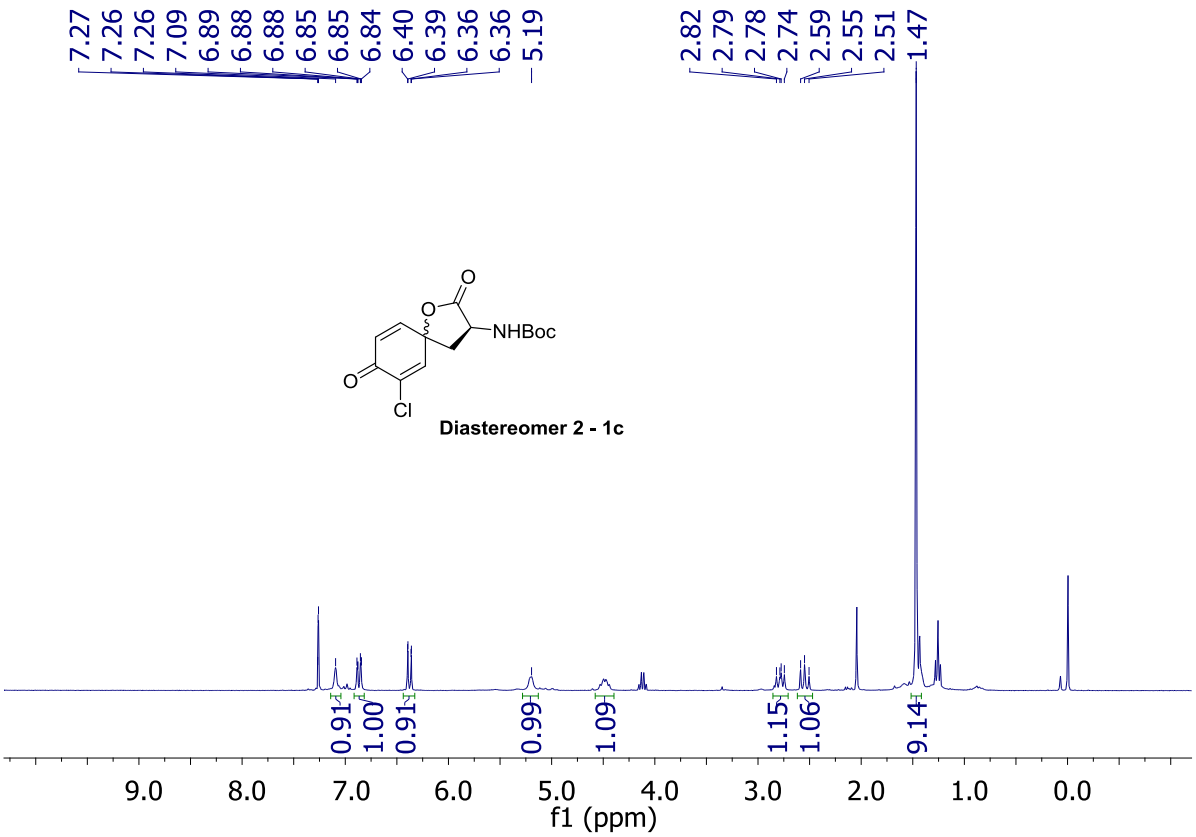
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2.79
2.78
2.74
2.59
2.55
2.51
1.47
O
O
NHBoc
O
Cl
Diastereomer 2 - 1c
0.91
1.00
0.91
0.99
1.09
1.15
1.06
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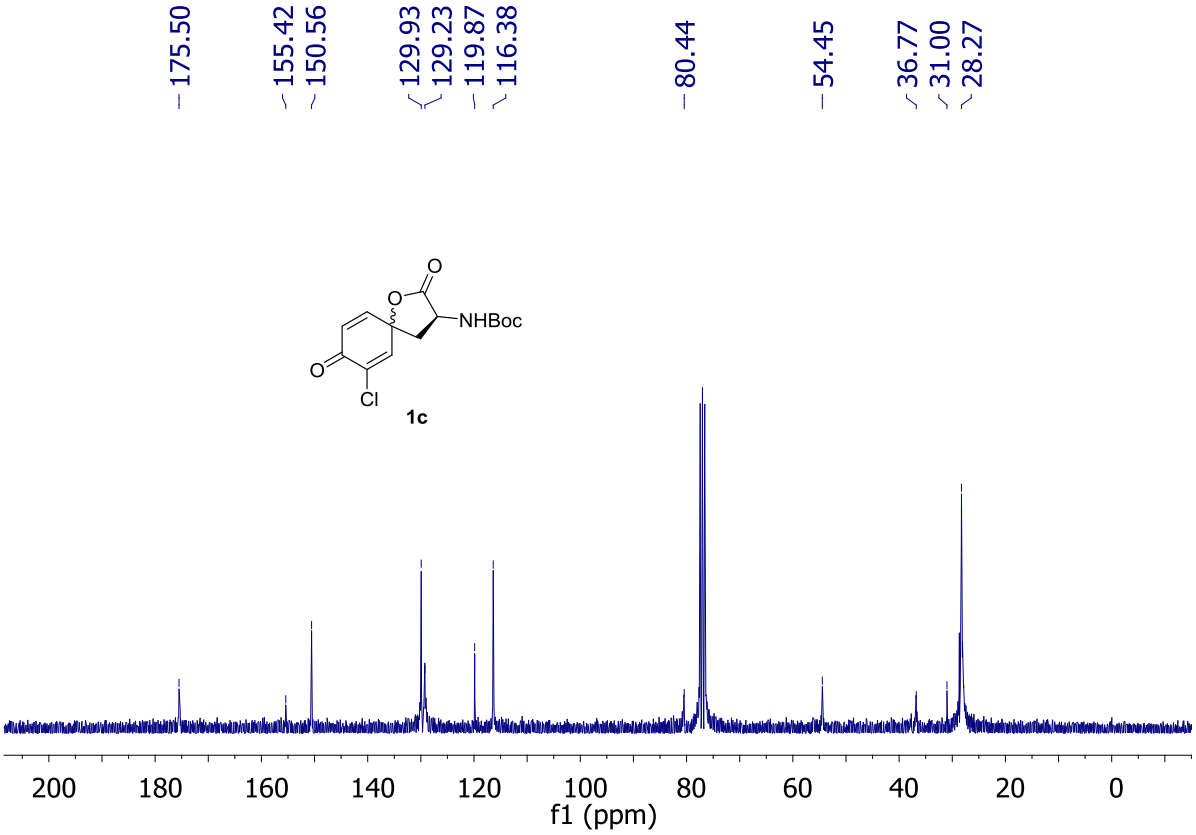
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20
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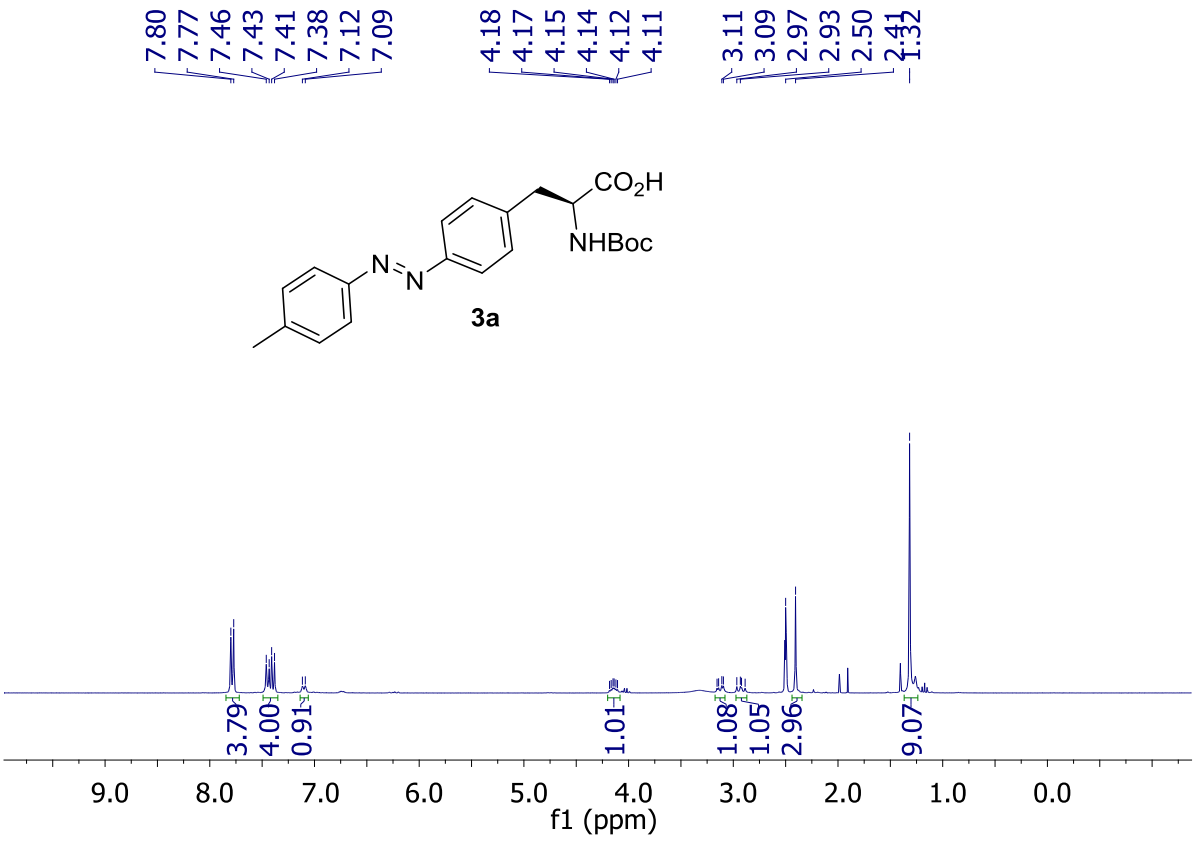
f1 (ppm)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3.09
2.97
2.93
2.50
2.41
1.32
CO2H
NHBoc
N
N
3a
3.79
4.00
0.91
1.01
1.08
1.05
2.96
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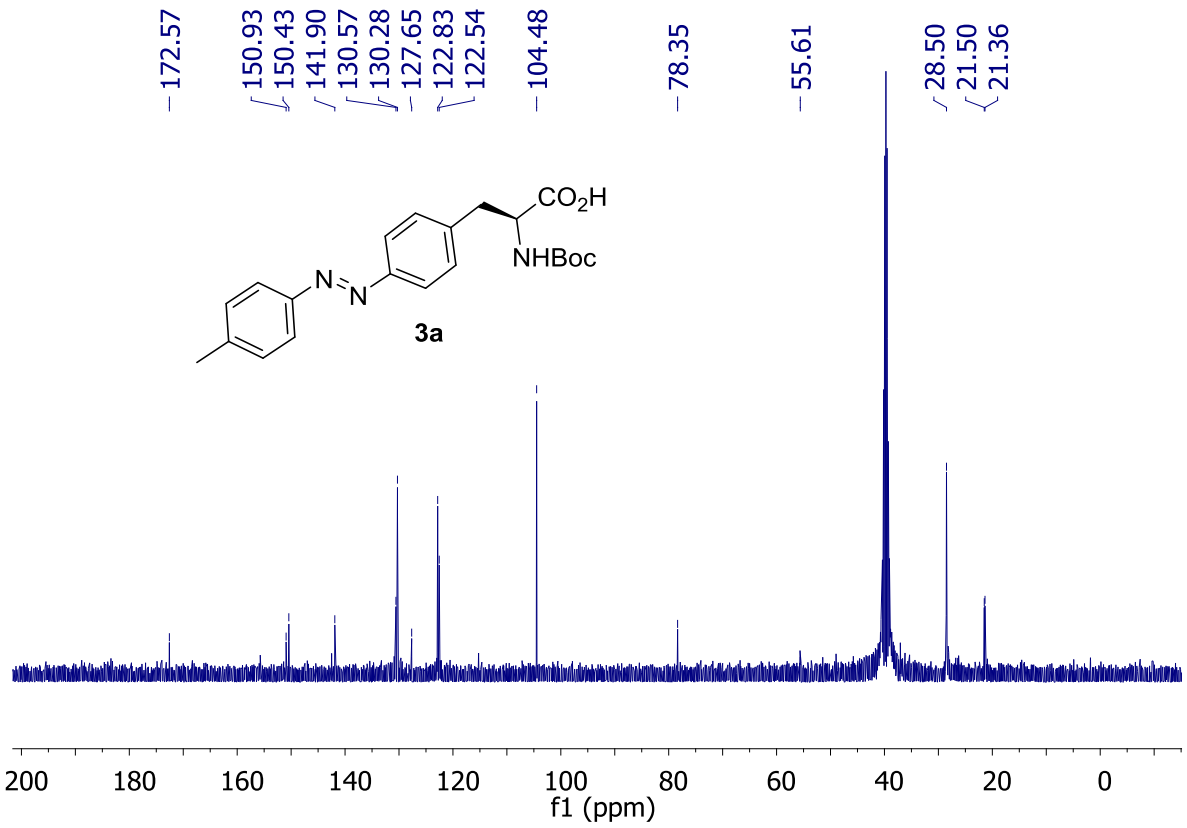
f1 (ppm)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21.50
21.36
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NHBoc
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N
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180
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140
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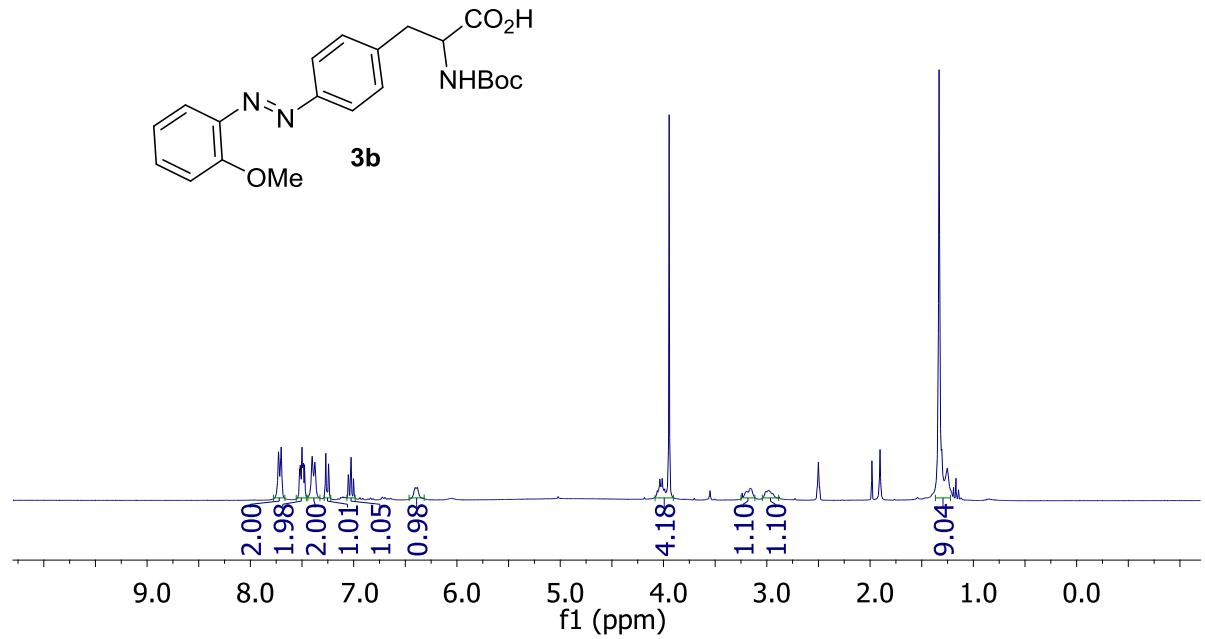
100
80
60
40
20
0
f1 (ppm)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NHBoc
N
N
OMe
3b
2.00
1.98
2.00
1.01
1.05
0.98
4.18
1.10
1.10
9.04
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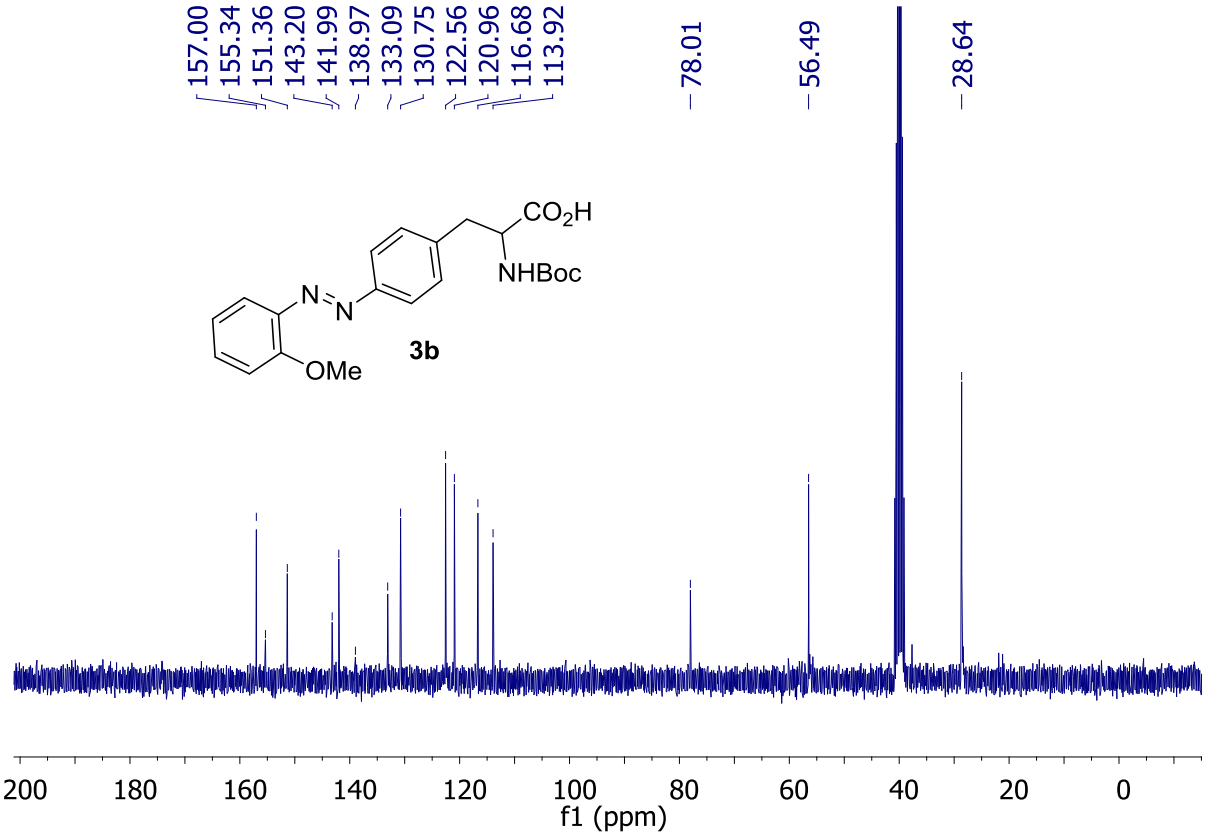
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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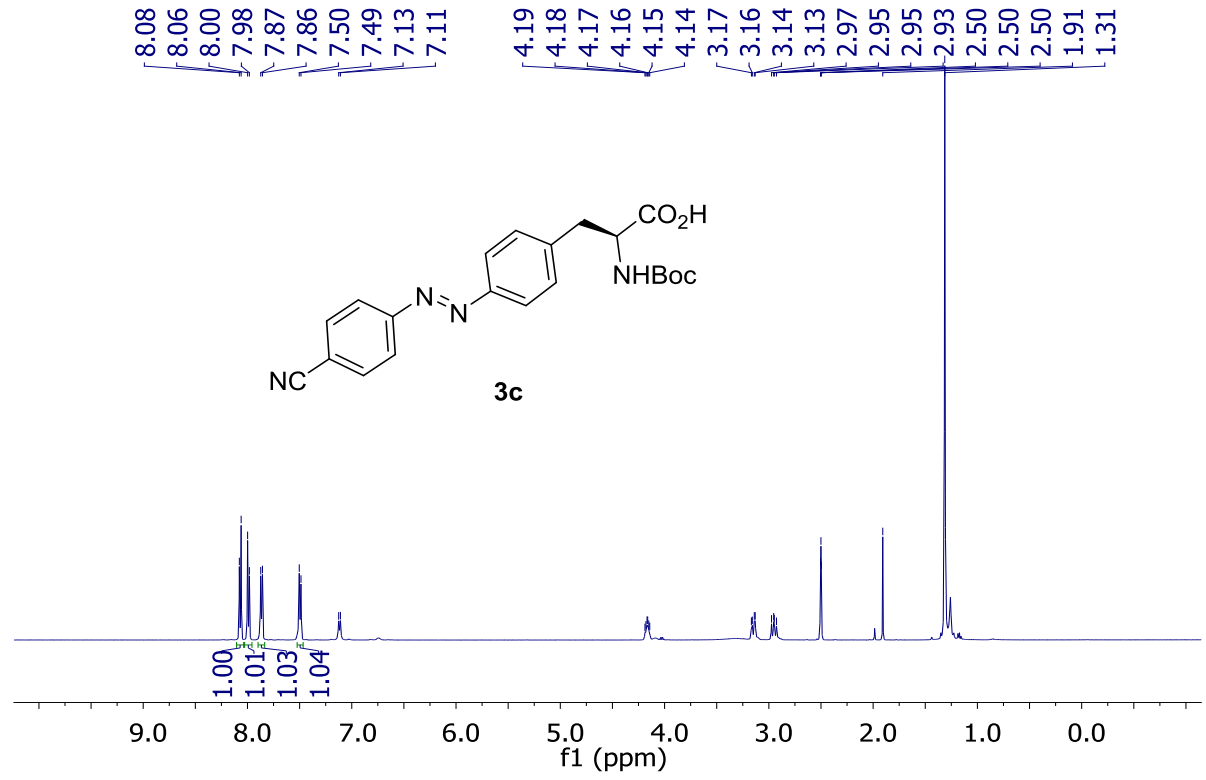
151.36
143.20
141.99
138.97
133.09
130.75
122.56
120.96
116.68
113.92
78.01
56.49
28.64
CO2H
NHBoc
N=N
OMe
3b
200
180
160
140
120
100
80
60
40
20
0
f1 (ppm)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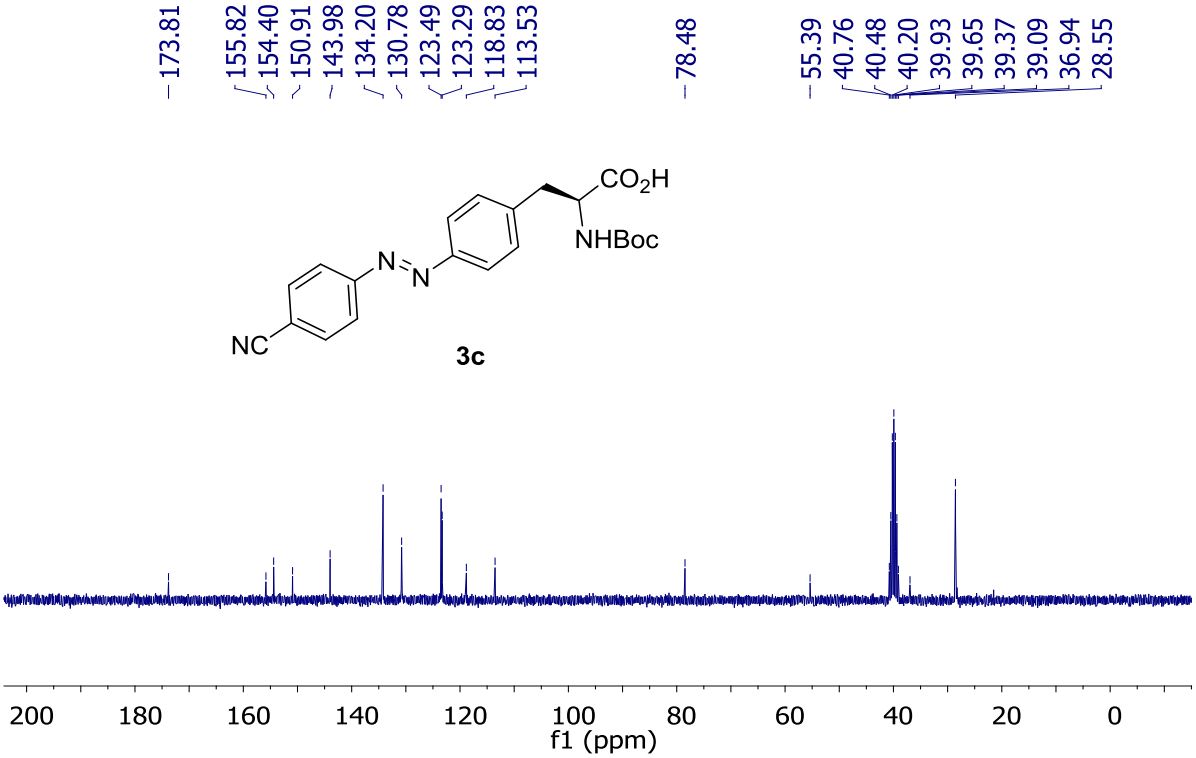
7.86
7.50
7.49
7.13
7.11
4.19
4.18
4.17
4.16
4.15
4.14
3.17
3.16
3.14
3.13
2.97
2.95
2.95
2.93
2.50
2.50
2.50
1.91
1.31
CO2H
NHBoc
N=N
NC
3c
1.00
1.01
1.03
1.04
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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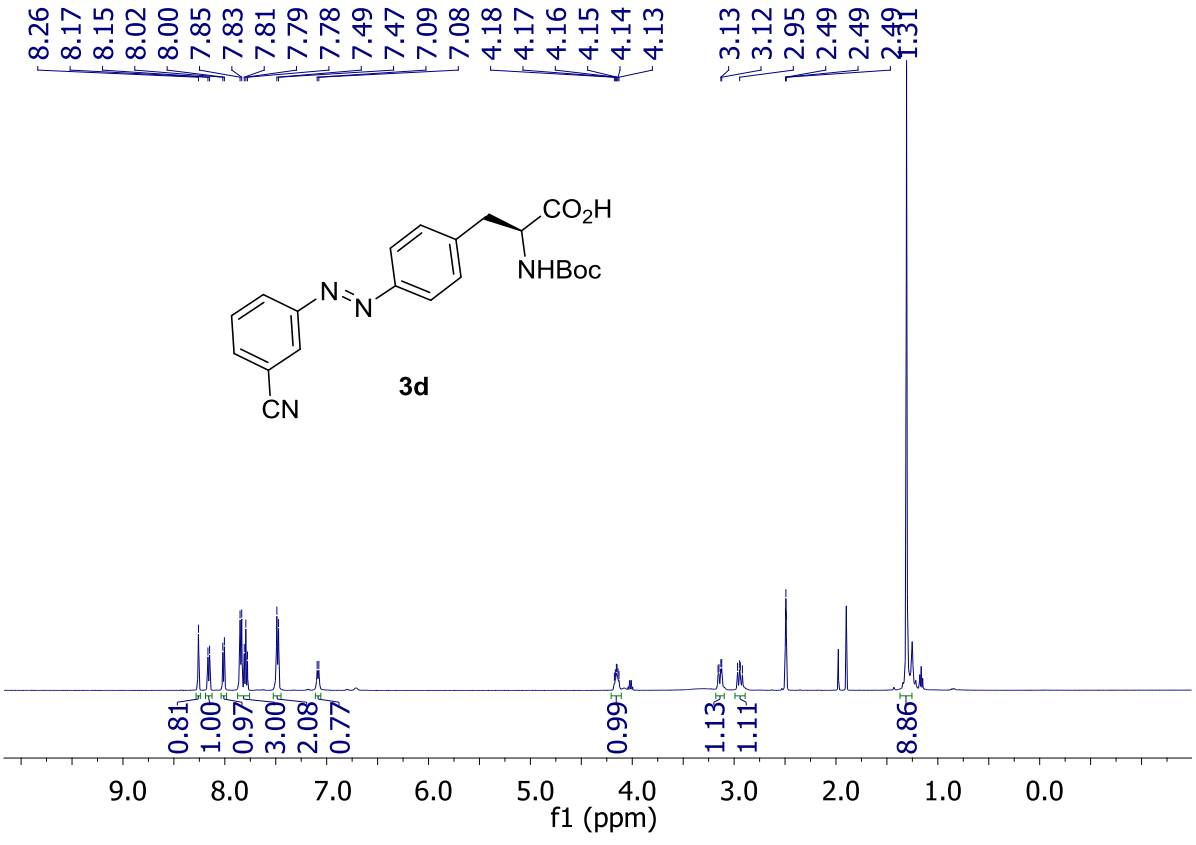
143.98
134.20
130.78
123.49
123.29
118.83
113.53
78.48
55.39
40.76
40.48
40.20
39.93
39.65
39.37
39.09
36.94
28.55
CO2H
NHBoc
N=N
NC
3c
200
180
160
140
120
100
80
60
40
20
0
f1 (ppm)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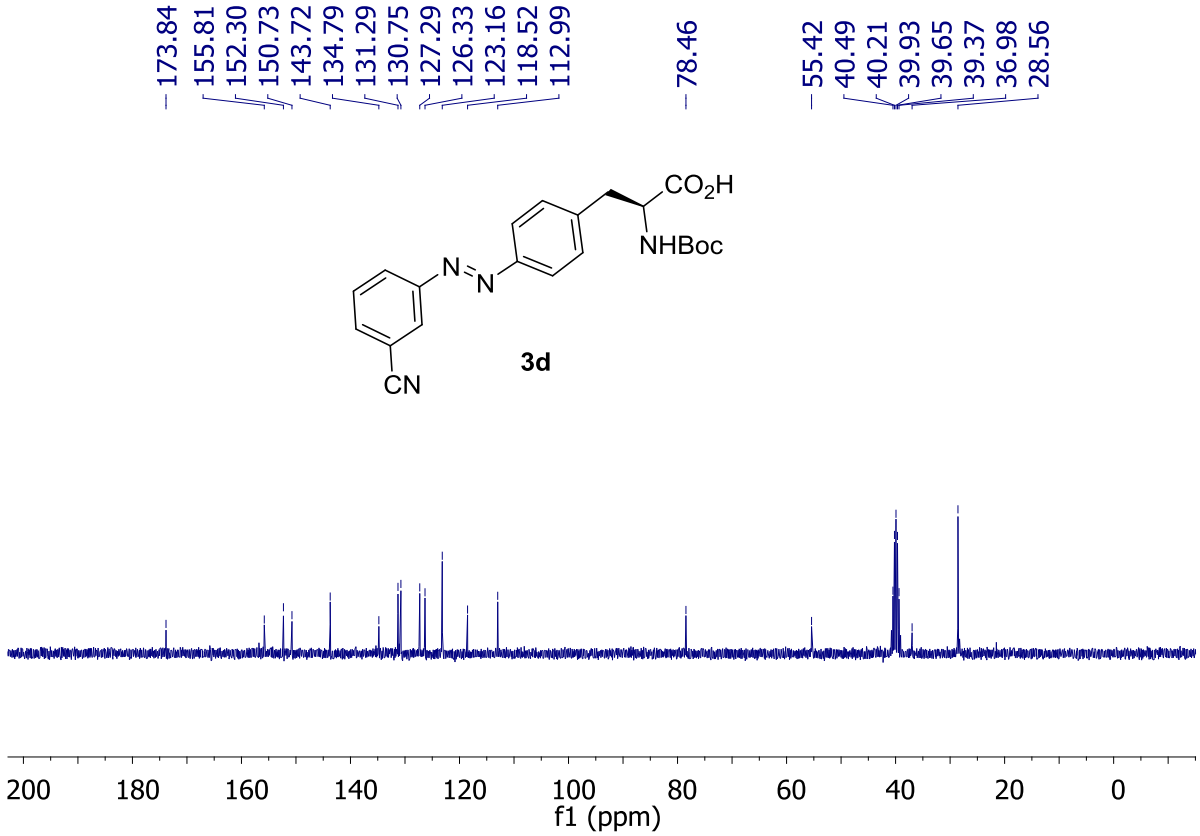
8.00
7.85
7.83
7.81
7.79
7.78
7.49
7.47
7.09
7.08
4.18
4.17
4.16
4.15
4.14
4.13
3.13
3.12
2.95
2.49
2.49
2.49
1.31
CO2H
NHBoc
N=N
CN
3d
0.81
1.00
0.97
3.00
2.08
0.77
0.99
1.13
1.11
8.86
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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131.29
130.75
127.29
126.33
123.16
118.52
112.99
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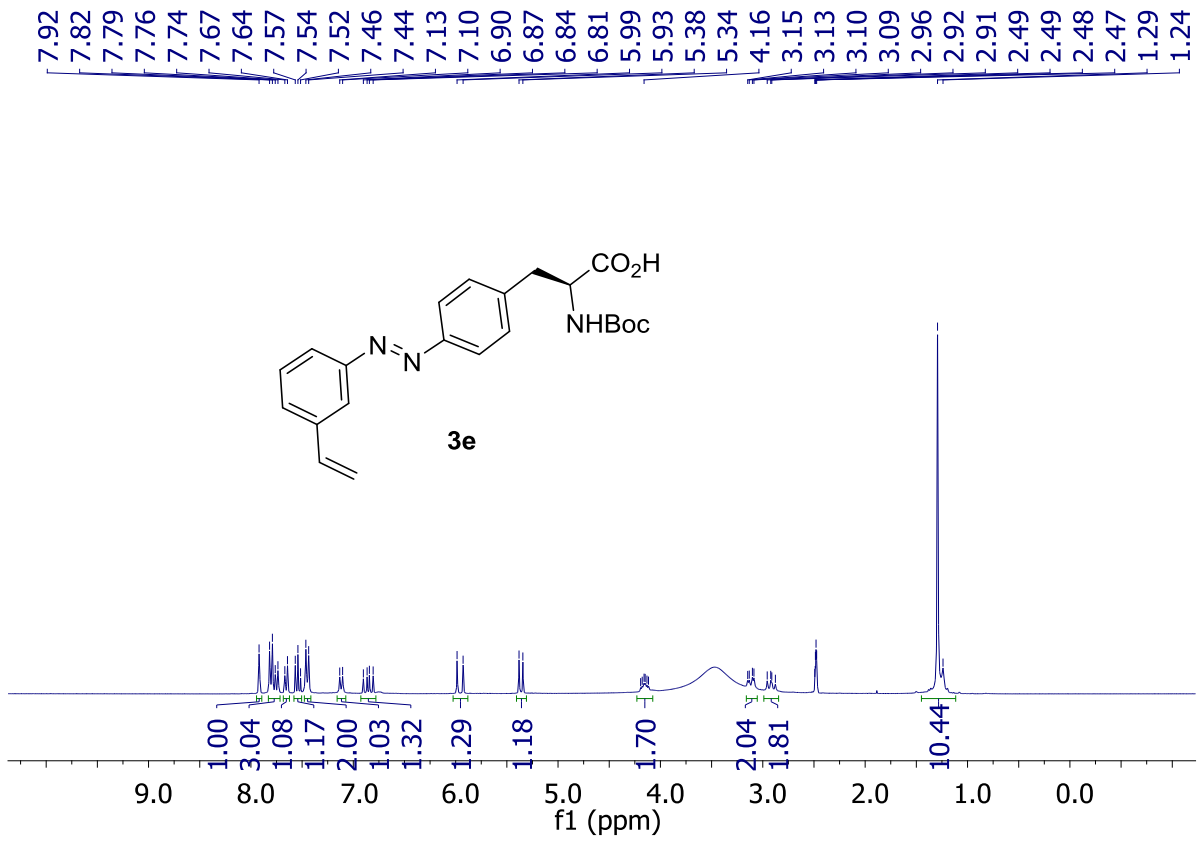
55.42
40.49
40.21
39.93
39.65
39.37
36.98
28.56
CO2H
NHBoc
N=N
CN
3d
200
180
160
140
120
100
80
60
40
20
0
f1 (ppm)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7.67
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7.57
7.54
7.52
7.46
7.44
7.13
7.10
6.90
6.87
6.84
6.81
5.99
5.93
5.38
5.34
4.16
3.15
3.13
3.10
3.09
2.96
2.92
2.91
2.49
2.49
2.48
2.47
1.29
1.24
CO2H
NHBoc
N=N
3e
1.00
3.04
1.08
1.17
2.00
1.03
1.32
1.29
1.18
1.70
2.04
1.81
10.44
9.0
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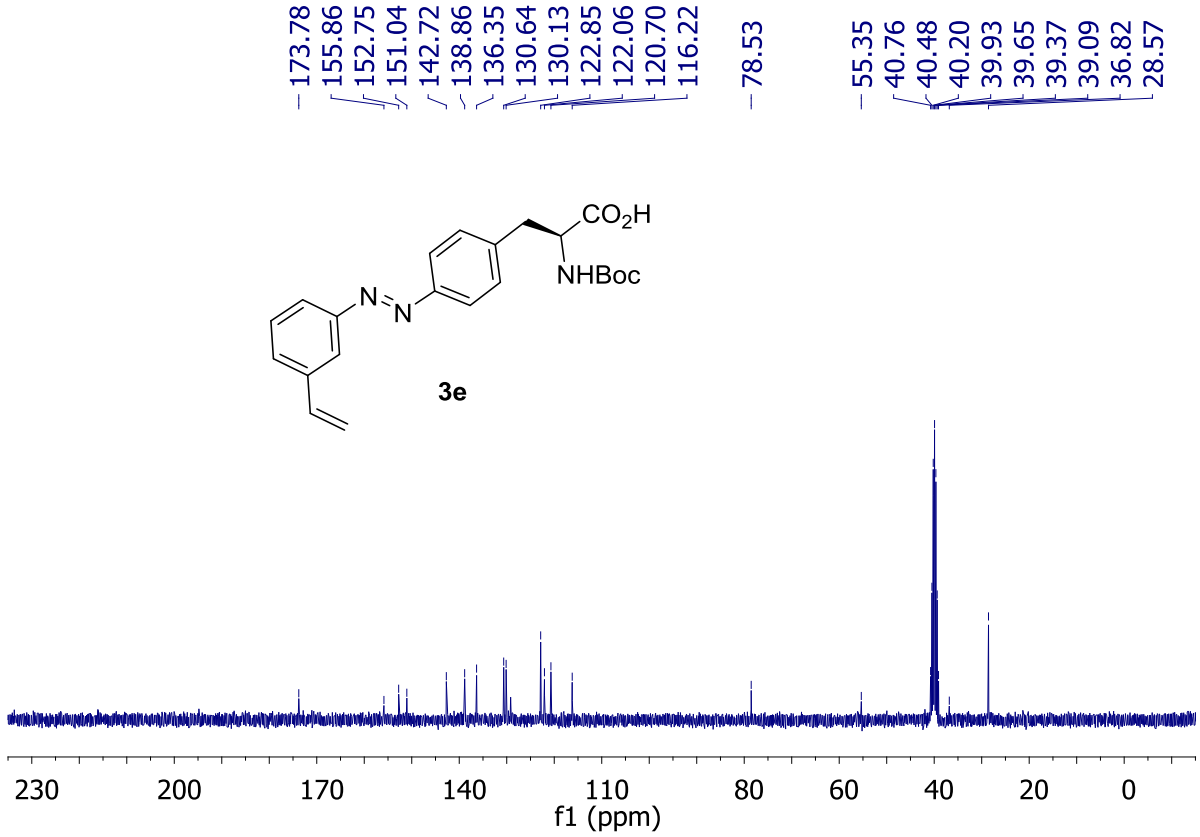
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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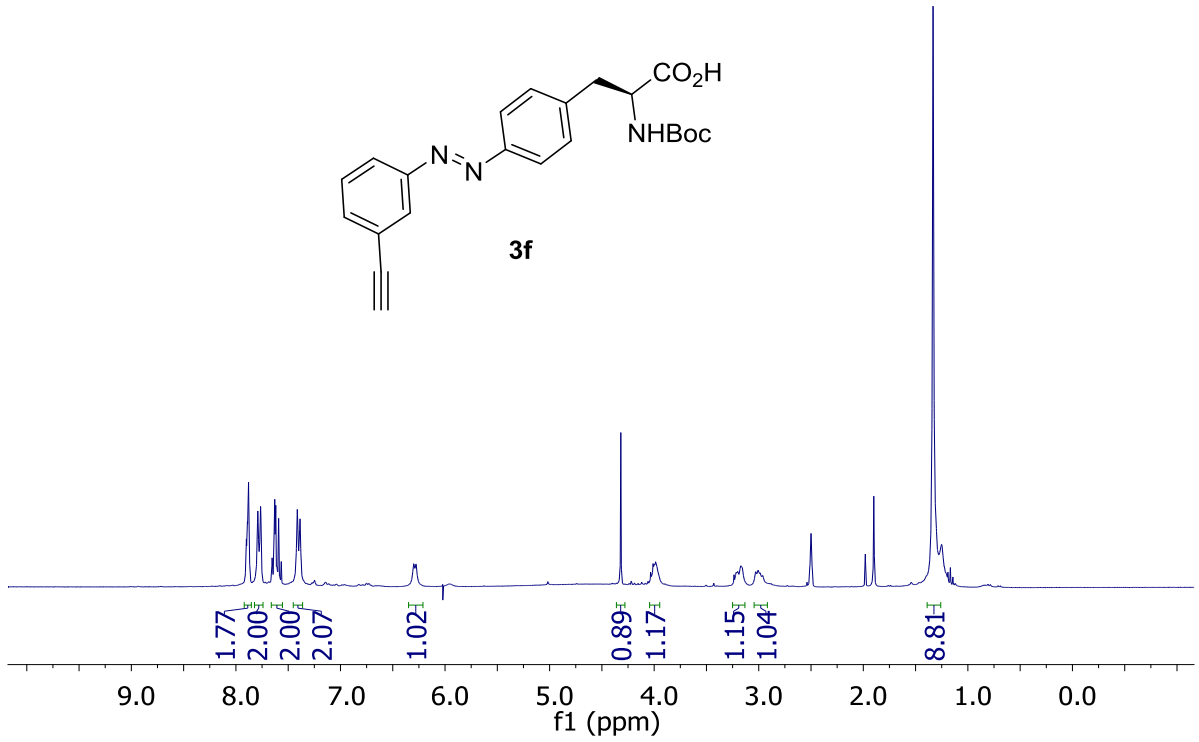
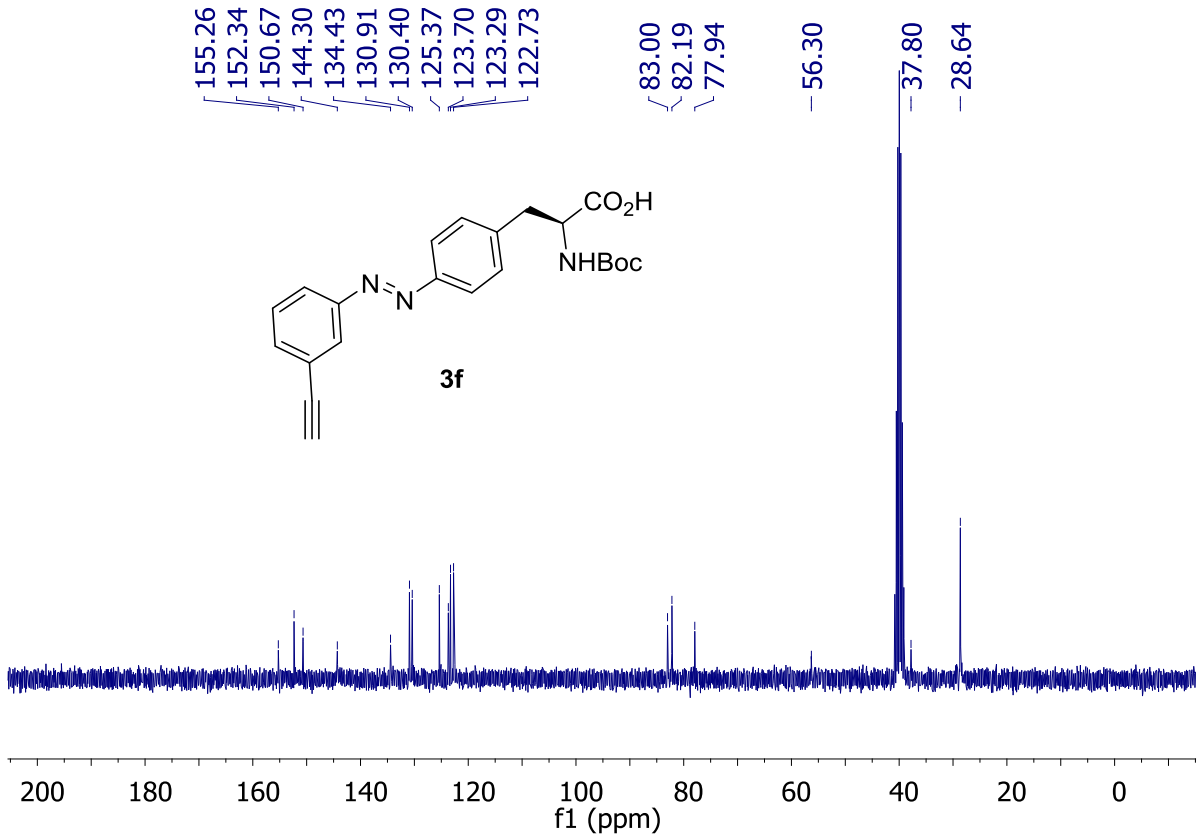
142.72
138.86
136.35
130.64
130.13
122.85
122.06
120.70
116.22
78.53
55.35
40.76
40.48
40.20
39.93
39.65
39.37
39.09
36.82
28.57
CO2H
NHBoc
N=N
3e
230
200
170
140
110
80
60
40
20
0
f1 (ppm)


CO2H
NHBoc
N=N
3f
1.77
2.00
2.00
2.07
1.02
0.89
1.17
1.15
1.04
8.81
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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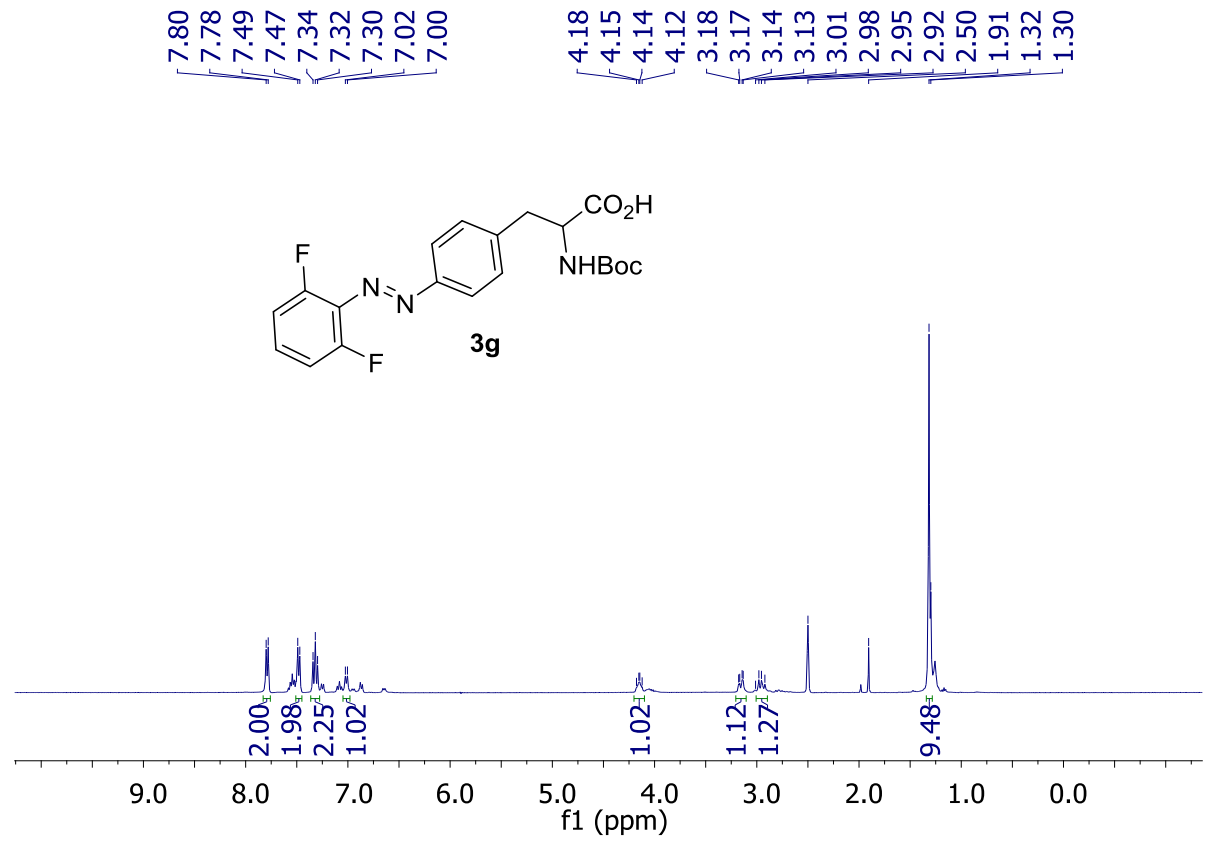
152.34
150.67
144.30
134.43
130.91
130.40
125.37
123.70
123.29
122.73
83.00
82.19
77.94
56.30
37.80
28.64
CO2H
NHBoc
N=N
3f
200
180
160
140
120
100
80
60
40
20
0
f1 (ppm)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7.47
7.34
7.32
7.30
7.02
7.00
4.18
4.15
4.14
4.12
3.18
3.17
3.14
3.13
3.01
2.98
2.95
2.92
2.50
1.91
1.32
1.30
CO2H
NHBoc
F
N=N
F
3g
2.00
1.98
2.25
1.02
1.02
1.12
1.27
9.48
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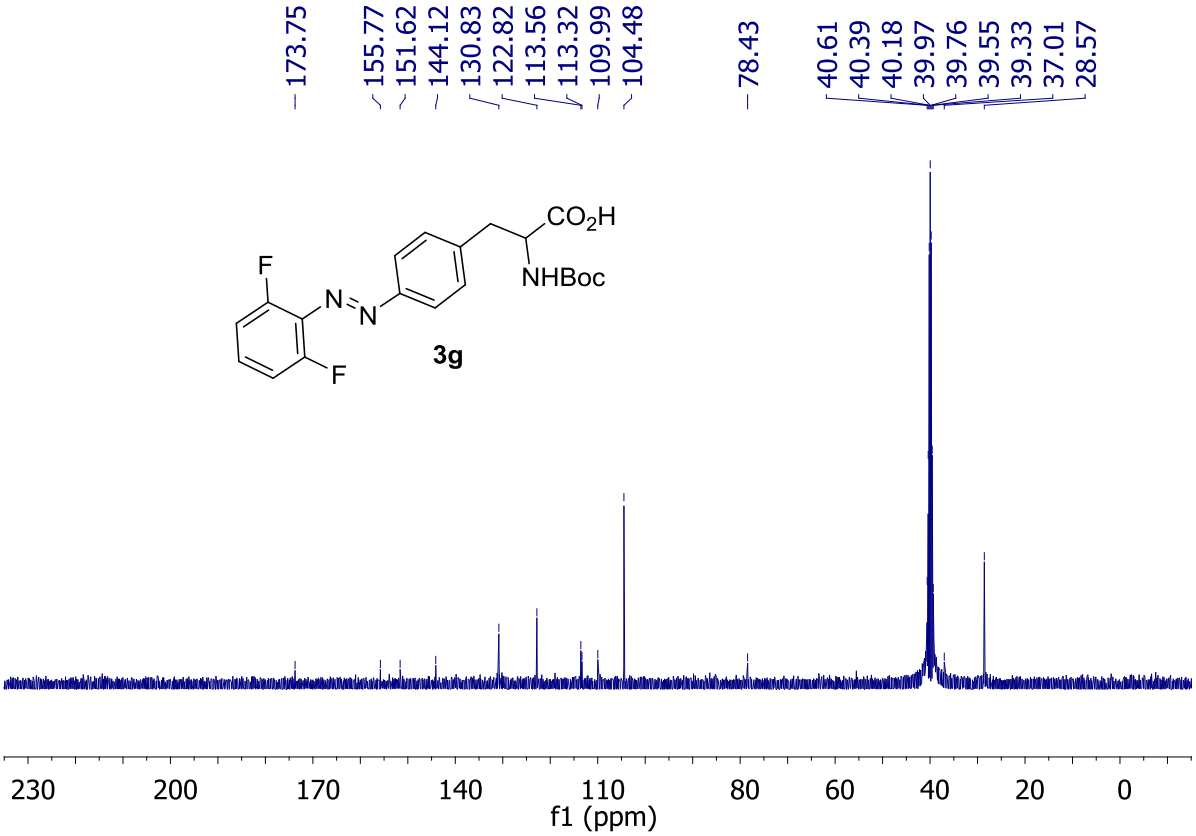
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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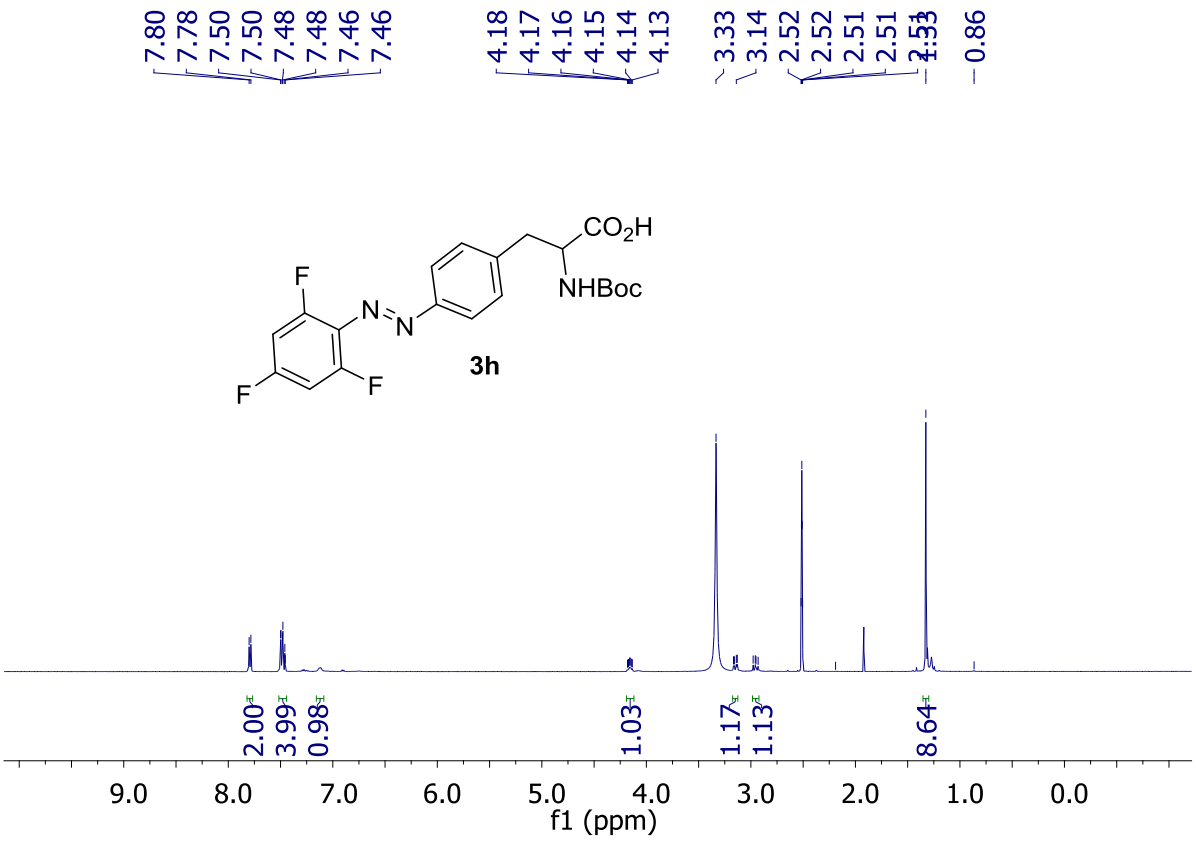
122.82
113.56
113.32
109.99
104.48
78.43
40.61
40.39
40.18
39.97
39.76
39.55
39.33
37.01
28.57
CO2H
NHBoc
F
N
N
F
3g
230
200
170
140
110
80
60
40
20
0
f1 (ppm)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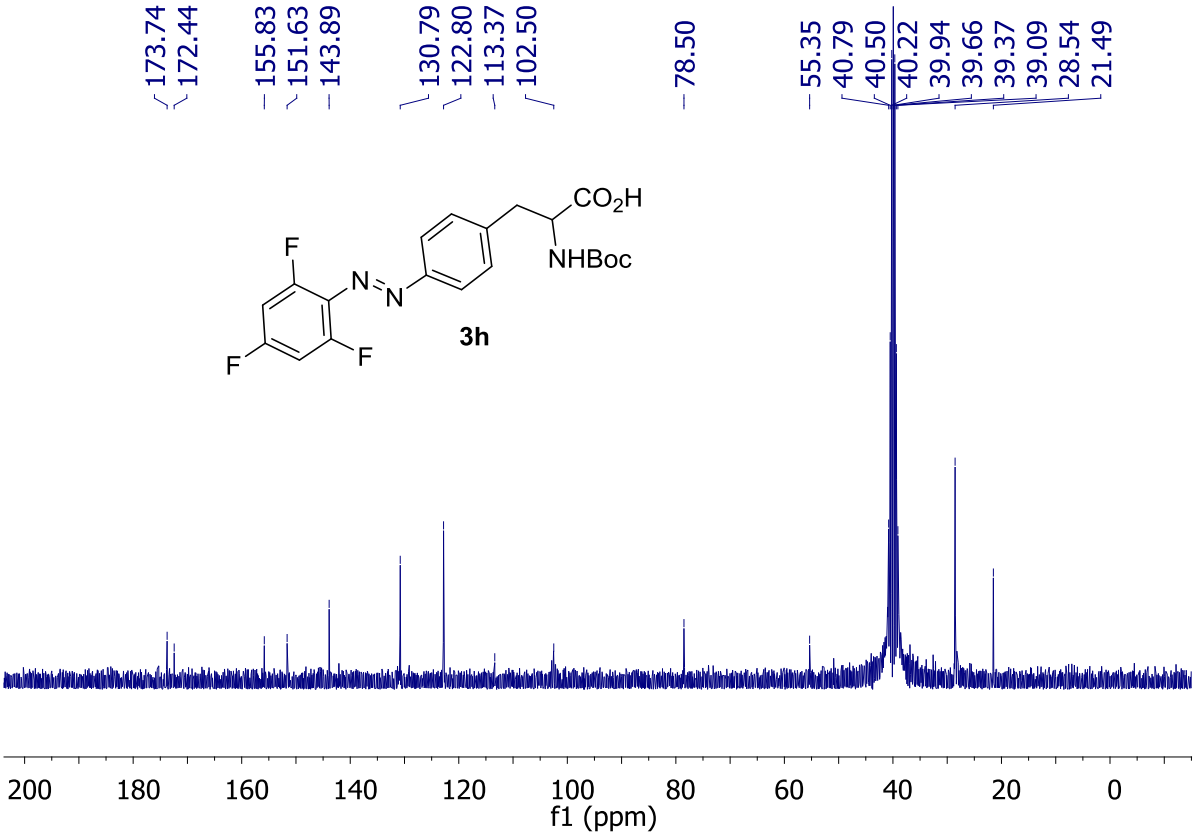
7.78
7.50
7.50
7.48
7.48
7.46
7.46
4.18
4.17
4.16
4.15
4.14
4.13
3.33
3.14
2.52
2.52
2.51
2.51
0.86
CO2H
NHBoc
F
N
N
F
F
3h
2.00
3.99
0.98
1.03
1.17
1.13
8.64
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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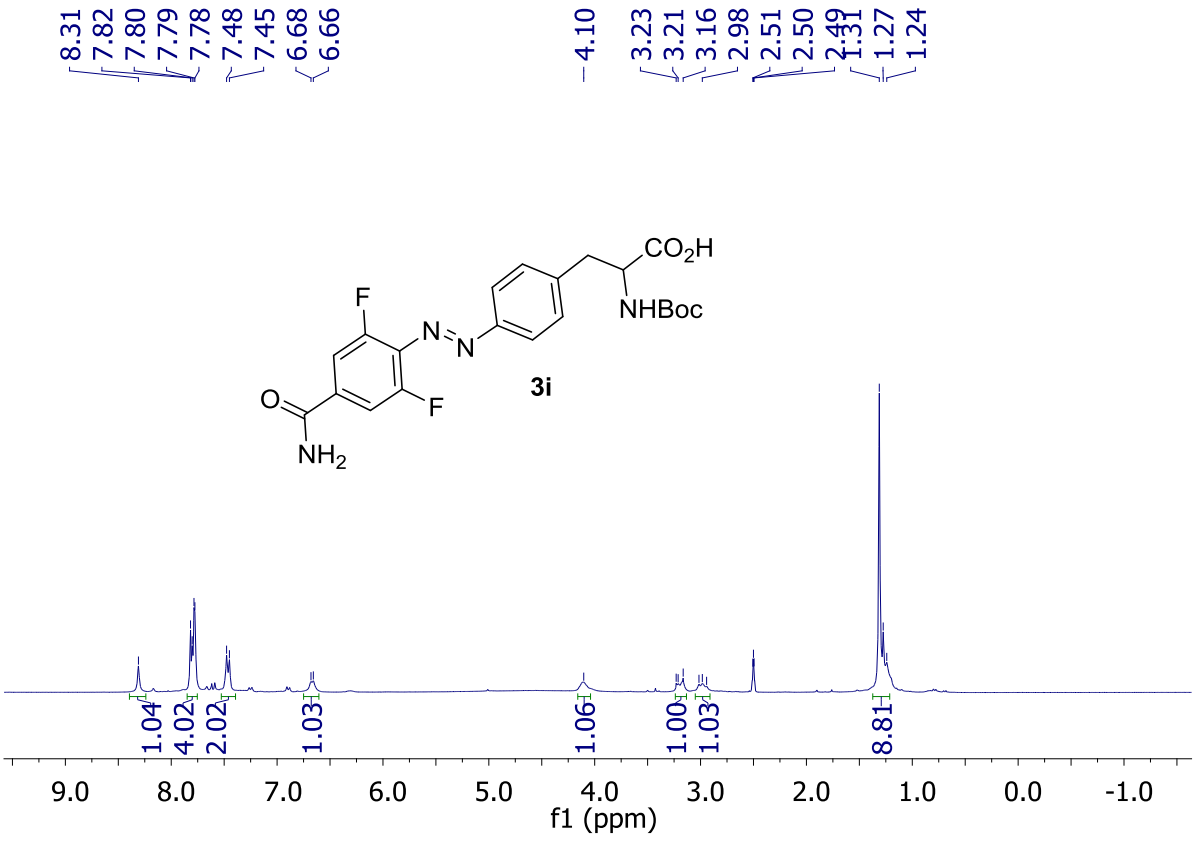
40.22
39.94
39.66
39.37
39.09
28.54
21.49
CO2H
NHBoc
F
N=N
F
F
3h
200
180
160
140
120
100
80
60
40
20
0
f1 (ppm)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6.66
4.10
3.23
3.21
3.16
2.98
2.51
2.50
2.49
1.31
1.27
1.24
CO2H
NHBoc
F
N=N
O
NH2
F
3i
1.04
4.02
2.02
1.03
1.06
1.00
1.03
8.81
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6.0
5.0
4.0
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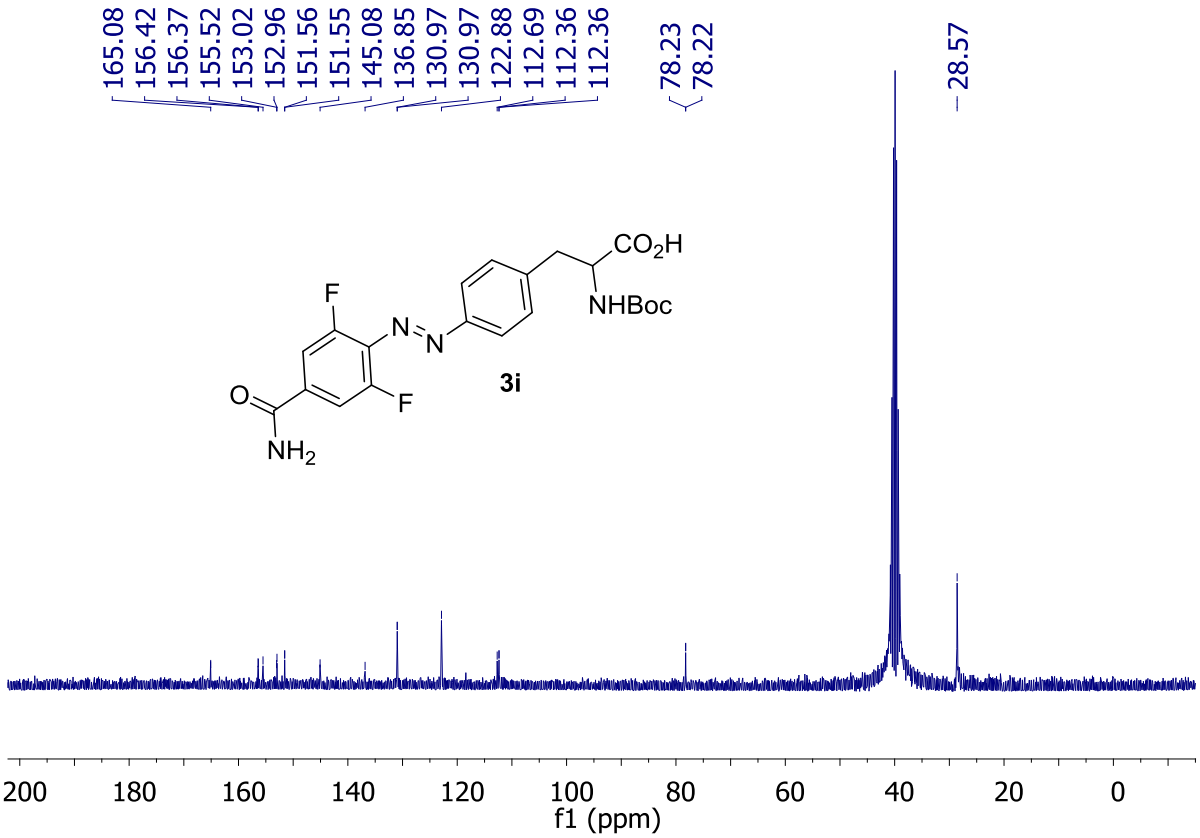
2.0
1.0
0.0
-1.0
f1 (ppm)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130.97
130.97
122.88
112.69
112.36
112.36
78.23
78.22
28.57
CO2H
NHBoc
F
N
N
F
O
NH2
3i
200
180
160
140
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100
80
60
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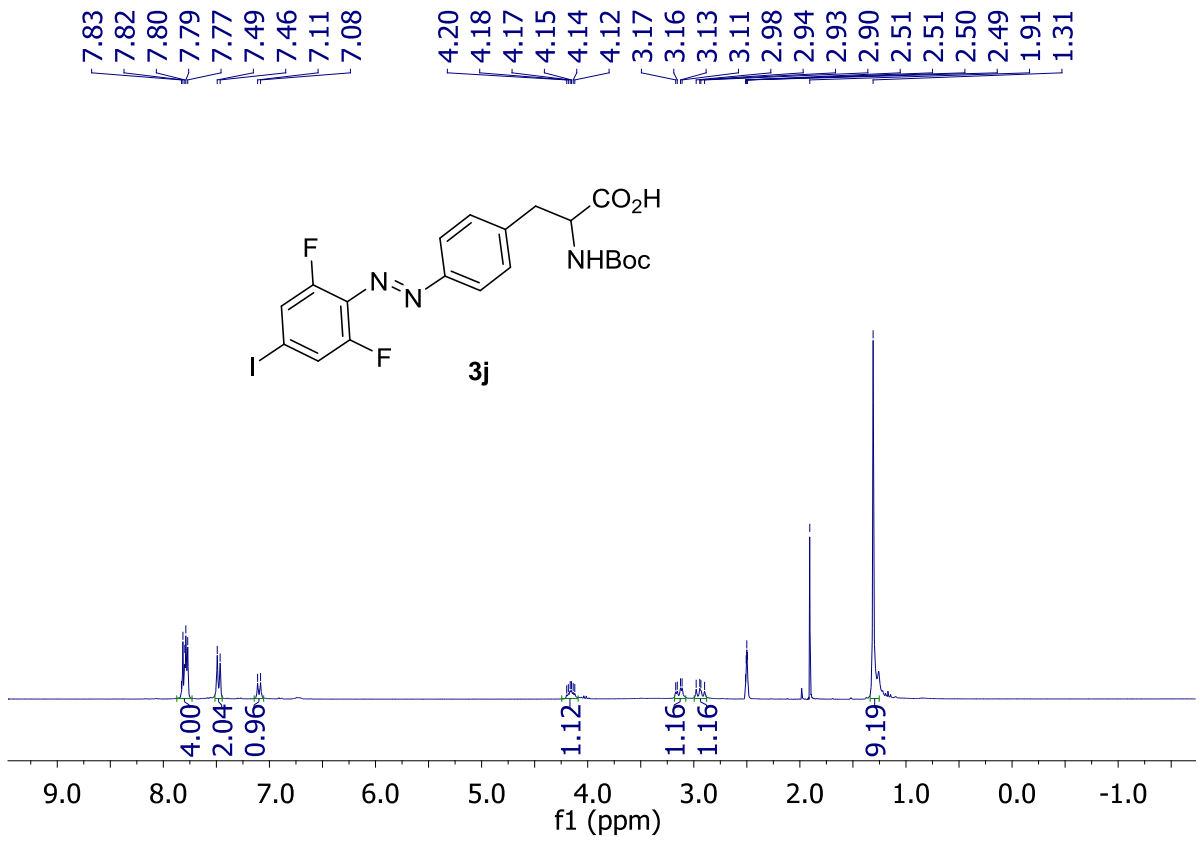
20
0
f1 (ppm)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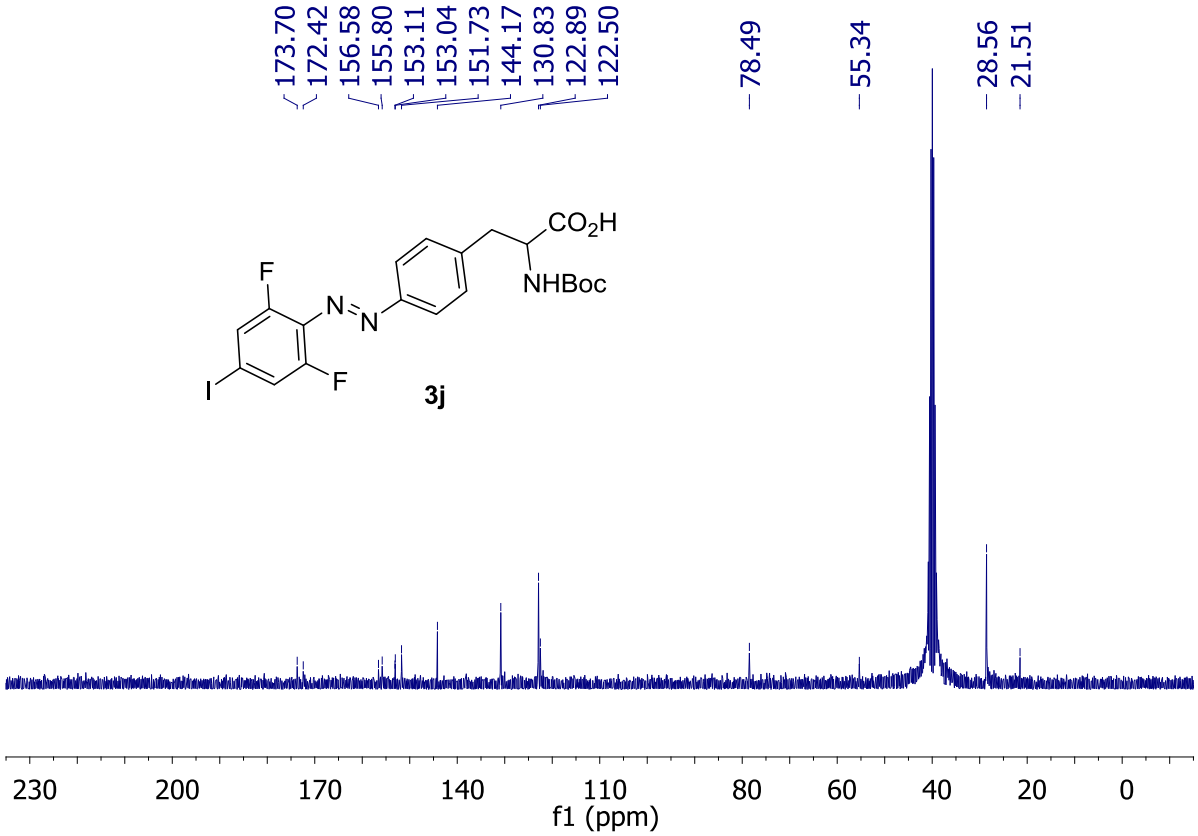
4.17
4.15
4.14
4.12
3.17
3.16
3.13
3.11
2.98
2.94
2.93
2.90
2.51
2.51
2.50
2.49
1.91
1.31
CO2H
NHBoc
F
N
N
F
I
3j
4.00
2.04
0.96
1.12
1.16
1.16
9.19
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
f1 (ppm)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144.17
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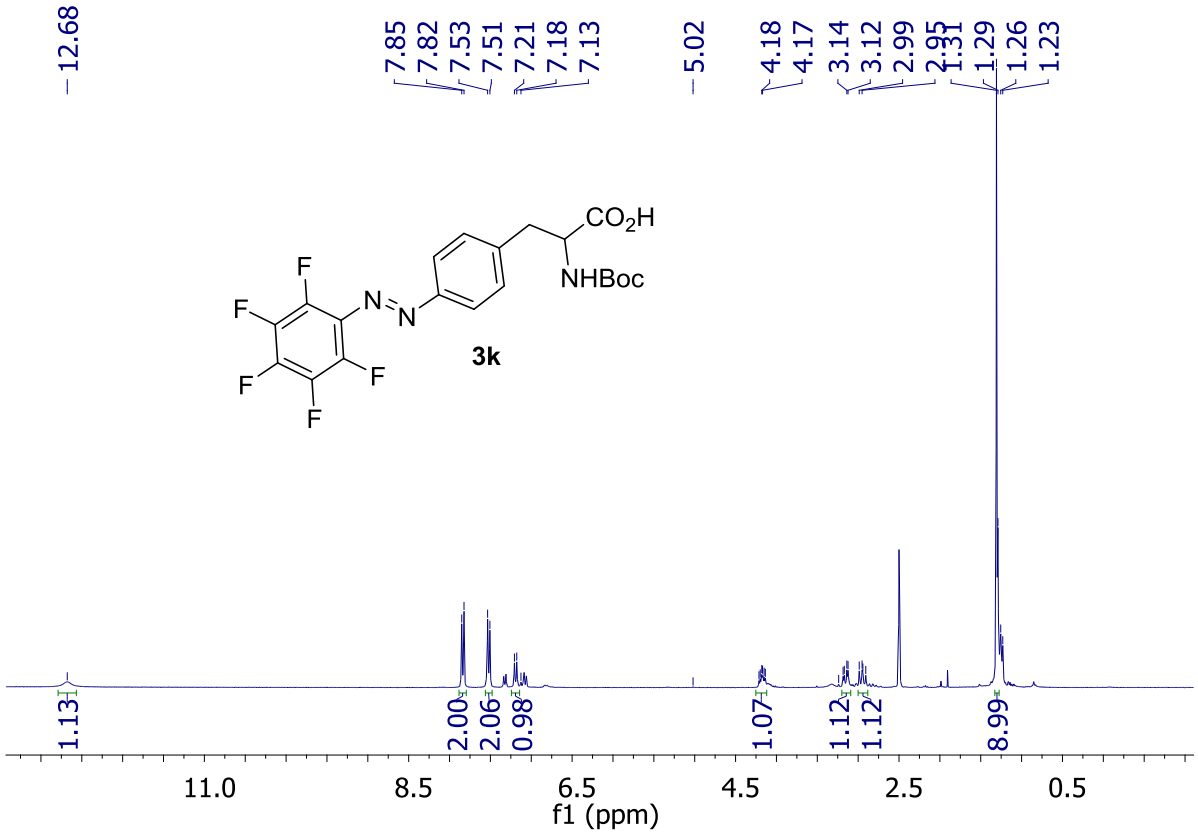
122.89
122.50
78.49
55.34
28.56
21.51
CO2H
NHBoc
F
N=N
F
I
3j
230
200
170
140
110
80
60
40
20
0
f1 (ppm)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7.21
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7.13
5.02
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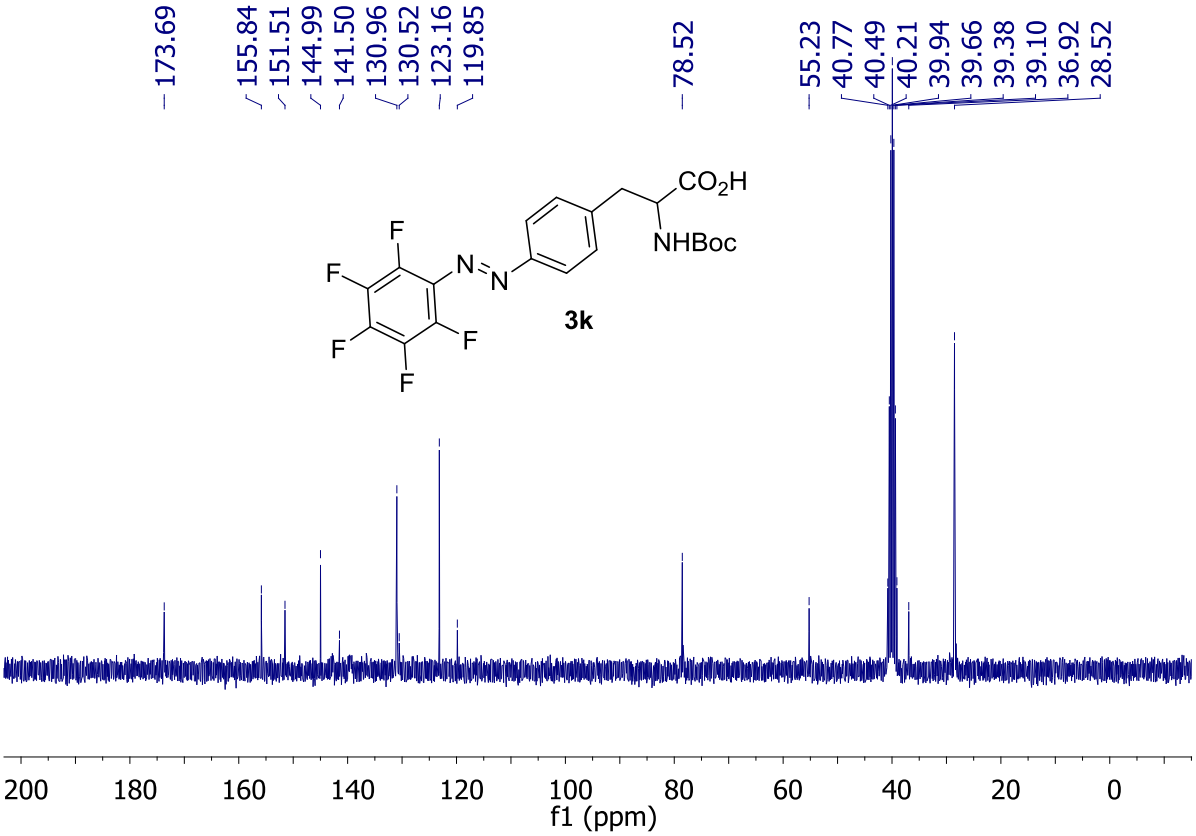
4.17
3.14
3.12
2.99
2.95
1.31
1.29
1.26
1.23
CO2H
NHBoc
F
F
F
F
F
N=N
3k
1.13
2.00
2.06
0.98
1.07
1.12
1.12
8.99
11.0
8.5
6.5
4.5
2.5
0.5
f1 (ppm)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123.16
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78.52
55.23
40.77
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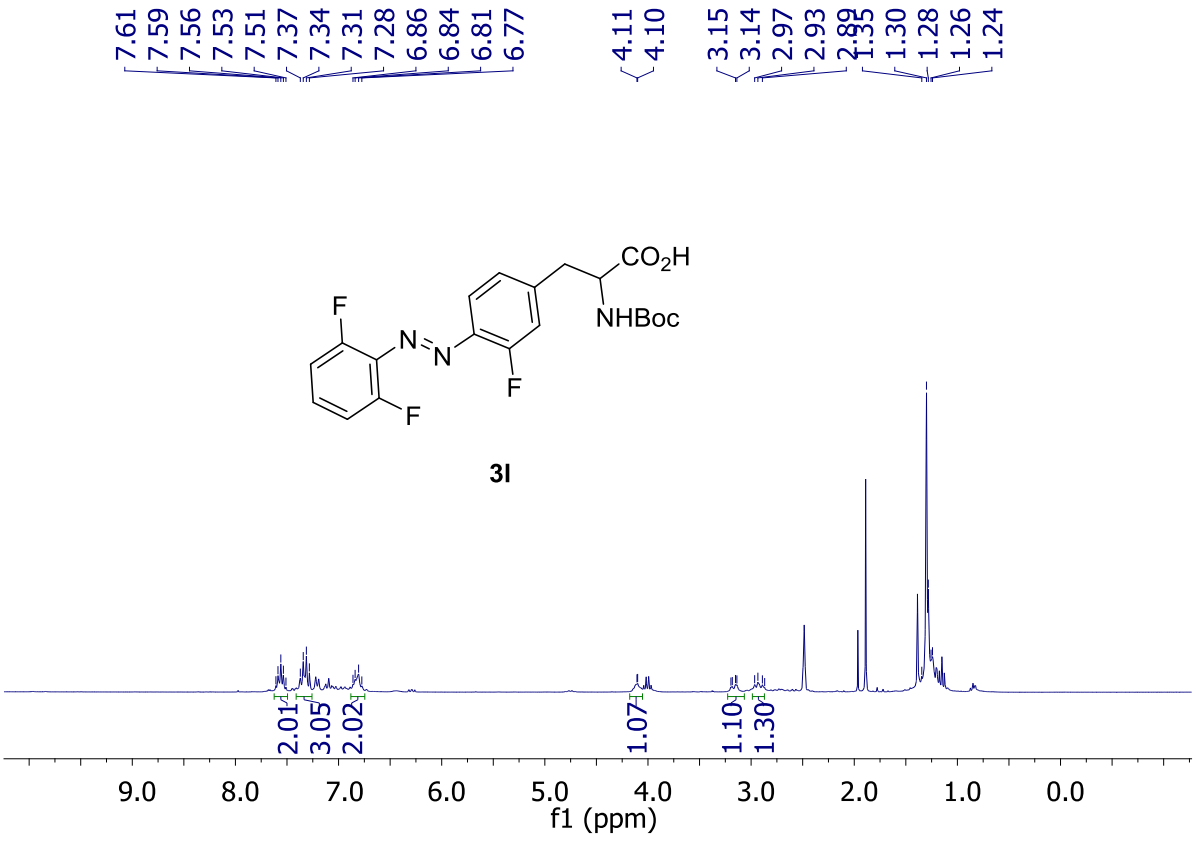
40.21
39.94
39.66
39.38
39.10
36.92
28.52
CO2H
NHBoc
F
F
F
F
F
N
N
3k
200
180
160
140
120
100
80
60
40
20
0
f1 (ppm)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6.84
6.81
6.77
4.11
4.10
3.15
3.14
2.97
2.93
2.89
1.35
1.30
1.28
1.26
1.24
CO2H
NHBoc
F
F
F
N
N
3l
2.01
3.05
2.02
1.07
1.10
1.30
9.0
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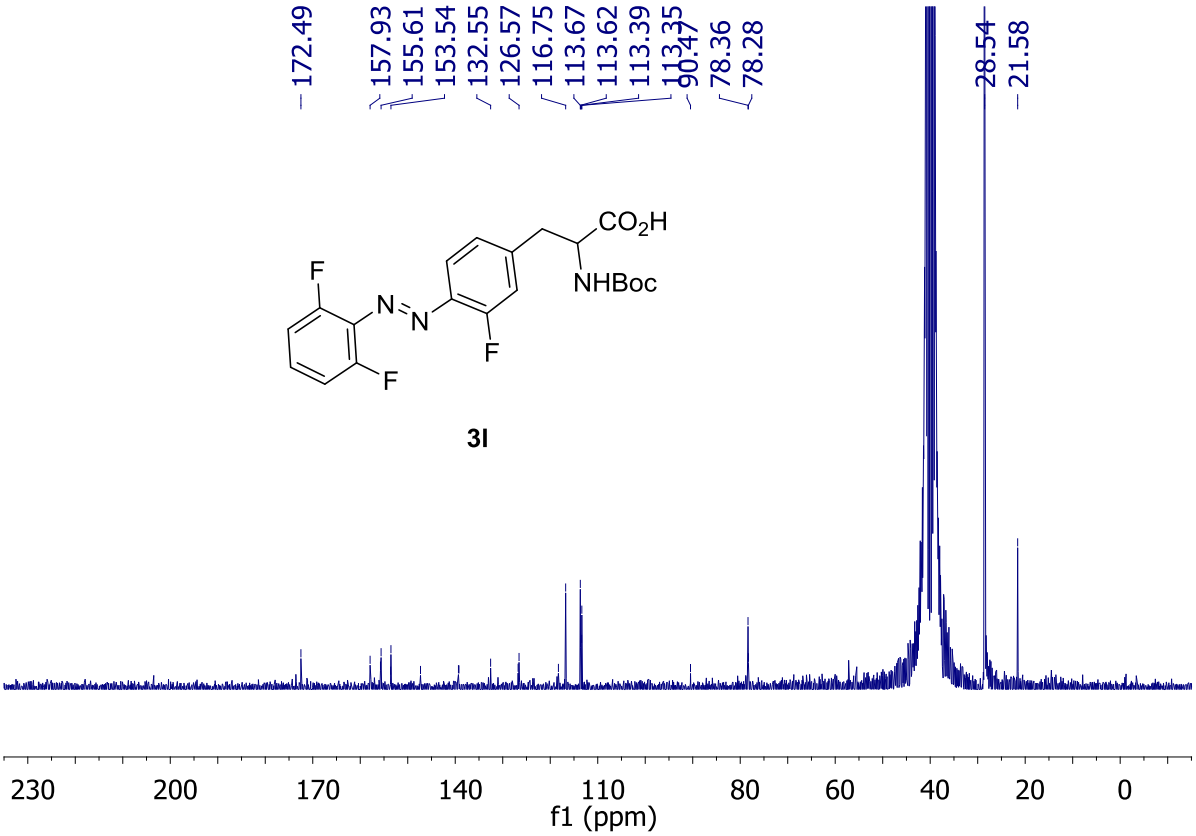
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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113.67
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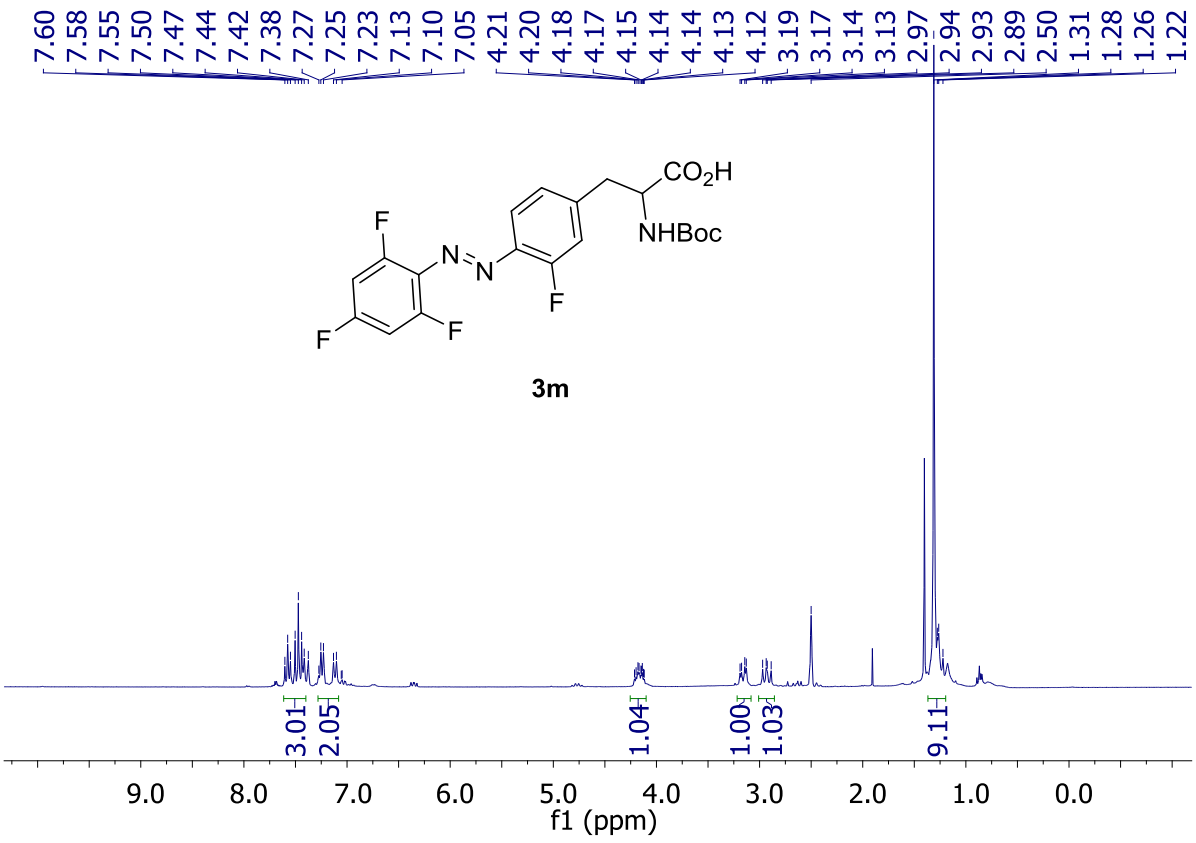
113.39
113.35
90.47
78.36
78.28
28.54
21.58
CO2H
NHBoc
F
N
N
F
F
230
200
170
140
110
80
60
40
20
0
f1 (ppm)

3l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7.13
7.10
7.05
4.21
4.20
4.18
4.17
4.15
4.14
4.14
4.13
4.12
3.19
3.17
3.14
3.13
2.97
2.94
2.93
2.89
2.50
1.31
1.28
1.26
1.22
CO2H
NHBoc
F
N
N
F
F
F
3.01
2.05
1.04
1.00
1.03
9.11
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8.0
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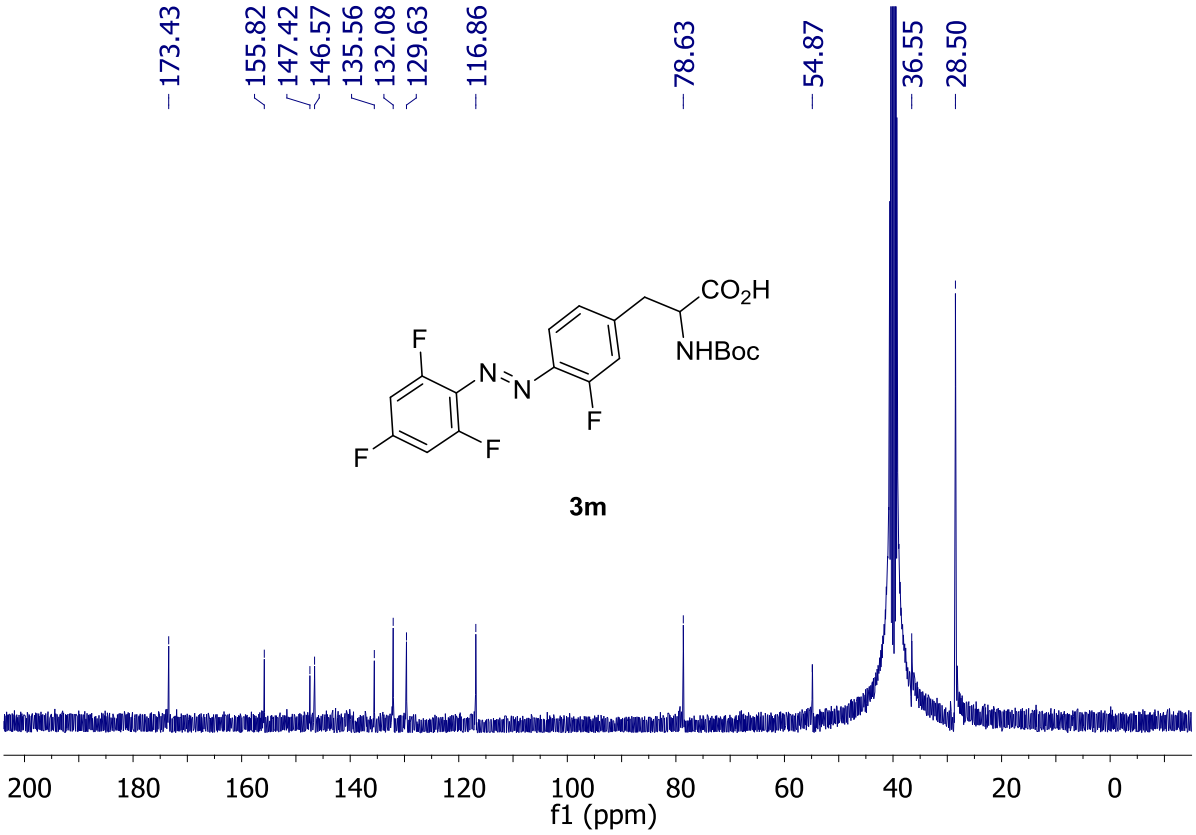
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)

3m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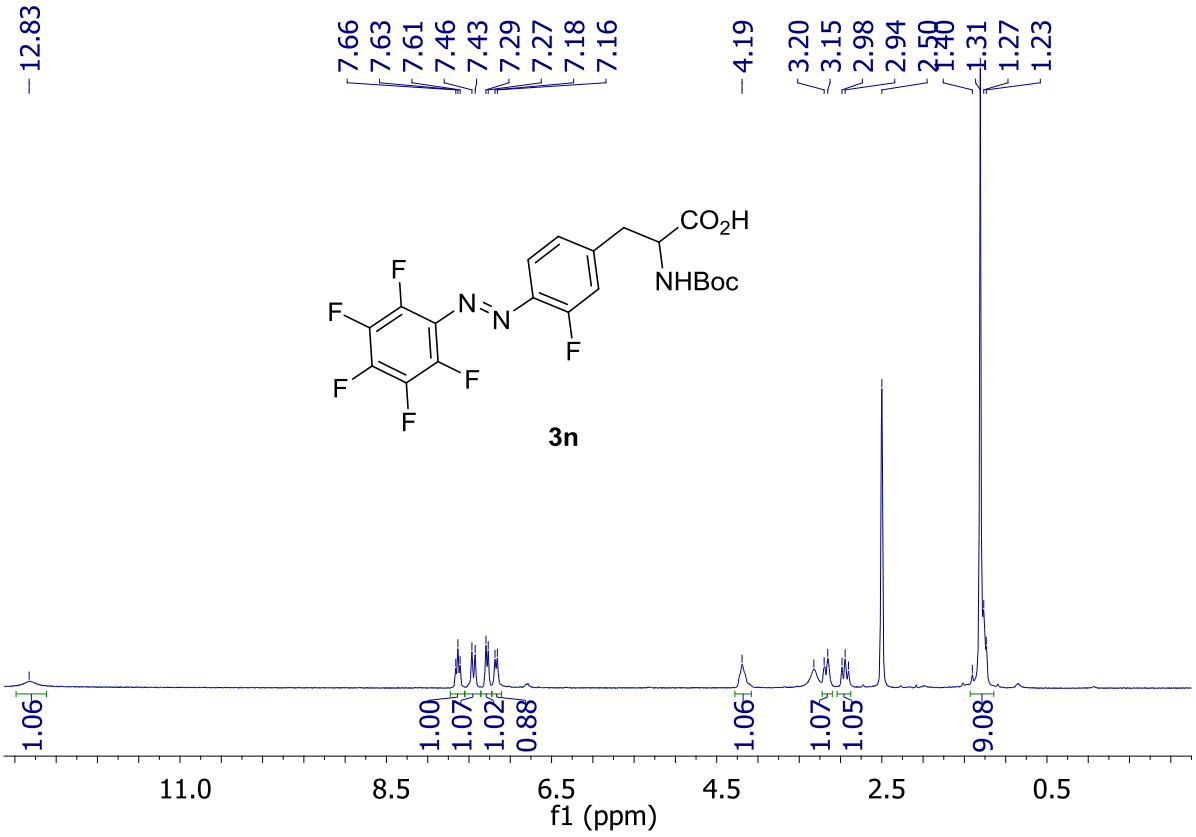
132.08
129.63
116.86
78.63
54.87
36.55
28.50
CO2H
NHBoc
F
N
N
F
F
F
3m
200
180
160
140
120
100
80
60
40
20
0
f1 (ppm)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7.27
7.18
7.16
4.19
3.20
3.15
2.98
2.94
2.50
1.40
1.31
1.27
1.23
CO2H
NHBoc
F
N
N
F
F
F
F
F
3n
1.06
1.00
1.07
1.02
0.88
1.06
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1.05
9.08
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4.5
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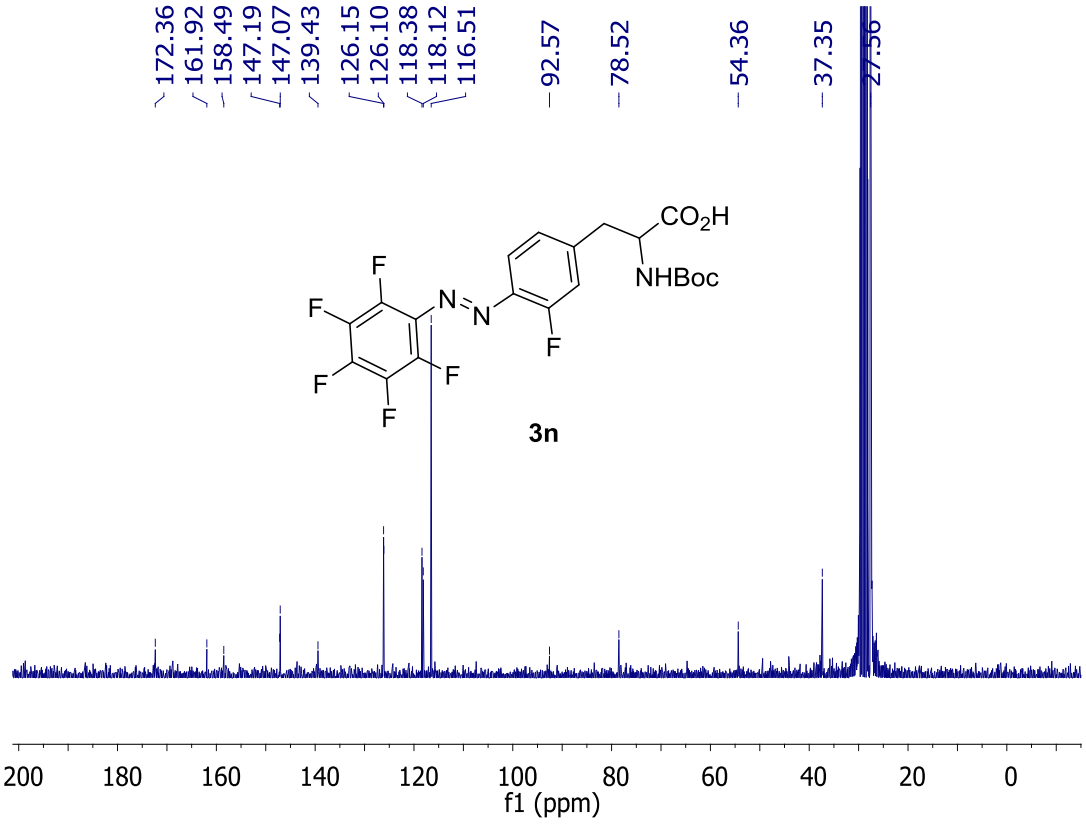
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118.12
116.51
92.57
78.52
54.36
37.35
27.56
CO2H
NHBoc
F
F
F
F
F
N
N
F
3n
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180
160
140
120
100
80
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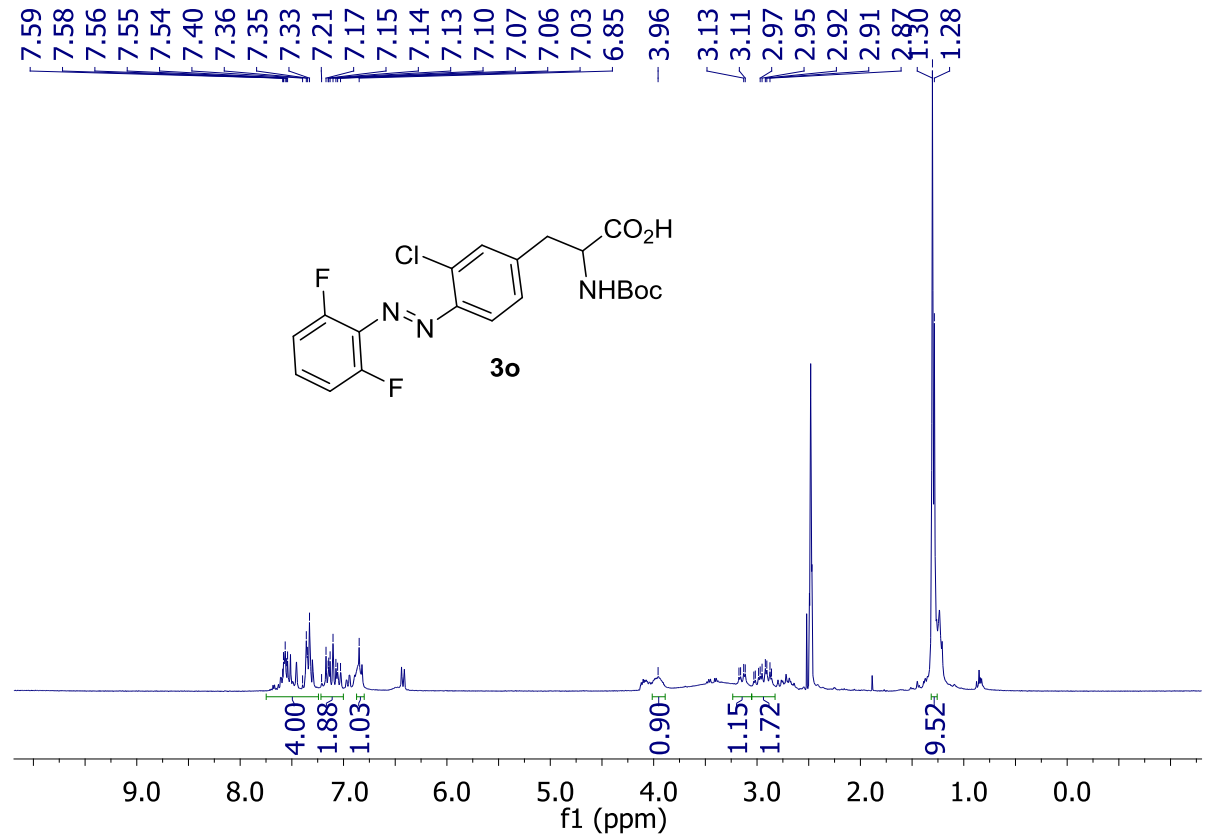
40
20
0
f1 (ppm)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7.14
7.13
7.10
7.07
7.06
7.03
6.85
3.96
3.13
3.11
2.97
2.95
2.92
2.91
1.30
1.28
CO2H
NHBoc
Cl
F
F
N
N
3o
4.00
1.88
1.03
0.90
1.15
1.72
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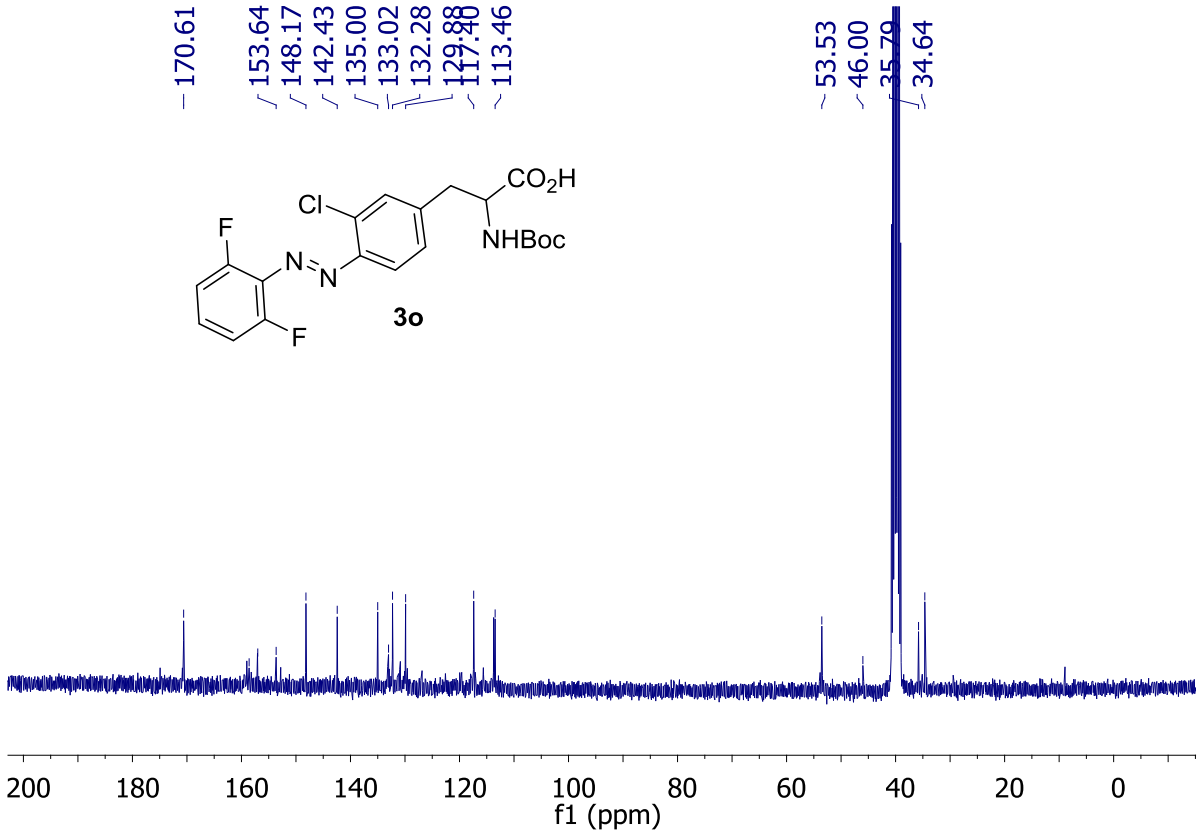
1.0
0.0
f1 (ppm)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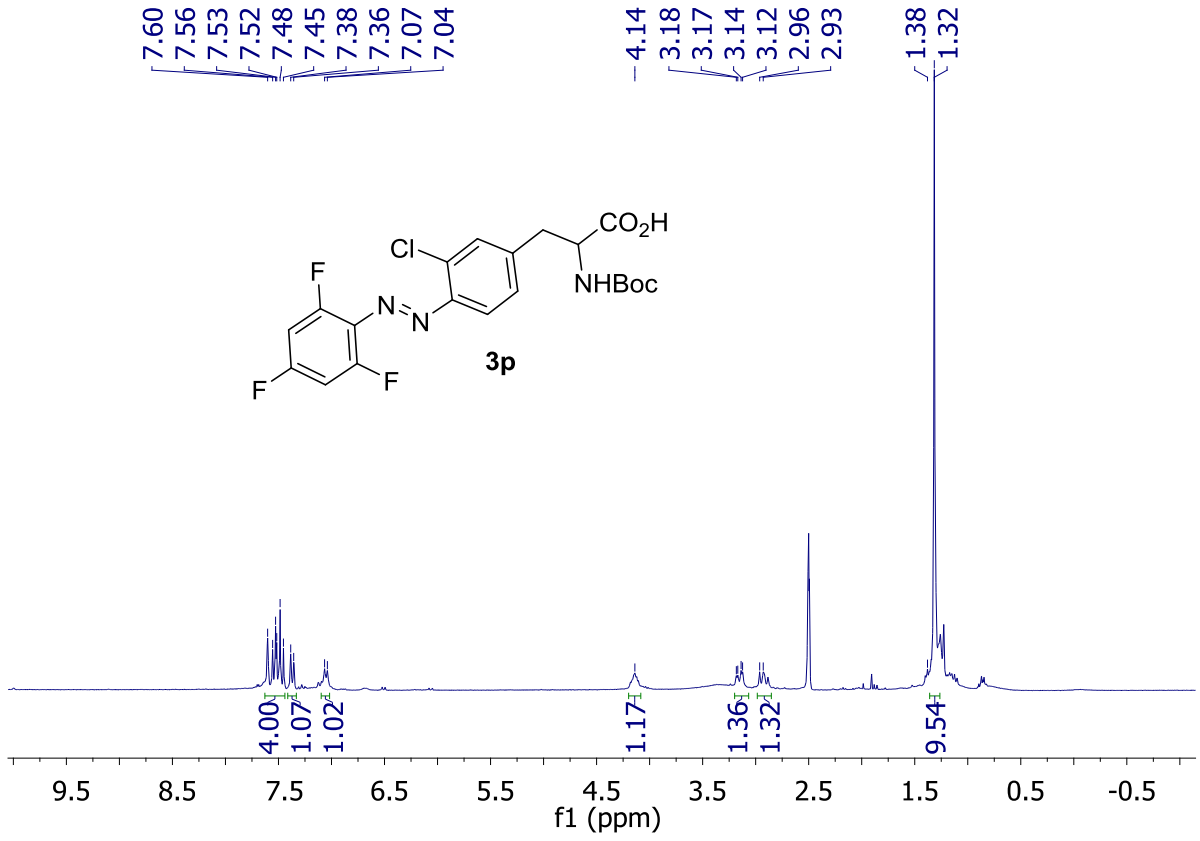
133.02
132.28
129.88
117.40
113.46
53.53
46.00
35.79
34.64
CO2H
Cl
NHBoc
F
N=N
F
3o
200
180
160
140
120
100
80
60
40
20
0
f1 (ppm)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7.45
7.38
7.36
7.07
7.04
4.14
3.18
3.17
3.14
3.12
2.96
2.93
1.38
1.32
CO2H
Cl
NHBoc
F
N=N
F
F
3p
4.00
1.07
1.02
1.17
1.36
1.32
9.54
9.5
8.5
7.5
6.5
5.5
4.5
3.5
2.5
1.5
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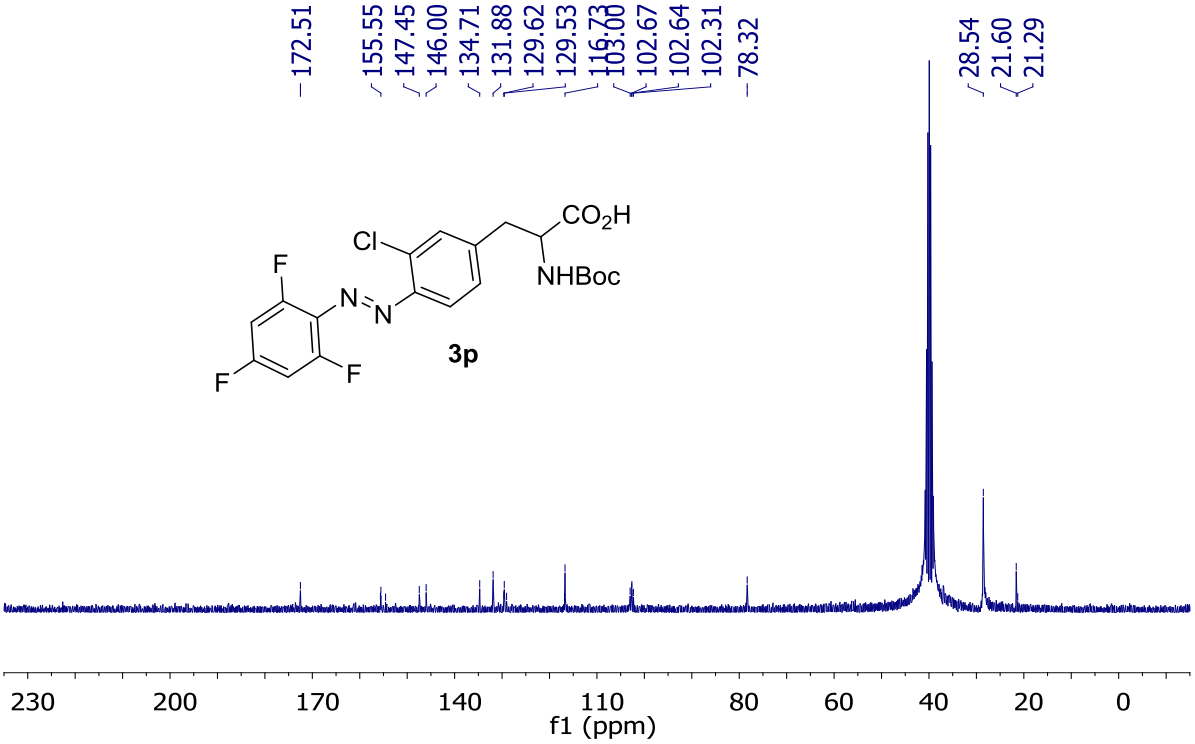
f1 (ppm)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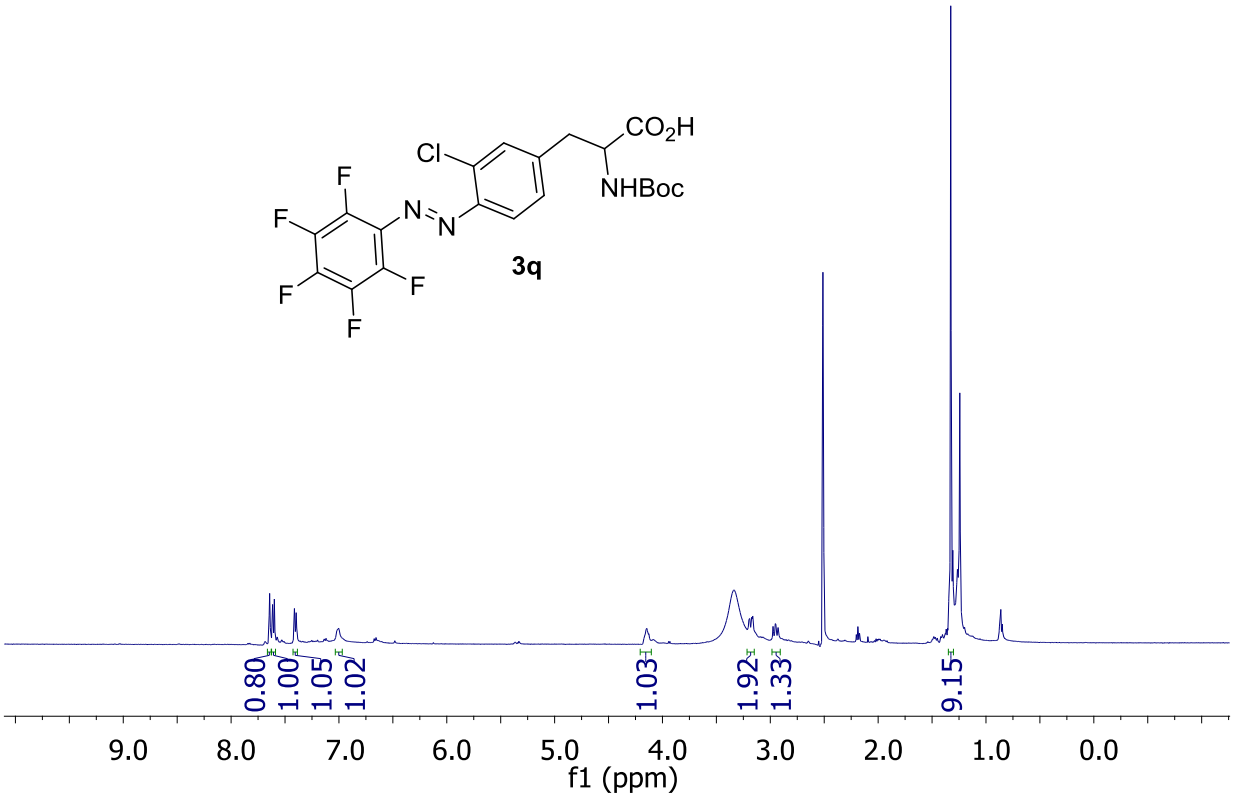
102.67
102.64
102.31
78.32
28.54
21.60
21.29
CO2H
Cl
NHBoc
F
N=N
F
F
3p
230
200
170
140
110
80
60
40
20
0
f1 (ppm)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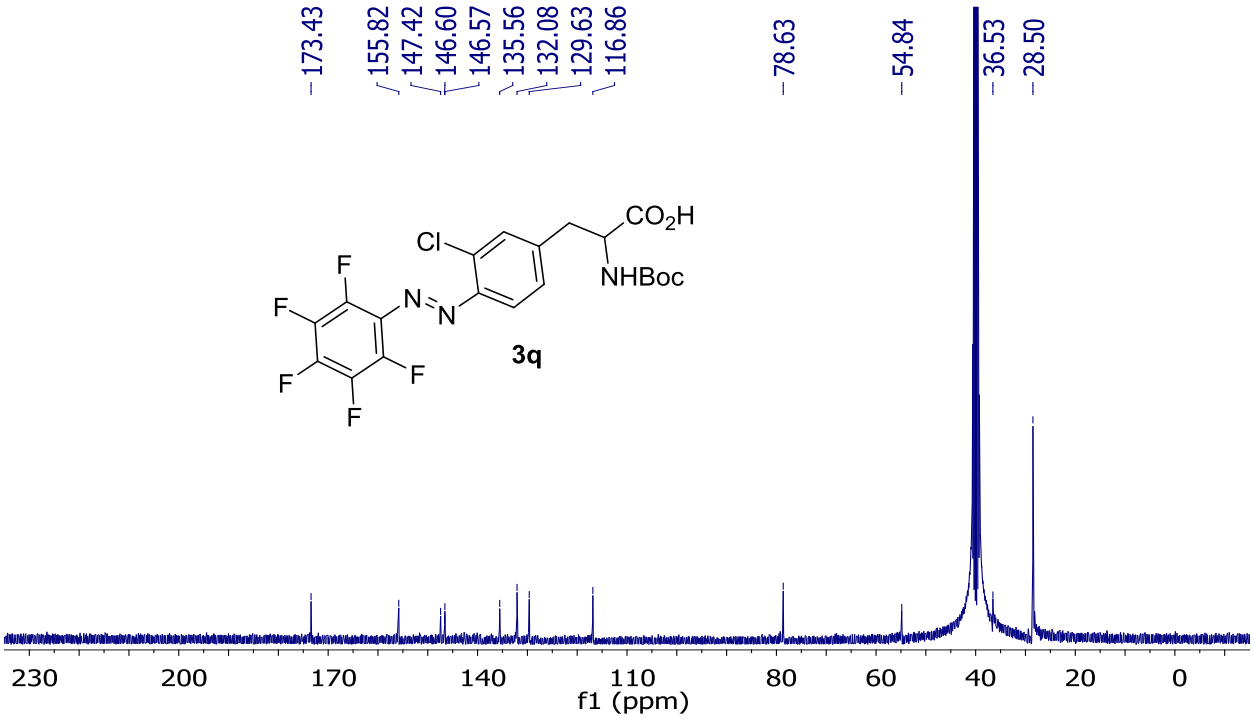
Cl
NHBoc
F
F
N=N
F
F
F
3q
0.80
1.00
1.05
1.02
1.03
1.92
1.33
9.15
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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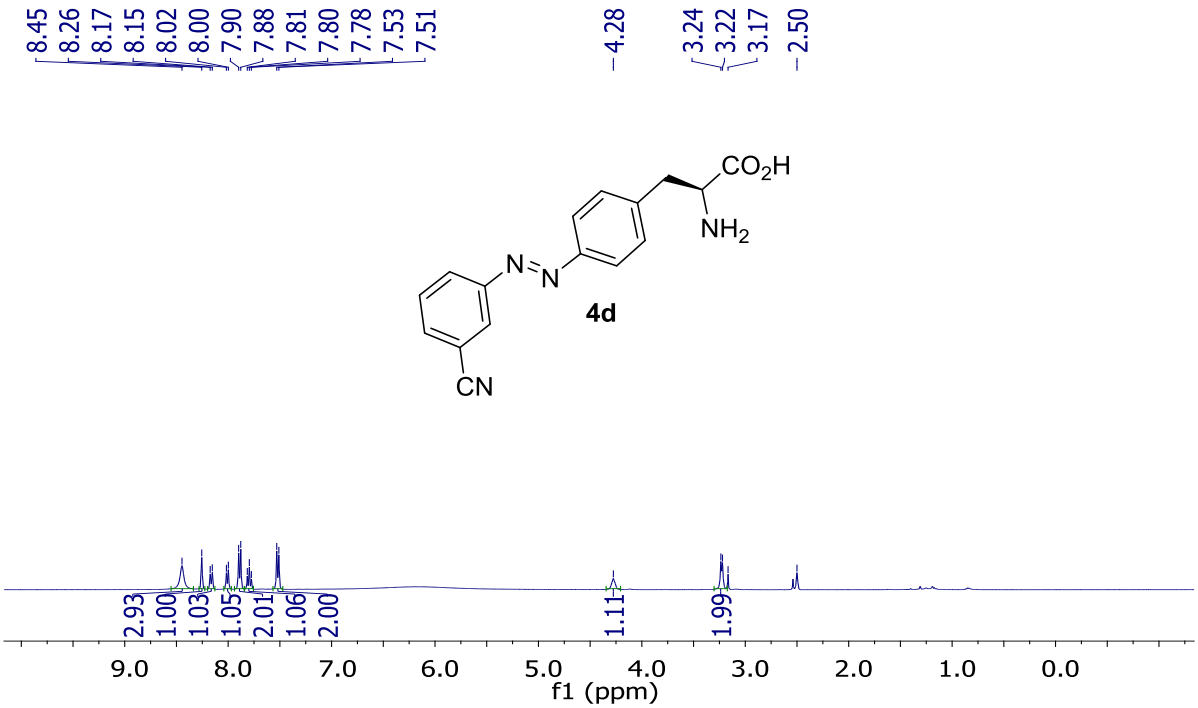
78.63
54.84
36.53
28.50
CO2H
Cl
NHBoc
F
F
N=N
F
F
F
3q
230
200
170
140
110
80
60
40
20
0
f1 (ppm)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8.26
8.17
8.15
8.02
8.00
7.90
7.88
7.81
7.80
7.78
7.53
7.51
4.28
3.24
3.22
3.17
2.50
CO2H
NH2
N=N
4d
CN
2.93
1.00
1.03
1.05
2.01
1.06
2.00
1.11
1.99
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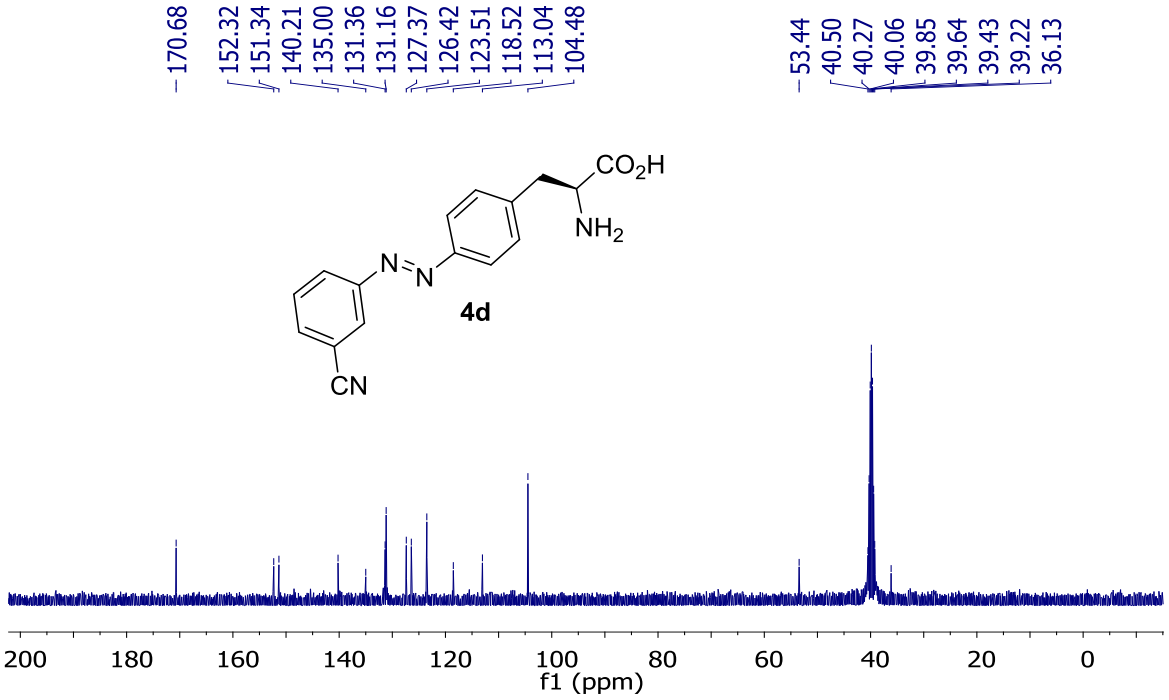
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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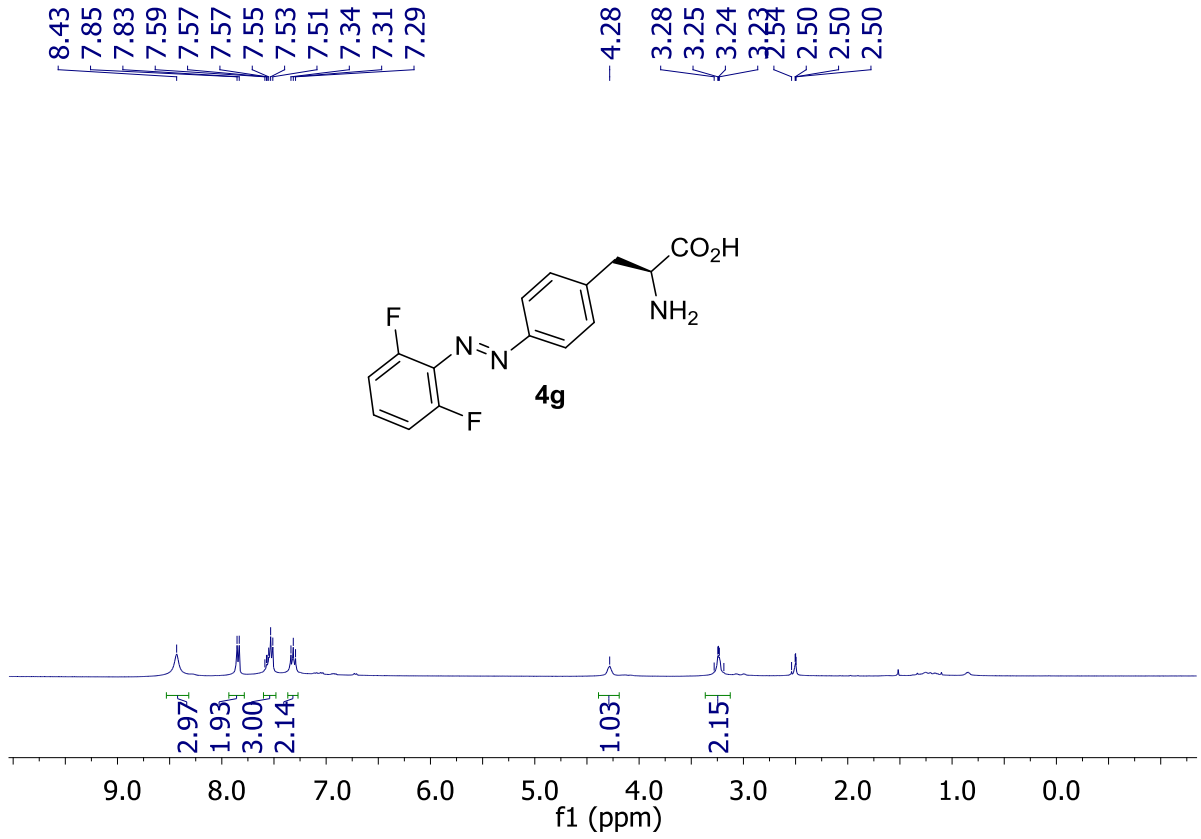
131.36
131.16
127.37
126.42
123.51
118.52
113.04
104.48
53.44
40.50
40.27
40.06
39.85
39.64
39.43
39.22
36.13
CO2H
NH2
N=N
CN
4d
200
180
160
140
120
100
80
60
40
20
0
f1 (ppm)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7.85
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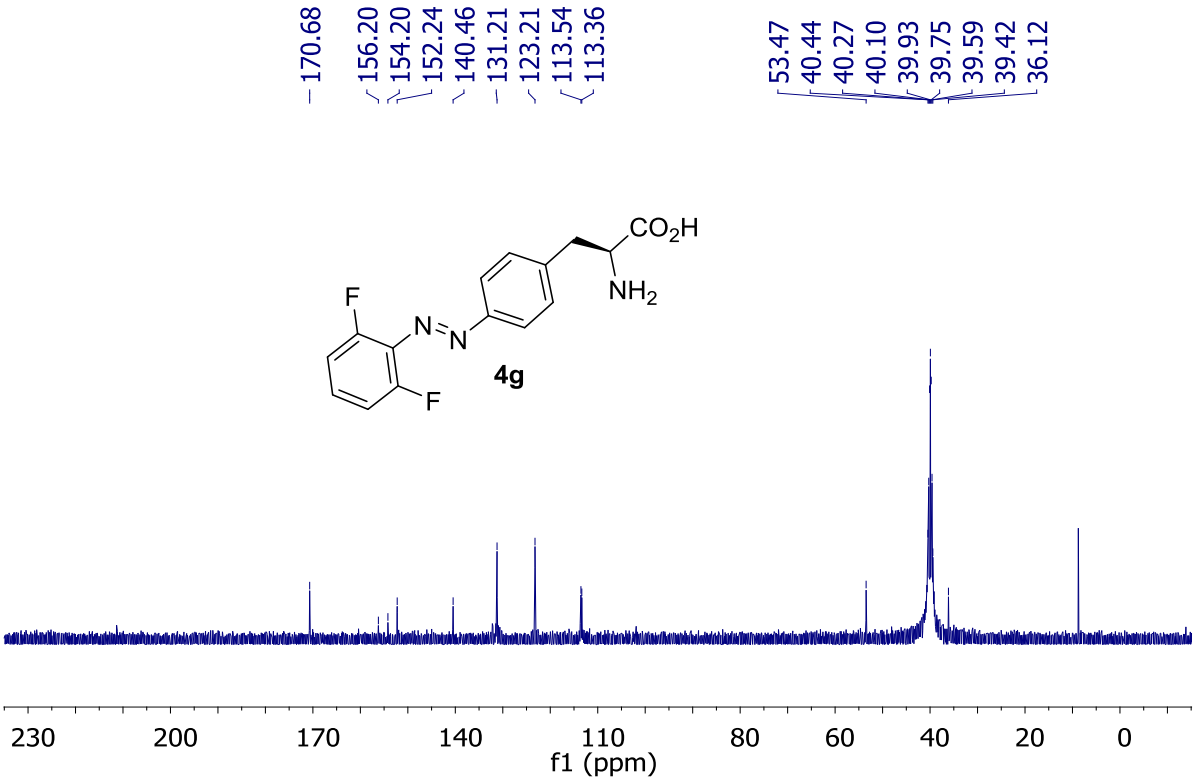
7.57
7.55
7.53
7.51
7.34
7.31
7.29
4.28
3.28
3.25
3.24
3.23
2.54
2.50
2.50
2.50
CO2H
NH2
F
N=N
F
4g
2.97
1.93
3.00
2.14
1.03
2.15
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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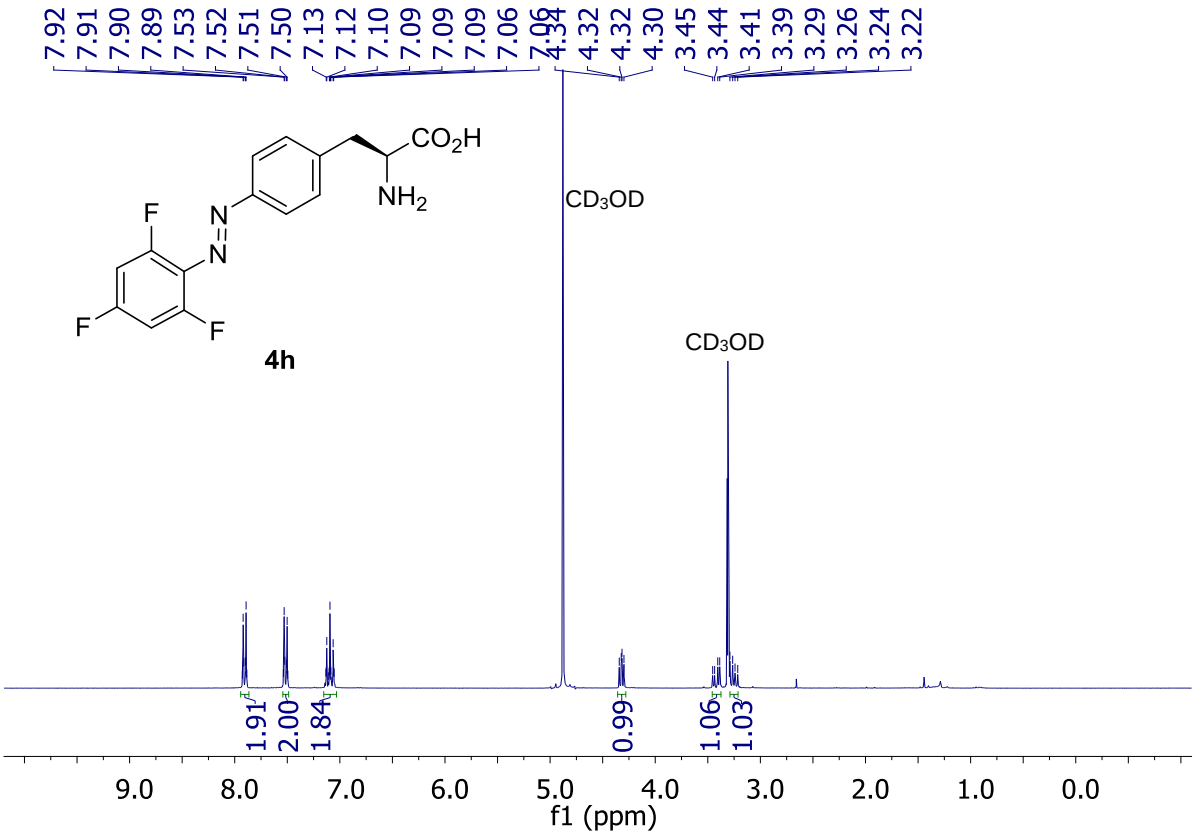
40.10
39.93
39.75
39.59
39.42
36.12
CO2H
NH2
F
N
N
F
4g
230
200
170
140
110
80
60
40
20
0
f1 (ppm)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7.90
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7.51
7.50
7.13
7.12
7.10
7.09
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7.09
7.06
7.06
4.34
4.32
4.32
4.30
3.45
3.44
3.41
3.39
3.29
3.26
3.24
3.22
CO2H
NH2
F
N
N
F
F
4h
CD3OD
CD3OD
1.91
2.00
1.84
0.99
1.06
1.03
9.0
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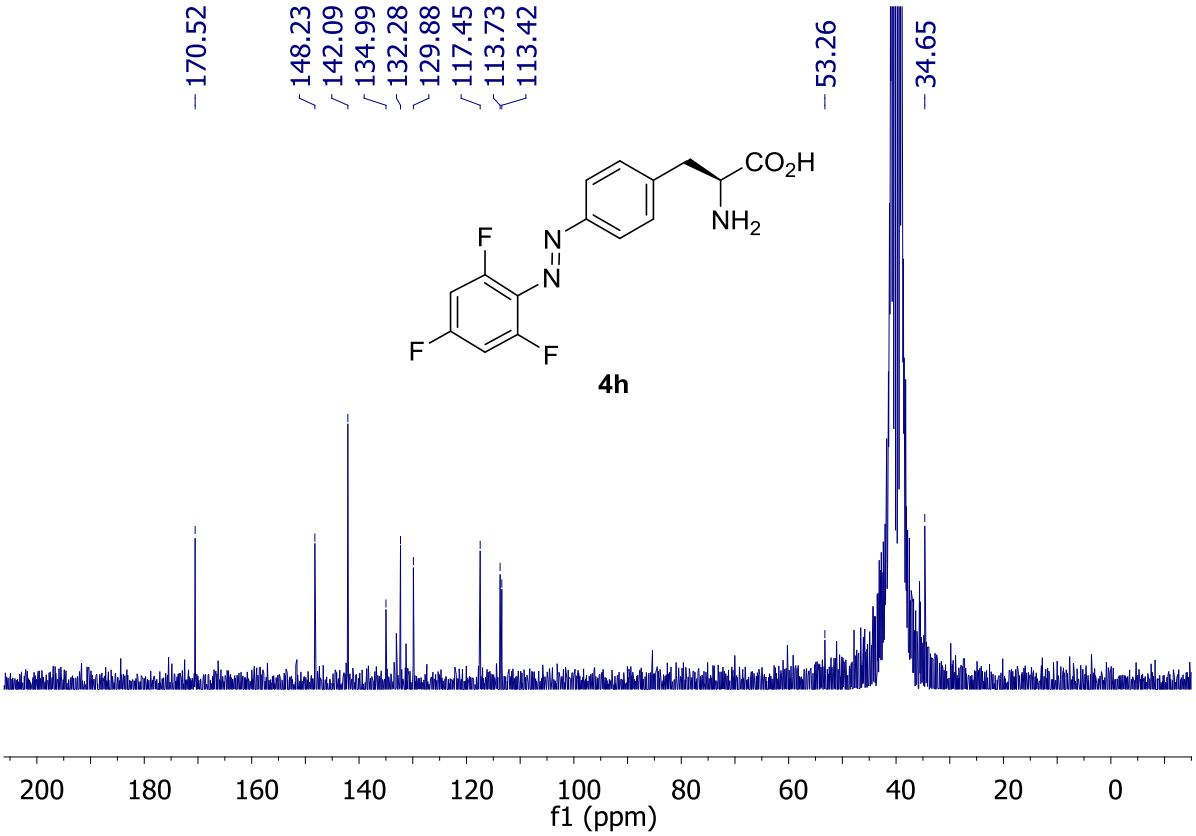
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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148.23
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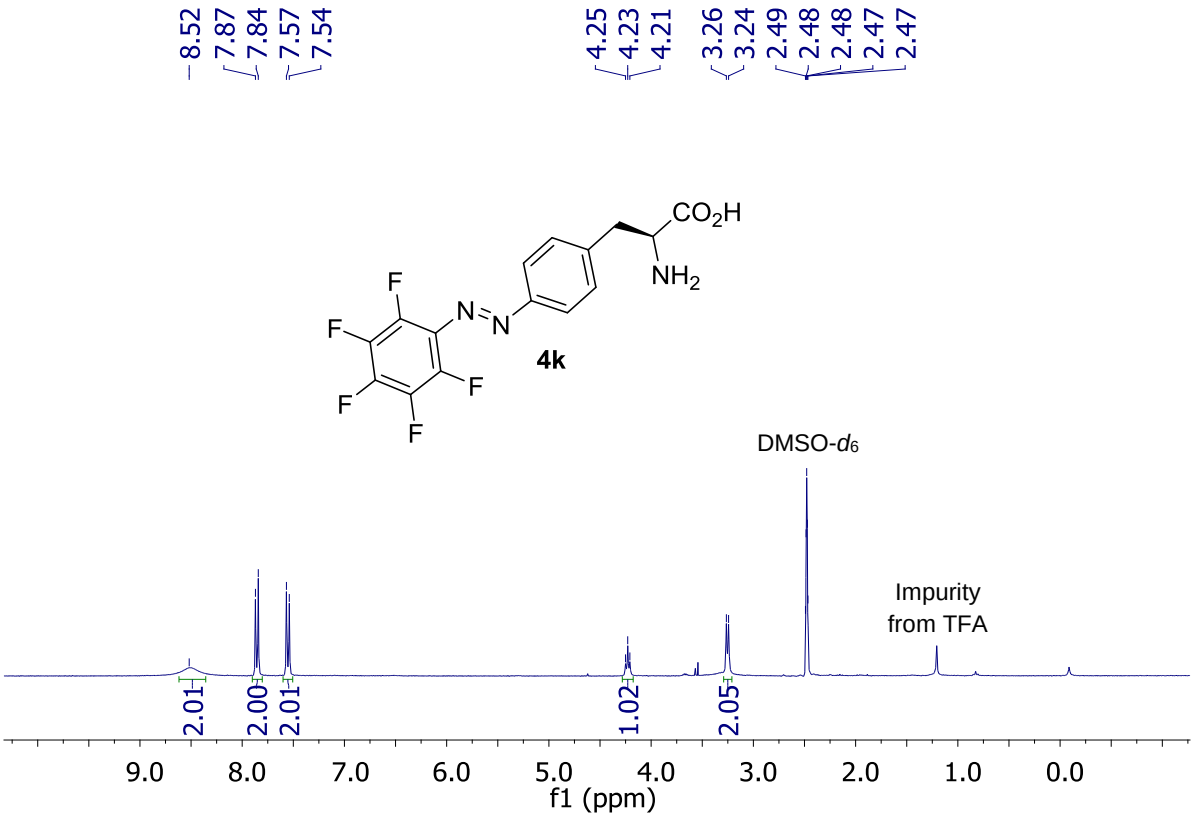
134.99
132.28
129.88
117.45
113.73
113.42
53.26
34.65
CO2H
NH2
F
N
N
F
F
4h
200
180
160
140
120
100
80
60
40
20
0
f1 (ppm)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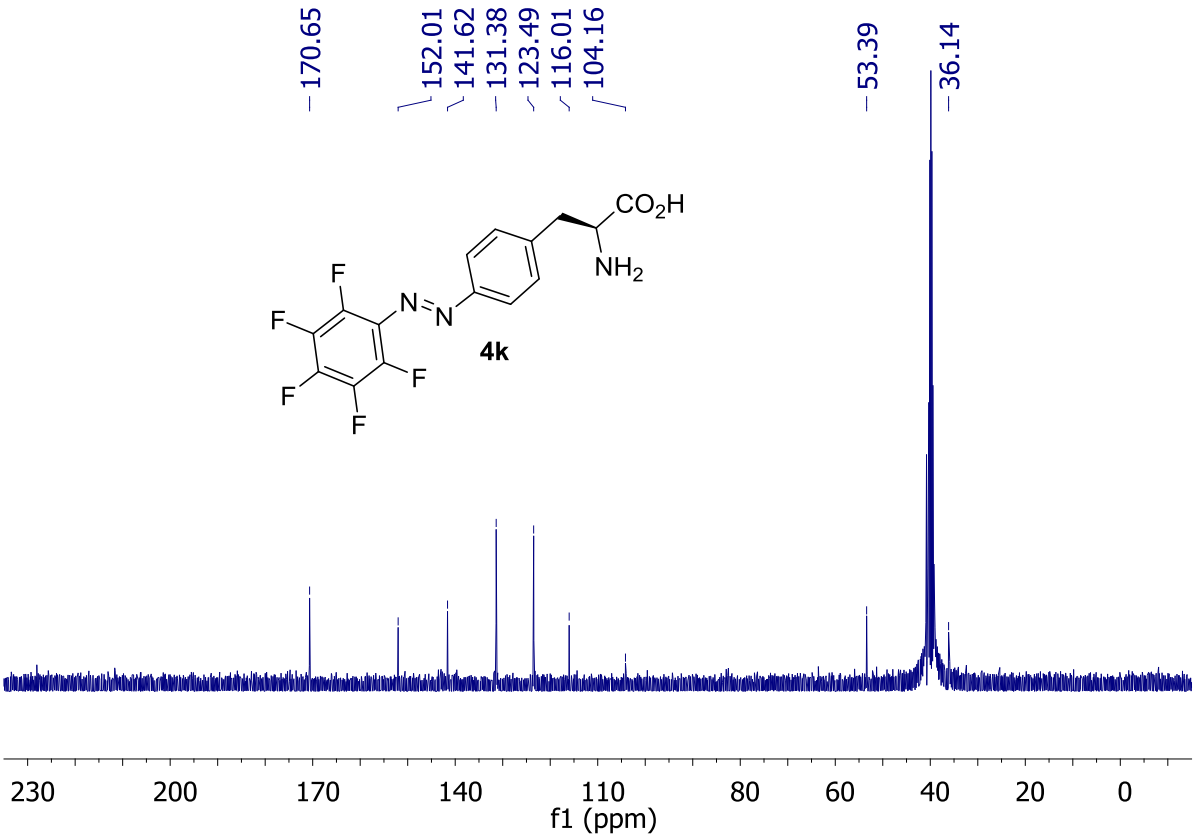
7.54
4.25
4.23
4.21
3.26
3.24
2.49
2.48
2.48
2.47
2.47
CO2H
NH2
F
N
N
F
F
F
F
4k
DMSO-d6
Impurity
from TFA
2.01
2.00
2.01
1.02
2.05
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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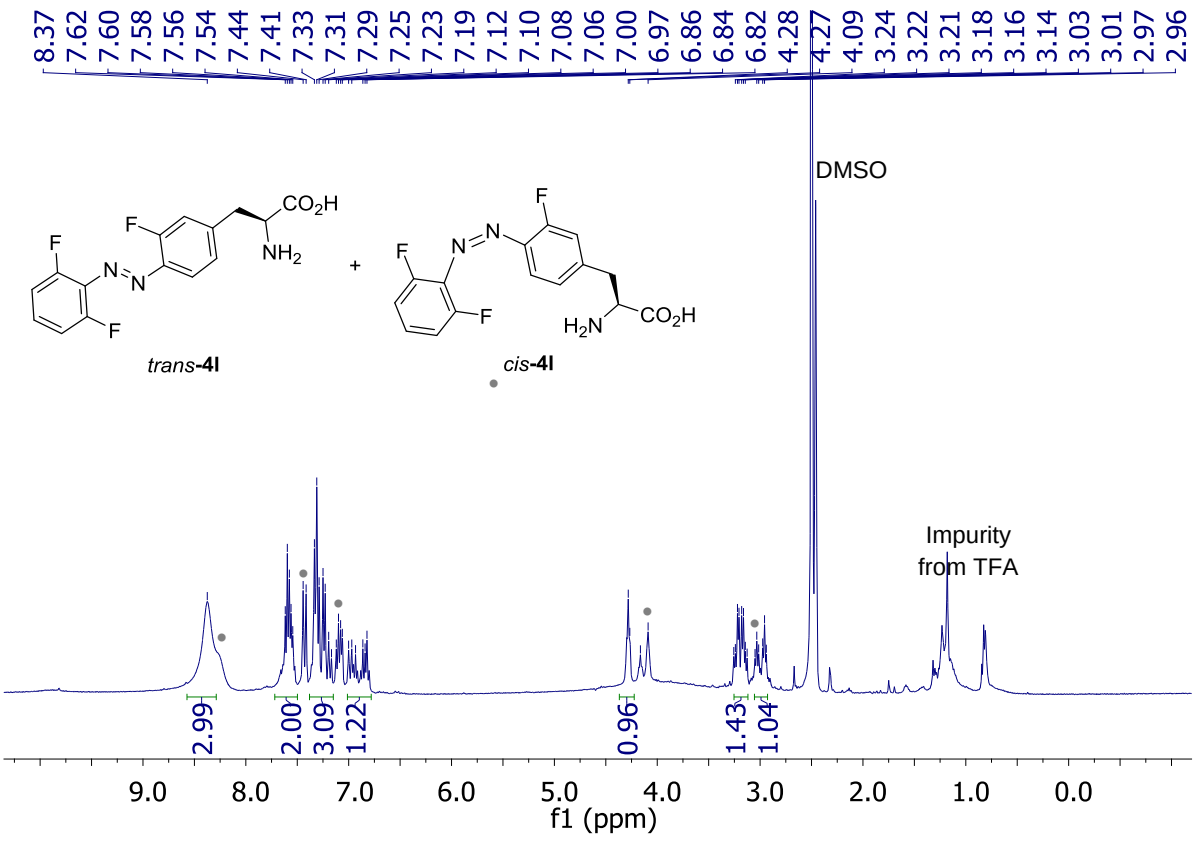
116.01
104.16
53.39
36.14
CO2H
NH2
F
N=N
4k
230
200
170
140
110
80
60
40
20
0
f1 (ppm)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7.29
7.25
7.23
7.19
7.12
7.10
7.08
7.06
7.00
6.97
6.86
6.84
6.82
4.28
4.27
4.09
3.24
3.22
3.21
3.18
3.16
3.14
3.03
3.01
2.97
2.96
DMSO
CO2H
NH2
trans-4l
+
cis-4l
H2N
Impurity
from TFA
2.99
2.00
3.09
1.22
0.96
1.43
1.04
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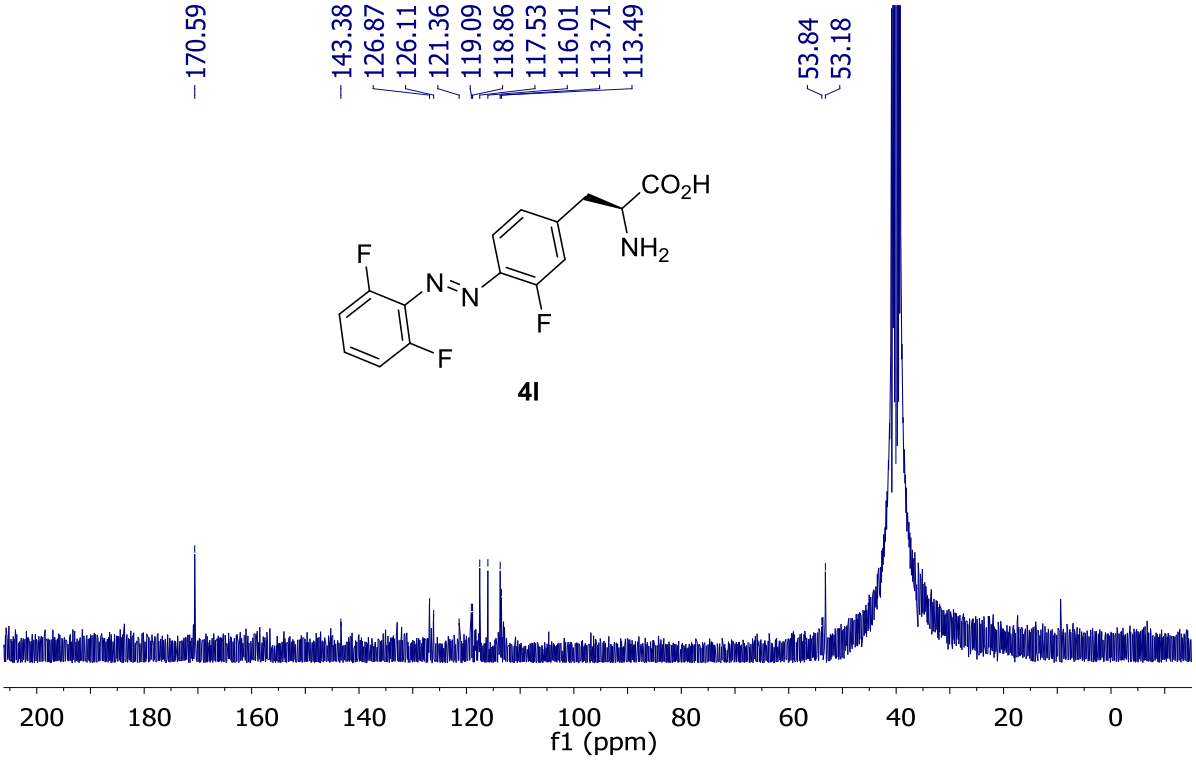
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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119.09
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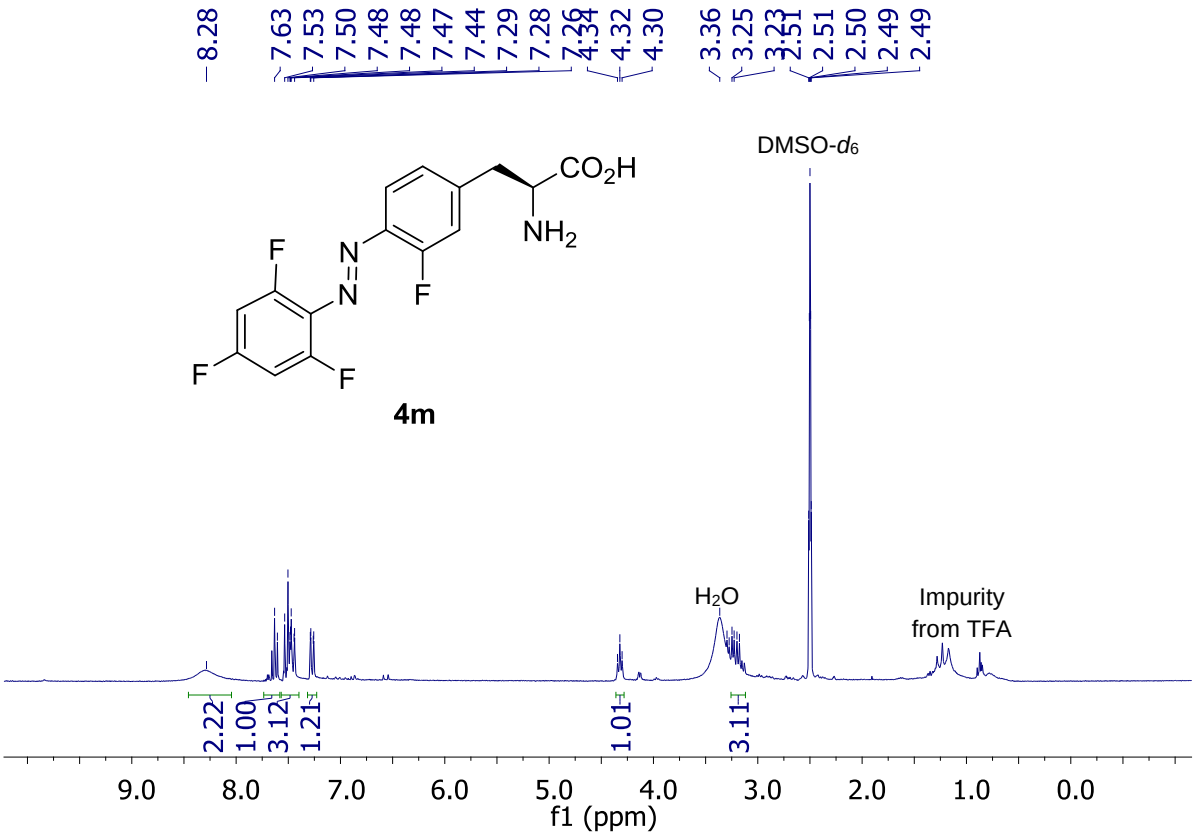
117.53
116.01
113.71
113.49
53.84
53.18
CO2H
NH2
F
N=N
F
F
4l
200
180
160
140
120
100
80
60
40
20
0
f1 (ppm)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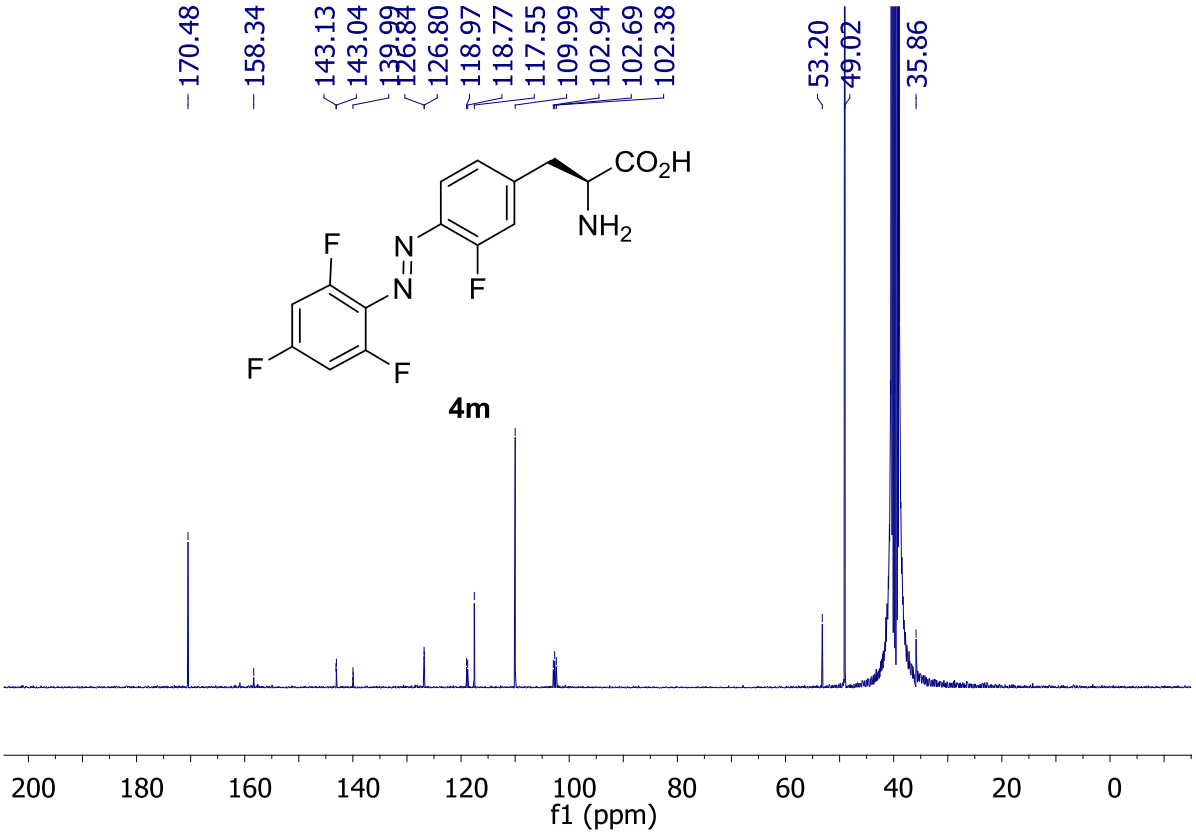
7.48
7.47
7.44
7.29
7.28
4.34
4.32
4.30
3.36
3.25
3.23
2.51
2.51
2.50
2.49
2.49
DMSO-d6
CO2H
NH2
F
N
N
F
F
F
4m
H2O
Impurity
from TFA
2.22
1.00
3.12
1.21
1.01
3.11
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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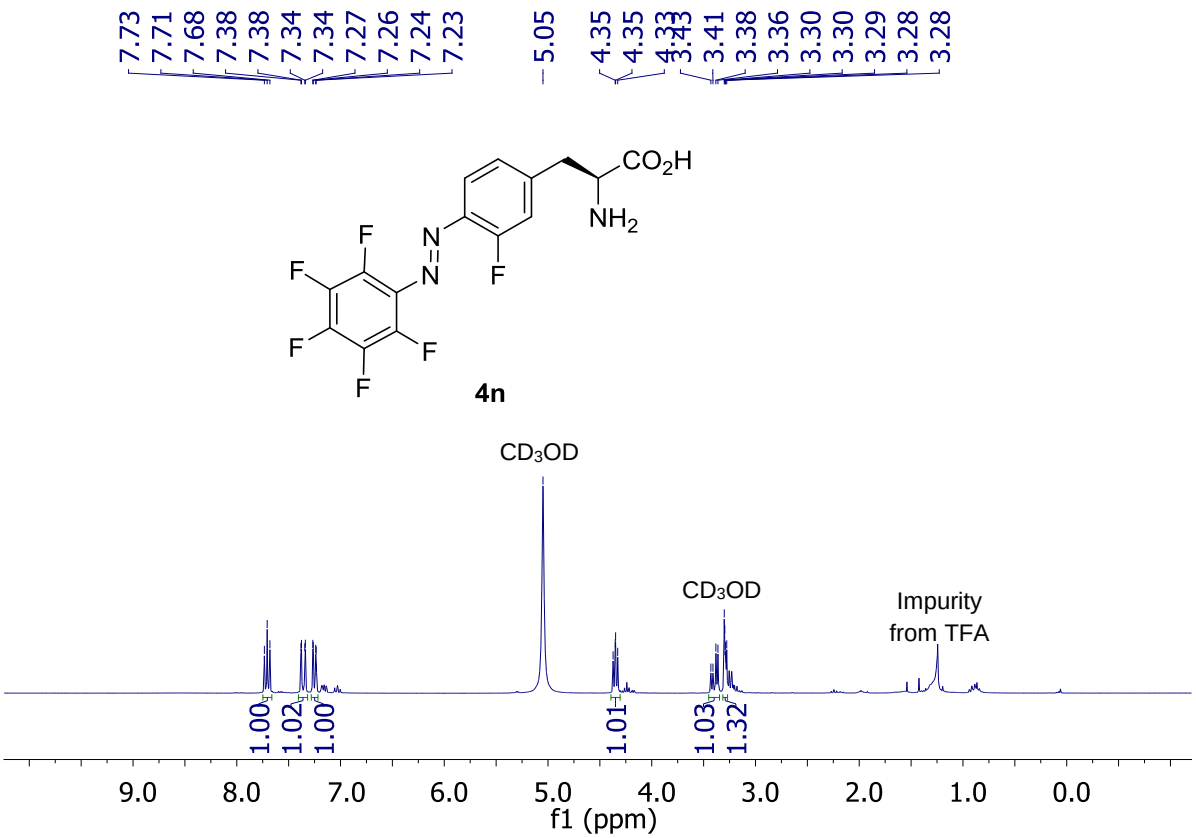
126.84
126.80
118.97
118.77
117.55
109.99
102.94
102.69
102.38
53.20
49.02
35.86
CO2H
NH2
F
N
N
F
F
F
4m
200
180
160
140
120
100
80
60
40
20
0
f1 (ppm)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7.27
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7.24
7.23
5.05
4.35
4.35
4.33
3.43
3.41
3.38
3.36
3.30
3.30
3.29
3.28
3.28
CO2H
NH2
F
N
N
F
F
F
F
F
4n
CD3OD
CD3OD
Impurity
from TFA
1.00
1.02
1.00
1.01
1.03
1.32
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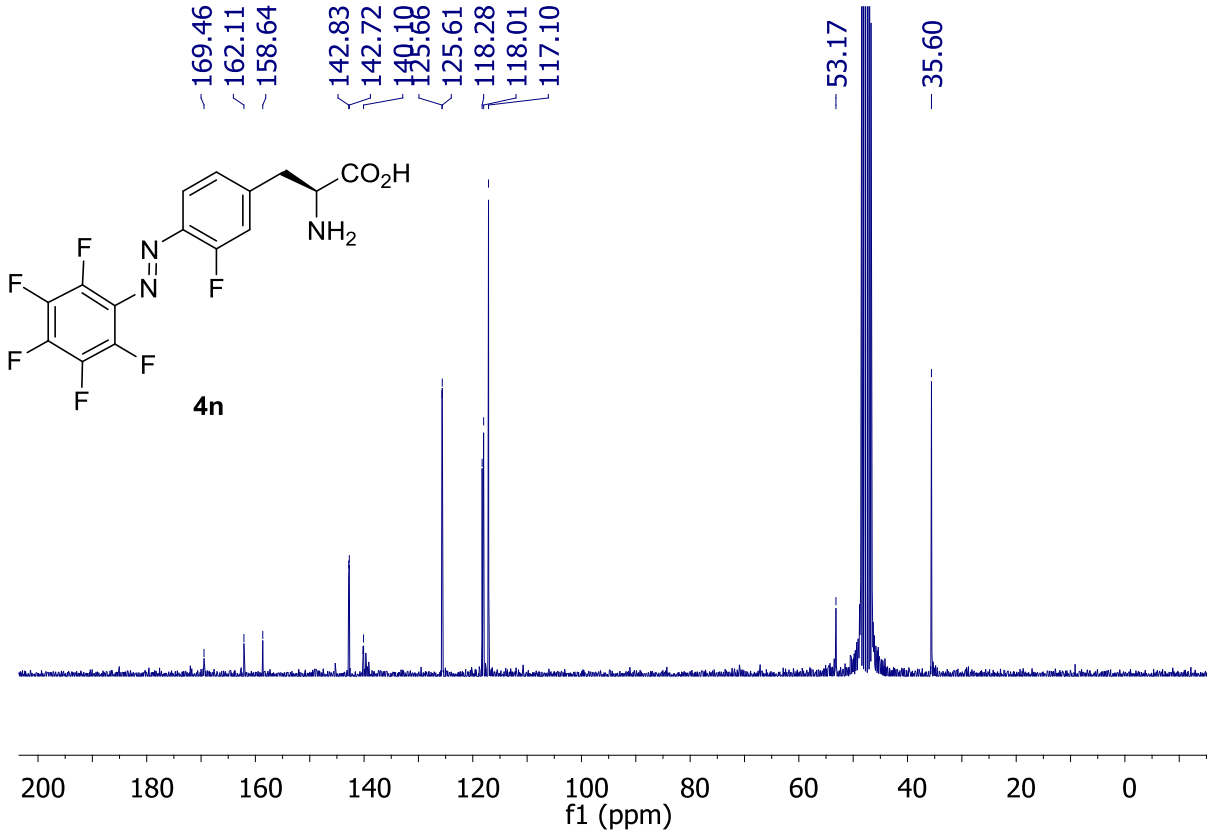
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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142.72
140.10
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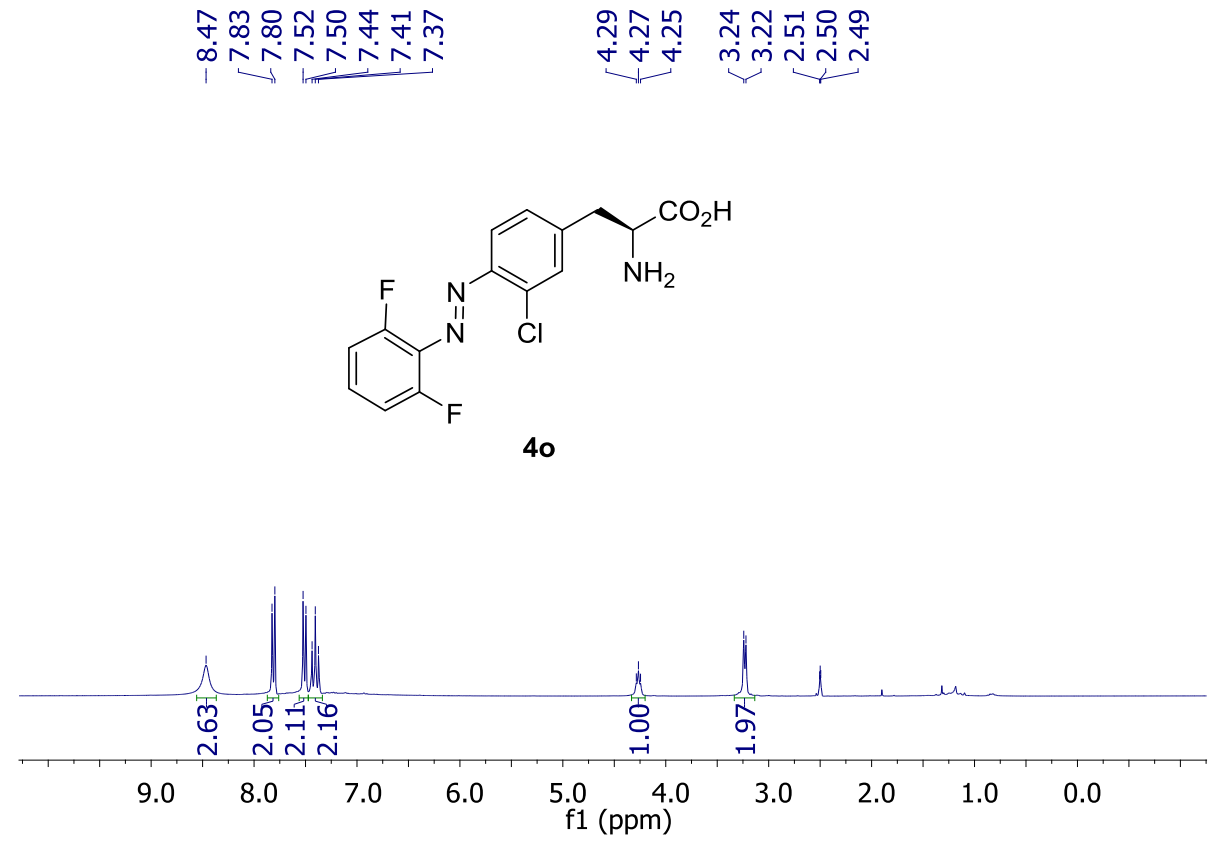
125.61
118.28
118.01
117.10
53.17
35.60
CO2H
NH2
F
N
N
F
F
F
F
F
4n
200
180
160
140
120
100
80
60
40
20
0
f1 (ppm)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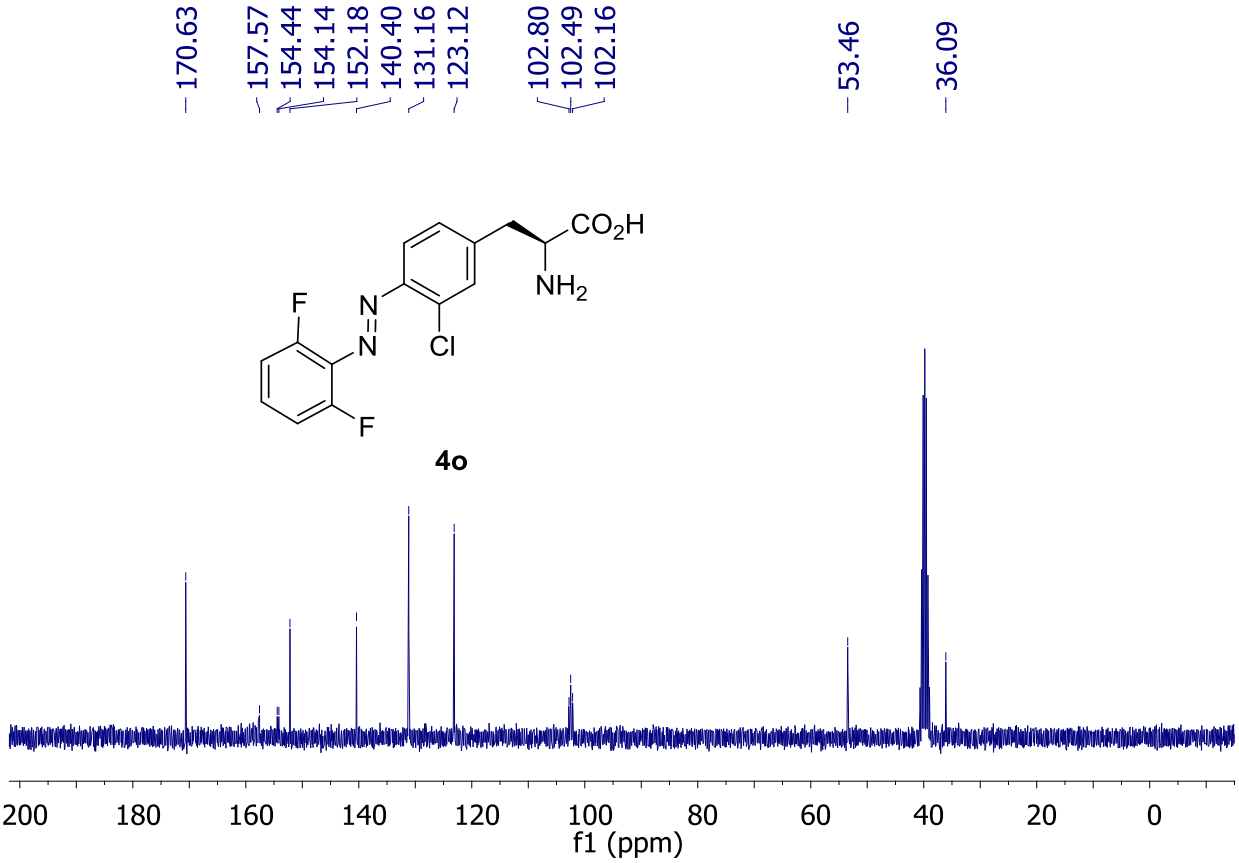
7.44
7.41
7.37
4.29
4.27
4.25
3.24
3.22
2.51
2.50
2.49
CO2H
NH2
F
N
N
Cl
F
4o
2.63
2.05
2.11
2.16
1.00
1.97
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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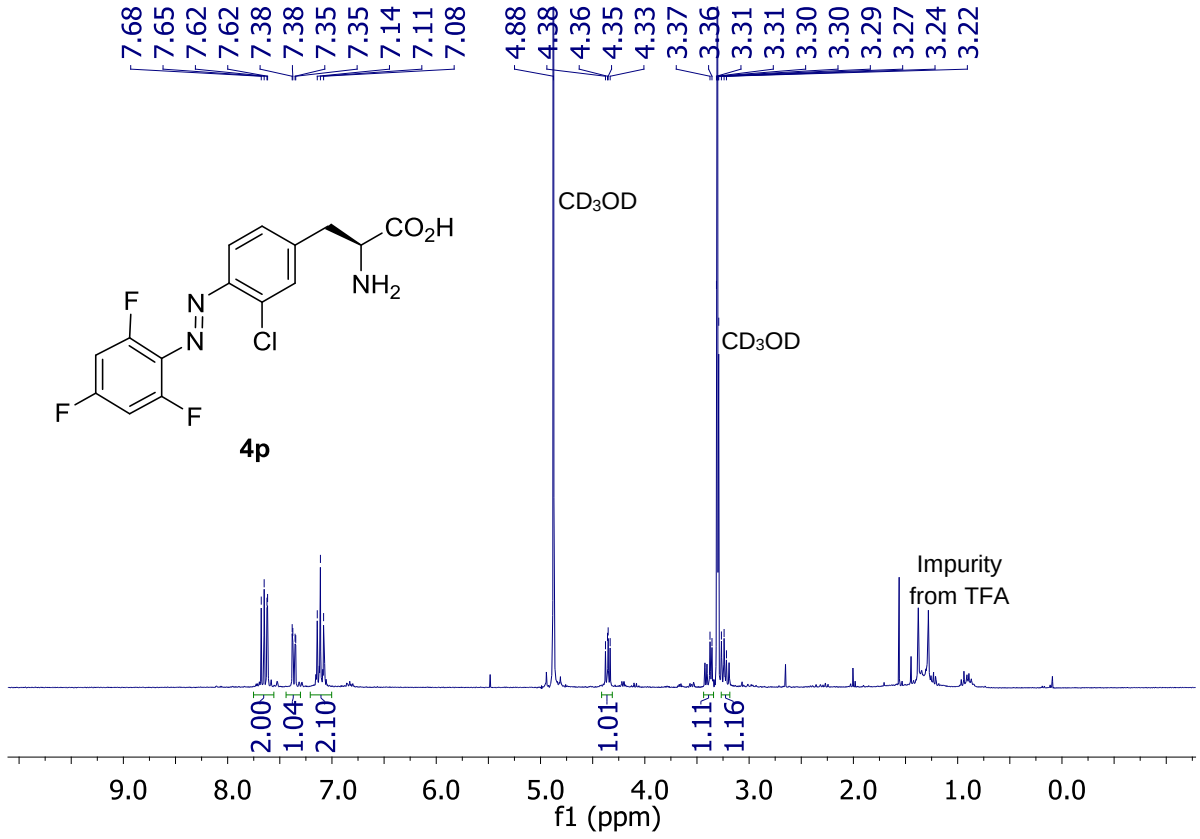
154.44
154.14
152.18
140.40
131.16
123.12
102.80
102.49
102.16
53.46
36.09
CO2H
NH2
F
N
N
Cl
F
4o
200
180
160
140
120
100
80
60
40
20
0
f1 (ppm)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7.38
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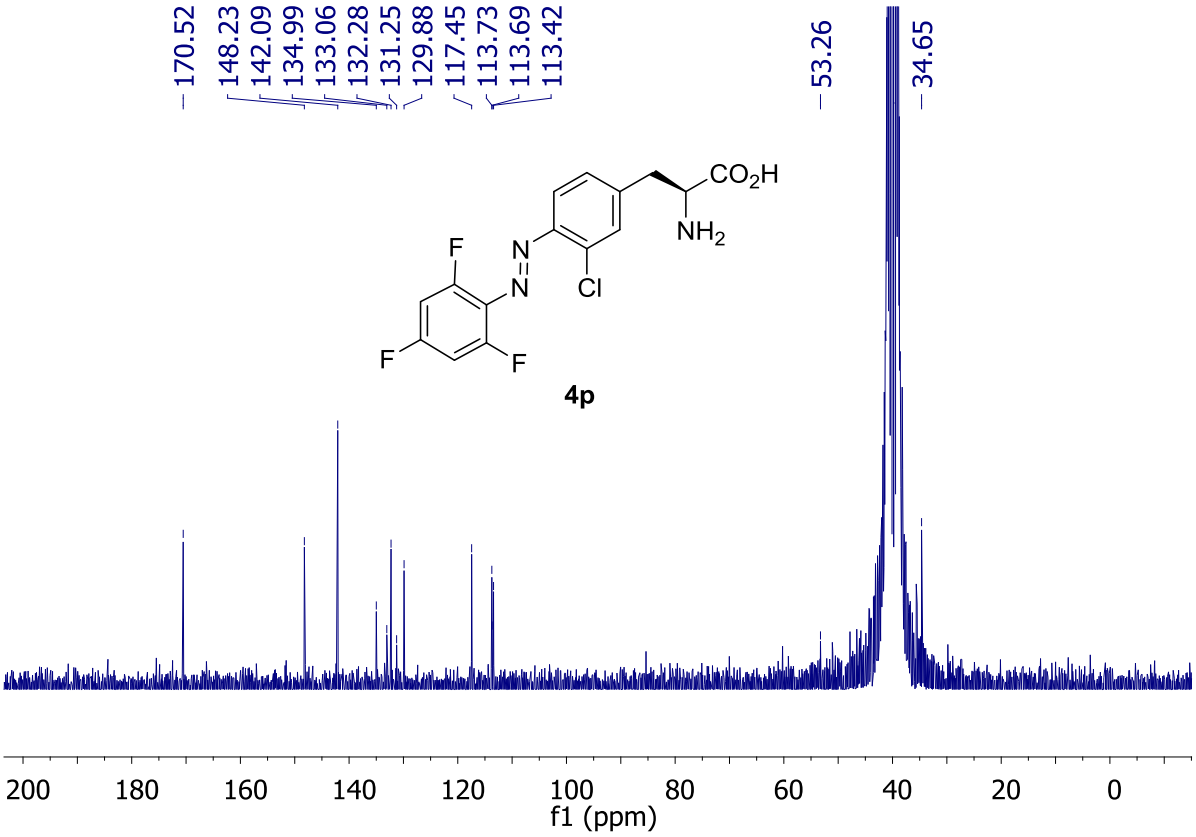
7.35
7.14
7.11
7.08
4.88
4.38
4.36
4.35
4.33
3.37
3.36
3.31
3.31
3.30
3.30
3.29
3.27
3.24
3.22
CD3OD
CD3OD
CO2H
NH2
F
N
N
Cl
F
F
4p
Impurity
from TFA
2.00
1.04
2.10
1.01
1.11
1.16
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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134.99
133.06
132.28
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129.88
117.45
113.73
113.69
113.42
53.26
34.65
CO2H
NH2
F
N
N
Cl
F
F
4p
200
180
160
140
120
100
80
60
40
20
0
f1 (ppm)