# Supporting Information

# The synthesis of specifically isotope labelled Fluorotryptophan and its use in mammalian cell-based protein expression for $^{19}$F-NMR applications

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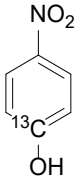
# 1. Synthesis of compound **1**

## 1.1. General Information

NMR spectra were recorded on Bruker BioSpin AV NEO 400. The chemical shifts are reported in parts per million (ppm) on the delta (δ) scale. The solvent peak was used as a reference value, for $^{1}H$ NMR: $CDCl_3$ = 7.27 ppm, $(CD_3)_2SO$ = 2.50 ppm, $D_2O$ = 4.79 ppm; for $^{13}C$ NMR: $CDCl_3$ = 77.16 ppm, (CD3)2SO = 39.52 ppm. The coupling data are reported as follows: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet. All the $^{13}C$ spectra are proton decoupled. High resolution mass spectra were collected on an Orbitrap Exploris 120 (Thermo Fisher Scientific, Bremen, Germany) and on timsTOF fleX ESI/MALDI dual source - Qq-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in the positive- and/or negative ion mode. The sum formulas of the detected ions were determined using Bruker Compass DataAnalysis 5.3 based on the mass accuracy ($\Delta m/z \leq 5$ ppm) and isotopic pattern matching (SmartFormula algorithm). Dry solvents were purchased from ThermoFisher Scientific. Analytical TLC was performed on pre-coated (25 mm) silica gel 60 with fluorescent indicator plates. Visualization was done under UV (254 nm) and by staining with ninhydrin or phosphomolybdic acid staining solutions with subsequent heating using a heat-gun. Column chromatography was done using Silica 60 (0.04-0.063) from Merk. All reactions were performed in flame-dried glassware under a positive pressure of argon with magnetic stirring unless noted otherwise. All reagents were purchased from commercial sources and used without further purification, unless otherwise noted. [2- $^{13}C$] acetone was purchased from Sigma Aldrich and stored into at – 25°C. $D_2O$ was purchased from Eurisotop and stored at 25 °C. 7.6 N DCl was purchased by Eurisotop. Yields refer to isolated compounds. Microwave reactions were performed in a Biotage® Initiator+. Microwave reaction vials, caps with septum and stir bars were also purchased from Biotage.

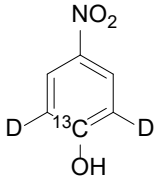
## 1.2. Synthetic Protocols and Spectral Data



**[1- $^{13}$C] 4-nitrophenol[1] (3):** Sodium nitromalonaldehyde (3.25 g, 23.37 mmol) was weighed into a 500 mL round-bottom flask and dissolved in 200 mL of $H_2O$. The mixture was placed in an ice bath, and a magnetic stir bar was added. 1 g of [2-$^{13}$C] acetone (16.93 mmol, 1.24 mL) was removed from the freezer at -25°C and promptly added to the mixture at 0°C using a syringe. While stirring the mixture at 0°C, an aqueous NaOH solution (4.4 g, 0.11 mol in 20 mL) was slowly added via a dropping funnel. After the addition was complete, the flask was tightly closed with a glass stopper and stirred for 6 days at 4°C. The resulting dark brown solution was cooled to 0°C, and 52 mL of 6 M HCl were slowly added. The mixture was then transferred to a separating funnel, and 50 mL of ethyl acetate were added. The phases were separated, and the aqueous phase was extracted again 5 times with 30 mL of ethyl acetate each. The combined organic phases were dried over $MgSO_4$, filtered, and evaporated under reduced pressure using a rotavapor, yielding a brown oil. The crude product was purified using a silica gel chromatography column eluted with heptane–ethyl acetate (7:3). The reaction yielded 1.47 g (63%) of [1-$^{13}$C] 4-nitrophenol **3** as a yellow solid.

**$^1$H NMR** (400 MHz, $CDCl_3$) δ 8.19 (t, *J* = 9.4 Hz, 2H, CH arom.), 6.93 (dd, *J* = 9.2, 2.4 Hz, 2H, CH aromatic), 5.49 (d, *J* = 3.4 Hz, 1H, OH).

**$^{13}$C NMR** (101 MHz, $CDCl_3$) δ 161.11 (s, $^{13}$C-OH), 126.43 (s), 142.03 (s), 115.79 (d, *J* = 65.9 Hz).

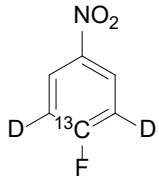


**[1-$^{13}$C, 2,6-$^2H_2$] 4-nitrophenol[1] (4):** A 2-5 mL microwave reaction vial was loaded with 200 mg of [1-$^{13}$C] 4-nitrophenol **3** (1.44 mmol), 1 mL of $D_2O$, and 1 mL of DCl 7.6 N. The mixture was irradiated for 1.5 h at 170 °C in a Biotage® Initiator+ and then cooled to RT. The solvent was evaporated under reduced pressure, and the resulting solid was purified via silica column chromatography eluted with ethyl acetate-heptane (6:4). The product **4** was isolated in 98% yield as a yellow solid. $^1$H-NMR spectroscopy analysis indicated 97.5% of deuterium incorporation at positions 2 and 6.

**$^1$H NMR** (400 MHz, $CDCl_3$) δ 8.19 (d, *J* = 9.6 Hz, 2H), 6.92 (dd, *J* = 9.6, 2.5 Hz, 2.5% residual CH position 3 and 5).

**$^{13}$C NMR** (101 MHz, $CDCl_3$) δ 161.18 (s, $^{13}$C-OH), 126.30 (s).

**HRMS** calcd for $C_5{}^{13}CH_2D_2NO_3$ $[M - H]^-$ 141.03557, found 141.0353.

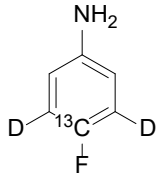



**[4-$^{13}$C, 3,5-$^{2}H_2$]4-fluoronitrobenzene[2] (5):** To a 50 mL two-neck round-bottom flask, 1.52 g (9.99 mmol) of CsF and 0.68 g (1.55 mmol) of N,N′-1,3-Bis(2,6-diisopropylphenyl)-2-chloro imidazolium chloride were added and stirred at 140 °C under vacuum for 3 hours. The solid mixture was then allowed to cool to room temperature, and 142 mg (0.99 mmol) of [1-$^{13}$C, 2,6-$^{2}H_2$]4-nitrophenol **4** were added. The flask was equipped with a reflux condenser and a septum, and placed under argon atmosphere. Next, 7 mL of dry toluene were added, and the mixture was stirred at 110 °C for 24 hours. Upon completion, the reaction mixture was allowed to cool to room temperature and filtered through Celite, eluting with dichloromethane. The dichloromethane was removed under reduced pressure (>500 mbar, 40 °C). The residual toluene was evaporated at a pressure above 90 mbar at 40 °C after forming an azeotrope with methanol, using a rotary evaporator (product bp = 205 °C, lit.). The resulting crude brown oil was further purified using column chromatography with heptane/ethyl acetate (20:1) as the eluent. This step resulted in the isolation of 110 mg (76%) of [4-$^{13}$C, 3,5-$^{2}H_2$]4-fluoronitrobenzene **5** as a yellow oil, which solidifies below 20 °C. $^{1}$H-NMR spectroscopy analysis indicated a deuteration level of 96% at both positions 2 and 6.

**$^{1}$H NMR** (400 MHz, $CDCl_3$) δ 8.29 (dd, $J$ = 10.7, 4.6 Hz, 2H), 7.25 – 7.19 (m, 4% residual CH position 3 and 5).

**$^{13}$C NMR** (101 MHz, $CDCl_3$) δ 166.36 (d, $J$ = 258.0 Hz, $^{13}$C-F), 126.36 (d, $J$ = 10.0 Hz, CH arom.), 117.49 – 115.06 (m, C-D arom.)

**HRMS (m/z)**: calcd for $C_5{}^{13}CH_2D_2FNO_2$ 144.0385, found 144.0377.

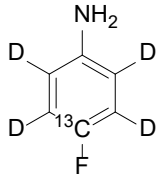


**4- $^{13}$C, 3,5-$^{2}H_2$]4-fluoroaniline[3] (6)**: 111 mg (0.77 mmol) of substrate **5** were dissolved in 4 mL of dry methanol in a flame dried Schlenk flask and 40 mg (0.38 mmol) of 10% Pd/C catalyst were added. The flask was purged with argon and then exposed to $H_2$ using a hydrogen balloon. The mixture was stirred under $H_2$ overnight and then filtered through a Celite pad, eluting with dichloromethane. The solvent was removed at reduced pressure (P > 300 mbar, bp product = 188 °C) and the resulting residue was dissolved in 10 mL of dichloromethane. The solution was washed with 10 mL of 1 M aqueous NaOH and 10 mL of brine. The organic phase was dried over $MgSO_4$, and the solvent evaporated, resulting in 108 mg (95%) of [4-$^{13}$C, 3,5-$^{2}H_2$]4-fluoroaniline **6** as a brown oil. $^{1}$H-NMR spectroscopy analysis indicated a deuteration level of 96% at both positions 2 and 6.

**$^{1}$H NMR** (400 MHz, $CDCl_3$) δ 6.90 – 6.82 (m, 4% residual CH position 3 and 5), 6.63 (dd, *J* = 10.5, 4.2 Hz, 2H, CH arom.), 3.54 (bs, 2H, $NH_2$).

**$^{13}$C NMR** (101 MHz, $CDCl_3$) δ 156.55 (d, *J* = 235.7 Hz, $^{13}$C-F).

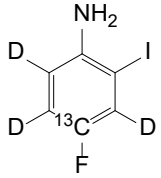
**HRMS:** calcd for $C_5{}^{13}CH_4D_2FN$ $[M + H]^+$ 115.07161, found 115.0714.



**[4- $^{13}$C, 2,3,5,6-$^{2}H_4$]4-fluoroanilinium chloride (7):** In a 2-5 mL microwave reaction vial, 114 mg of [4-$^{13}$C, 3,5-$^{2}H_2$] 4-fluoroaniline **6** (1 mmol) was mixed with 1 mL of $D_2O$ and 1 mL of DCl 7.6 N. The vial was irradiated for 1.5 h at 180 °C. After cooling to RT, the solvent is evaporated under vacuum. The resulting solid was then taken up in 10 mL of 1 M NaOH and extracted with dichloromethane (5 × 20 mL). The combined organic phases were dried over $MgSO_4$ and the solvent was evaporated, yielding 110 mg (95%) of [4-$^{13}$C, 2,3,5,6-$^{2}H_4$]4-fluoroaniline **7** as a pale brown oil. $^{1}$H-NMR spectroscopy indicated almost quantitative aryl deuteration with 97.5 % deuteration for each of the four positions.

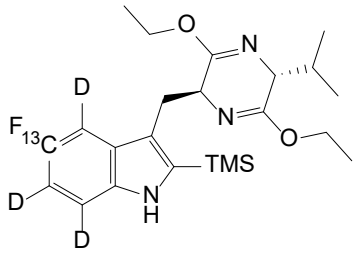
**$^{1}$H NMR** (400 MHz, $CDCl_3$) δ 6.86 (m, 2.5% residual CH position 3 and 5.), 6.64 (m, 2.5% residual CH position 2 and 6), 3.53 (bs, 2H, $NH_2$).

**$^{13}$C NMR** (101 MHz, $CDCl_3$) δ 156.43 (d, *J* = 235.7 Hz, $^{13}$C-F).




**[4- $^{13}$C, 1,3,5-$^{2}$H$_3$]4-fluoro-2-iodoaniline[4] (8)**: 581 mg of compound **7** (5.23 mmol), 1.33 g of $I_2$ (5.23 mmol), and 879 mg of $NaHCO_3$ were added to a brown screw-cap vial. To this, 6 mL of $H_2O$ and 4 mL of dichloromethane were added, and the mixture was stirred at room temperature for 30 hours. After this time, 413 mg (2.61 mmol) of sodium thiosulfate were added, and the mixture was stirred for an additional 15 minutes. The phases were then separated, and the aqueous phase was extracted three times with 10 mL portions of dichloromethane. The combined organic phases were washed with 20 mL of brine, dried over $MgSO_4$, and evaporated under vacuum. The product **8** was isolated as a yellow oil by short-path distillation at 120°C and 0 mbar, yielding 1.087 g (87.6%).

**$^{1}$H NMR** $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.42 – 7.34 (m, 2% residual CH position 3), 6.94 – 6.86 (m, 5% residual CH position 5), 6.73 – 6.67 (m, 5% residual CH position 6), 3.95 (bs, 2H).
**$^{13}$C NMR** (101 MHz, $CDCl_3$) δ 155.36 (d, $J$ = 240.8 Hz, $^{13}$C-F).
**HRMS** calcd for $C_5{}^{13}CH_2D_3FIN$ $[M + H]^+$ 241.9745; found 241.9743.

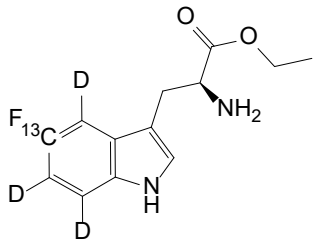



**(2R,5S)-3,6-Diethoxy-2-isopropyl-5-[5-$^{13}$C, 4,6,7-$^{2}$H$_3$, 5-fluoro-2-(trimethylsilyl)-3-indolyl]methyl-2,5 -dihydro pyrazine[5] (9):** In a 100 mL round-bottom flask equipped with a stirring bar were placed compound **8** (161 mg, 0.67 mmol), (2R,5S)-3,6-diethoxy-2-iso-propyl-5-[3-(trimethylsilyl)prop-2-ynyl]-2,5-dihydropyrazine **12** (237 mg, 0.73 mmol), palladium(II) acetate (6 mg, 0.027 mmol), lithium chloride (28.3 mg, 0.67 mmol), sodium carbonate (141 mg, 1.33 mmol), and DMF (10 mL). The reaction mixture was degassed and then heated at 100°C under argon until the starting compound **8** was no longer detected on analysis by TLC (30 hours). The DMF was removed under reduced pressure, and the residue was taken up in dichloromethane (50 mL). The suspension that resulted was allowed to pass through a Celite pad to remove insoluble solids. The solution was concentrated in vacuum, and the product was purified by flash chromatography (silica gel, hexane/EtOAc 98:2) to afford 197 mg of compound **9** as an oil in 70% yield.

**$^{1}$H NMR** (400 MHz, $CDCl_3$) δ 7.90 (s, 1H), 7.44 – 7.38 (m, 2% residual CH position 4), 7.25 – 7.21 (m, 10% residual CH position 6), 6.92 – 6.87 (m, 2% residual CH position 7), 4.29 – 3.98 (m, 5H), 3.88 (t, *J* = 3.4 Hz, 1H), 3.52 (dd, *J* = 14.3, 3.3 Hz, 1H), 2.82 (dd, *J* = 14.3, 9.7 Hz, 1H), 2.33 – 2.21 (m, 1H), 1.32 (t, *J* = 7.1 Hz, 3H), 1.22 (t, *J* = 7.1 Hz, 3H), 1.03 (d, *J* = 6.8 Hz, 3H), 0.68 (d, *J* = 6.8 Hz, 3H), 0.42 (s, 9H).

**$^{13}$C NMR** (101 MHz, $CDCl_3$) δ 163.45 (s), 163.01 (s), 157.45 (d. $^{13}$C-F, J = 233.49 Hz), 156.37 (s), 136.30 (s), 60.75 (s), 58.78 (s), 31.84 (d, *J* = 19.8 Hz), 19.19 (s), 16.72 (s), 14.47 (s), 14.39 (s) - 0.56 (s).

**HRMS** calcd. for $C_{22}{}^{13}CH_{31}D_3FN_3O_2Si$ $[M + H]^+$ 436.2699, found 436.2696.

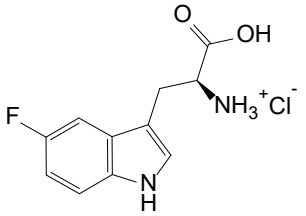



**[5- $^{13}$C, 4,6,7-$^{2}H_3$]-5-Fluoro-L-tryptophan ethyl ester[5] (10):** To a solution of optically pure compound **9** (235 mg, 0.53 mmol) in THF (8 mL) at 0°C was slowly added an aqueous solution of 6 N HCl (7 mL). The mixture was allowed to warm to room temperature and stirred for 4 h. Ice (10 g) was added to the solution, and the pH of the reaction mixture was adjusted to 8 with aqueous $NH_4OH$ (concentrated) at 0°C. The mixture was then extracted with dichloromethane (3 × 50 mL). The combined organic layers were dried over $MgSO_4$, and the solvent was removed under reduced pressure. The residue which resulted was purified by flash chromatography (silicagel, EtOAc) to afford 108 mg of compound **10** in 79% yield.

**$^{1}$H NMR** (400 MHz, $CDCl_3$) δ 8.07 (s, 1H), 7.13 (d, *J* = 2.0 Hz, 1H), 4.21 – 4.13 (m, 2H), 3.79 (m, 1H), 3.22 (dd, *J* = 14.4, 5.0 Hz, 1H), 3.04 (dd, *J* = 14.5, 7.4 Hz, 1H), 1.59 (s, 2H), 1.26 (t, *J* = 7.1 Hz, 3H).

**$^{13}$C NMR** (101 MHz, $CDCl_3$) δ 175.47 (s), 157.97 (d, *J* = 234.9 Hz), 124.77 (s), 111.85 (s), 61.14 (s), 55.08 (s), 30.84 (s), 14.32 (s).

**HRMS** calcd for $C_{12}{}^{13}CH_{12}D_3FN_2O_2$ $[M + H]^+$ 255.1412, found 255.1413.

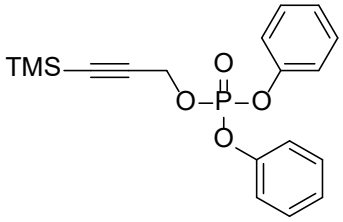


**[5- $^{13}$C, 4,6,7-$^{2}H_3$]-5-Fluoro-L-tryptophan[5] (1):** Into a 25 mL round-bottom flask were added compound **10** (131 mg, 0.51 mmol), aqueous 1 N NaOH (1.0 mL, 1 mmol), and ethanol (1.5 mL). The solution was heated to 50°C for 2 h. Analysis by TLC (silica gel) indicated that all the starting material had disappeared. Most of the EtOH was removed under reduced pressure. A piece of ice was dropped into the flask and the mixture was brought to pH 6-7 with an aqueous solution of 2 N HCl at 0°C. The water in the solution that resulted was removed under reduced pressure until a white precipitate appeared. The total volume of solution was reduced to 0.5 mL. Cold water (1 mL) was then added, and the precipitate that formed was collected by vacuum filtration, washed with cold water (3 × 1 mL), and dried to provide compound **1** (54 mg) in 94.6% yield.

**$^{1}$H NMR** (400 MHz, $D_2O$) δ 7.46 (m, 7% residual CH position 7), 7.36 (s, 1H), 7.07 – 7.02 (m, 4% residual CH position 4 and 6), 4.30 – 4.18 (m, 1H), 3.46 (dd, *J* = 15.4, 5.2 Hz, 1H), 3.41 – 3.32 (dd, *J* = 15.5, 7.38 Hz, 1H).

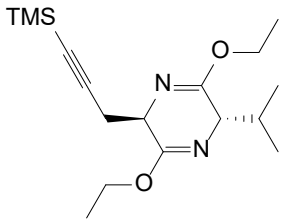
**$^{13}$C NMR** (101 MHz, MeOD) δ 157.72 (d, *J* = 233.0 Hz), 126.18 (s), 52.97 (s), 26.04 (s).

**HRMS** calcd for $C_{10}{}^{13}CH_8D_3FN_2O_2$ $[M + H]^+$ 227.10992, found 227.1095.



**3-(Trimethylsilyl)-2-propyn-1-yl-P,P-diphenylphosphinate[5] (13):** To a solution of 3-(trimethylsilyl)prop-2-yn-1-ol (1.4 g, 11.7 mmol) and diphenyl chlorophosphate (3.52 g, 13.1 mmol) in diethyl ether (30 mL) at -50°C was added powdered potassium hydroxide (4.18 g, 74 mmol). The mixture that resulted was allowed to warm to 0°C over 20 min and stirred at 0°C for 12 h. The reaction mixture was poured into ice-water (100 mL), and the ether layer was separated. The aqueous layer was extracted with ether (2 × 50 mL). The combined ether layer was washed with water until the water washing was no longer alkaline to pH paper. The ether solution was dried ($MgSO_4$), and the solvent was removed under reduced pressure. The colorless residue was chromatographed on silica gel (hexane/EtOAc 8:1) to provide pure **13** (3.6 g) in 92% yield.

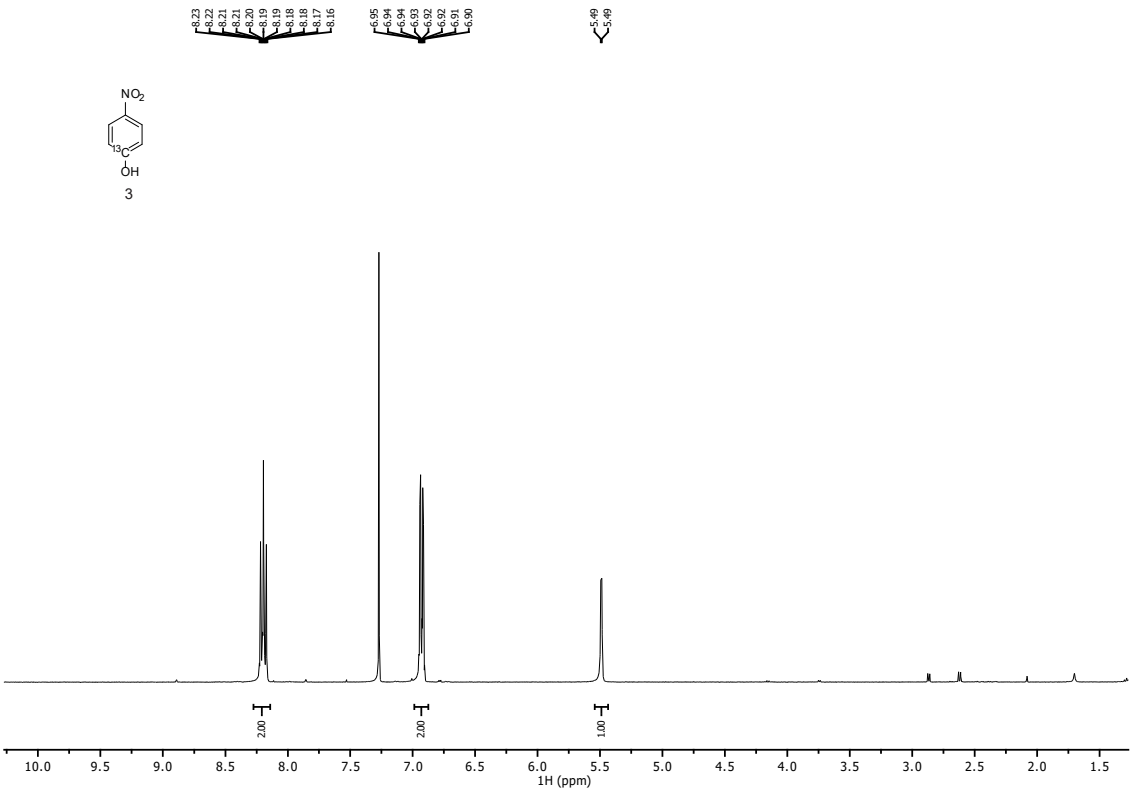
**$^1$H NMR** (400 MHz, $CDCl_3$) δ 7.38 – 7.19 (m, 10H), 4.86 (d, *J* = 10.7 Hz, 2H), 0.16 (s, 9H).

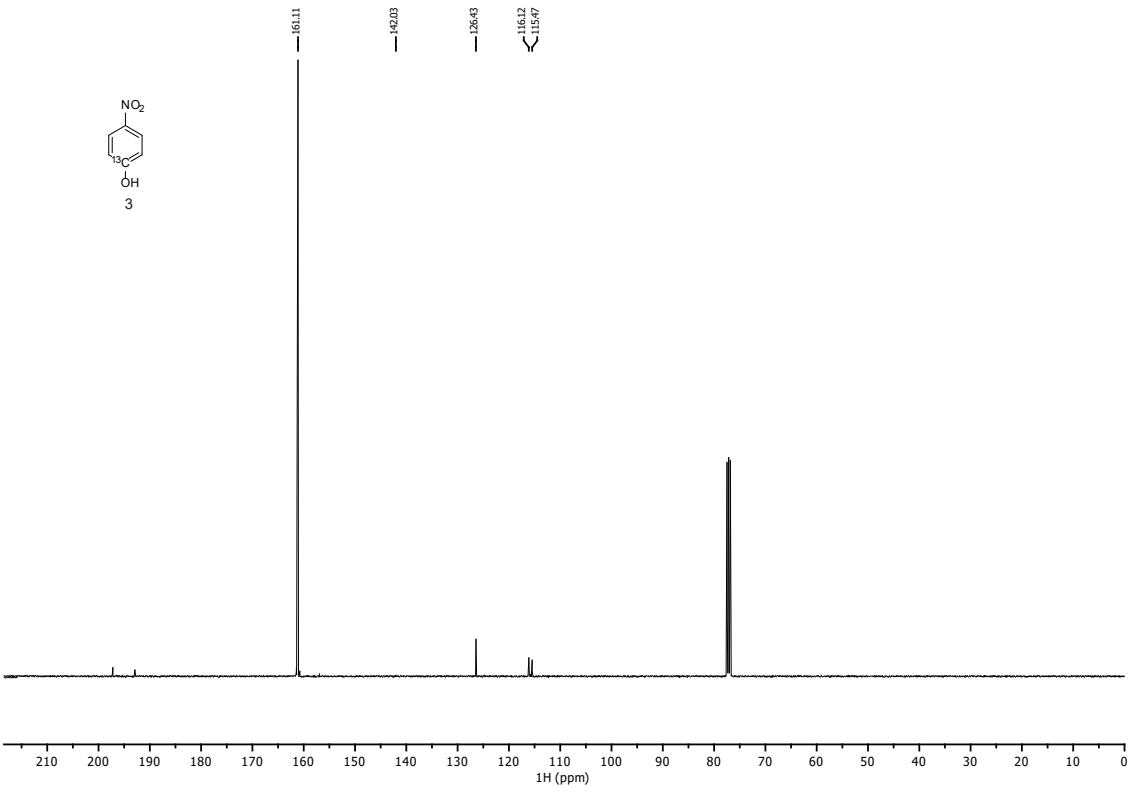


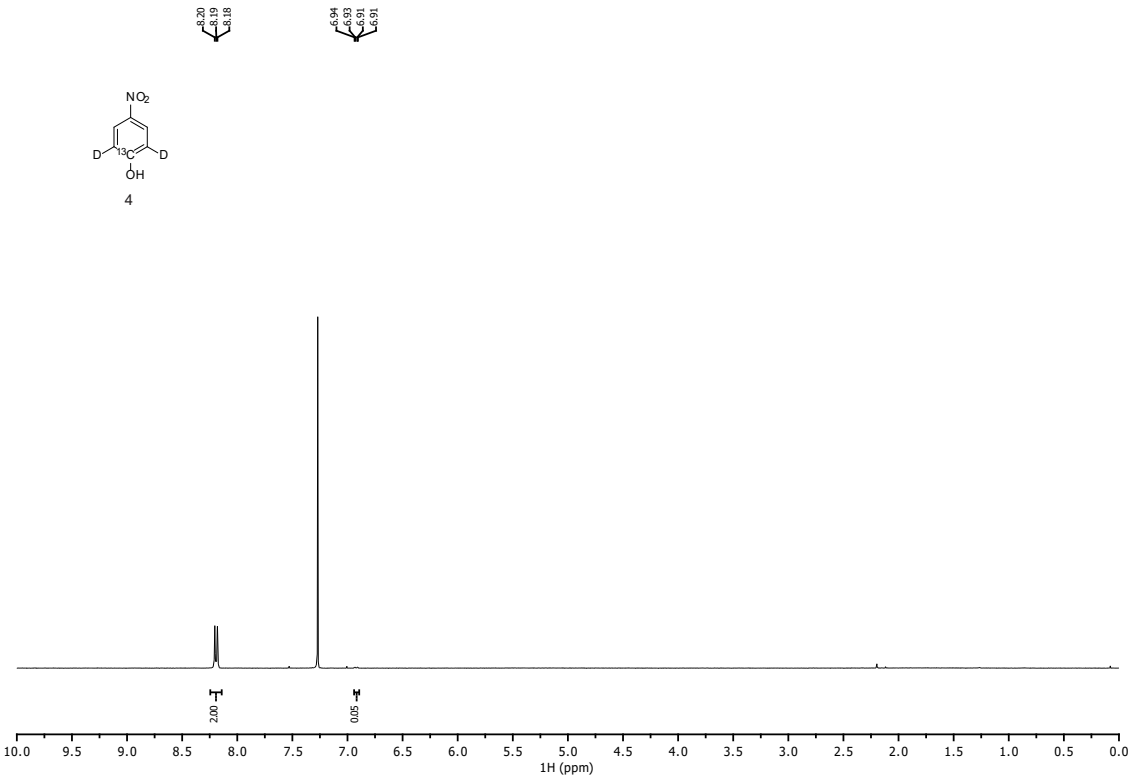

**(2R,5S)-3,6-Diethoxy-2-isopropyl-5-[3-(trimethylsilyl)-prop-2-ynyl]-2,5-dihydropyrazine[5] (12):** To a solution of (2R)-3,6-diethoxy-2-isopropyl-2,5-dihydropyrazine (0.827 g, 3.9 mmol) in dry THF (25 mL) under nitrogen was syringed n-BuLi (2.5 M, 1.72 mL, 4.3 mmol) dropwise at -78 °C. This solution was stirred at -78 °C for 30 min. To this solution was added slowly a solution of diphenyl 3-(trimethylsilyl)prop- 2-ynyl phosphate **13** (1.4 g, 39 mmol) in dry THF (20 mL) that was cooled to -78 °C. After the reaction mixture was allowed to stir for 6 h at -78 °C, it was allowed to slowly warm to room temperature. The solution was then quenched with the addition of water (2 mL). The THF was removed under reduced pressure, and the residue was partitioned between water (20 mL) and diethyl ether (60 mL). The organic layer was separated, and the aqueous layer was extracted with ether (3 × 30 mL). The combined organic layers were washed with brine and dried ($MgSO_4$). After removal of solvent under reduced pressure, the residue was purified by chromatography (silica gel, hexane/EtOAc 98:2) to afford **12** as an oil (1.0 g) in 80% yield.

**$^1$H NMR** (400 MHz, $CDCl_3$) δ 4.30 – 4.06 (m, 5H), 3.98 (t, *J* = 3.3 Hz, 1H), 2.74 (ddd, *J* = 42.2, 16.6, 4.5 Hz, 2H), 2.30 (dtd, *J* = 13.7, 6.8, 3.2 Hz, 1H), 1.30 (td, *J* = 7.1, 2.8 Hz, 6H), 1.06 (d, *J* = 6.9 Hz, 3H), 0.71 (d, *J* = 6.8 Hz, 3H), 0.11 (s, 9H).

## 1.3. NMR spectra

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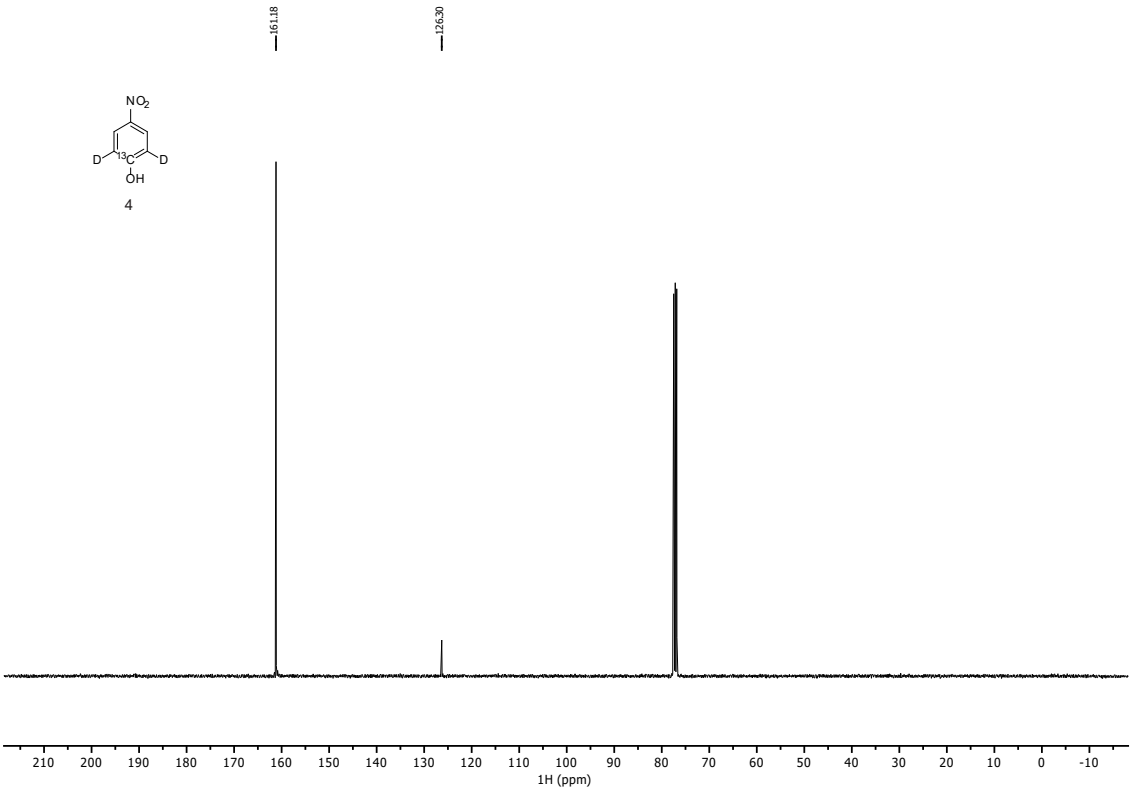
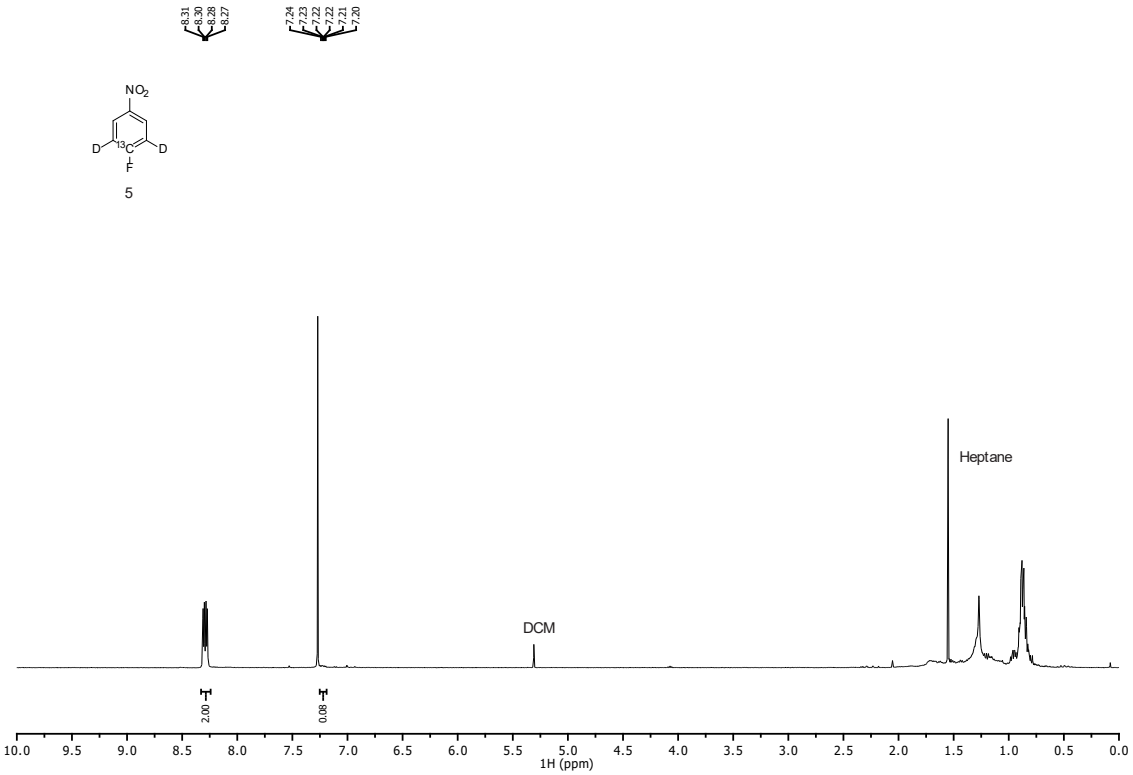
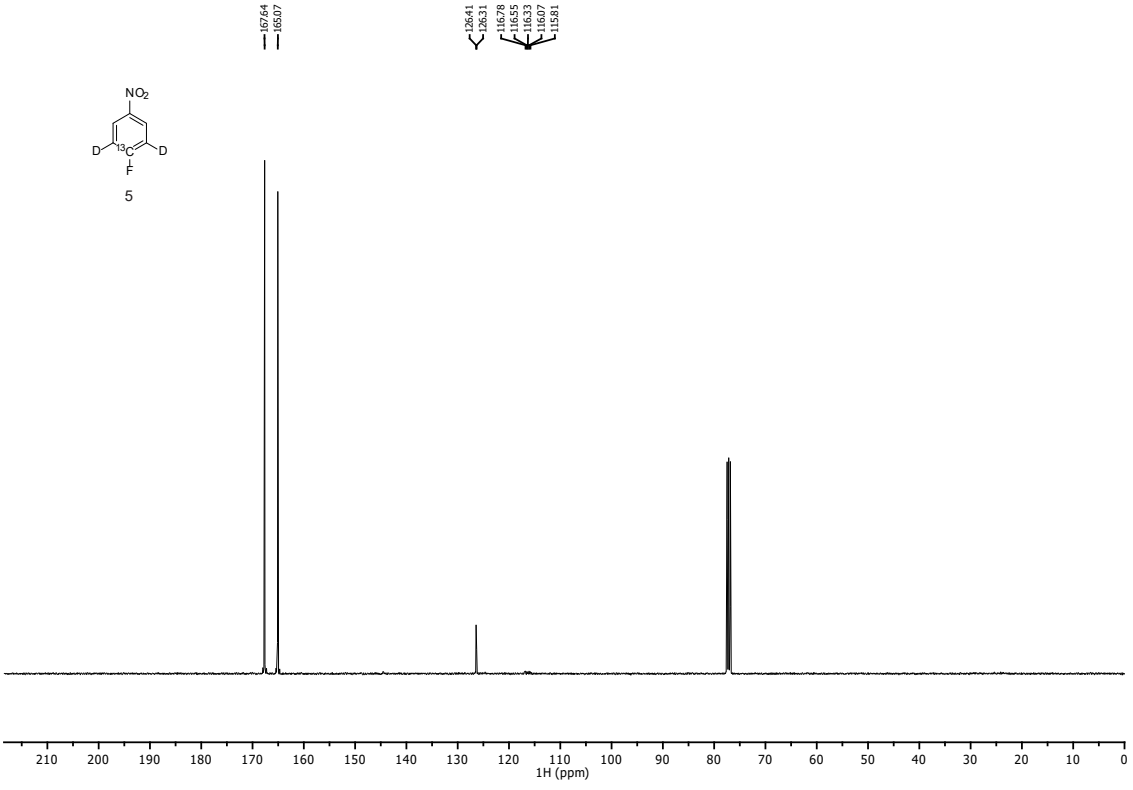
8.19
8.18
6.94
6.93
6.91
6.91
NO2
D
13C
D
OH
2.00
0.05
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
1H (ppm)

4


161.18
126.30
NO2
D
13C
D
OH
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
1H (ppm)

4


8.31
8.30
8.28
8.27
7.24
7.23
7.22
7.22
7.22
7.21
7.20
NO2
D
13C
D
F
5
Heptane
DCM
2.00
0.08
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
1H (ppm)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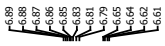
165.07
126.41
126.31
116.78
116.55
116.33
116.07
115.81
NO2
D
13C
D
F
5
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
1H (ppm)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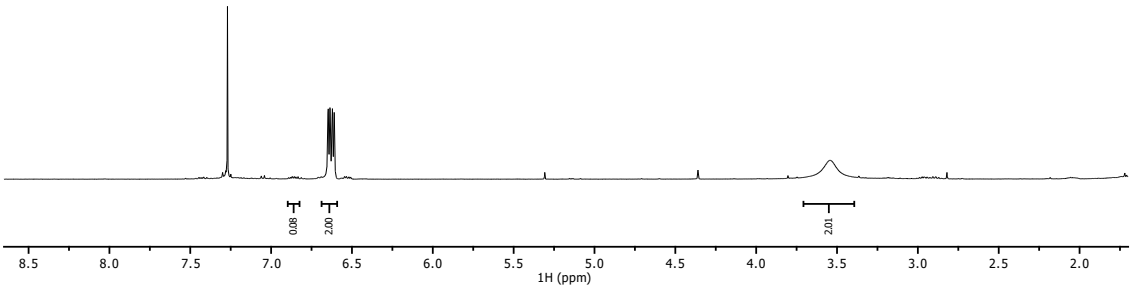
6.88
6.87
6.86
6.85
6.83
6.81
6.79
6.65
6.64
6.62
6.61

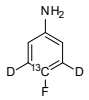

3.54


NH2
D
13C
D
F

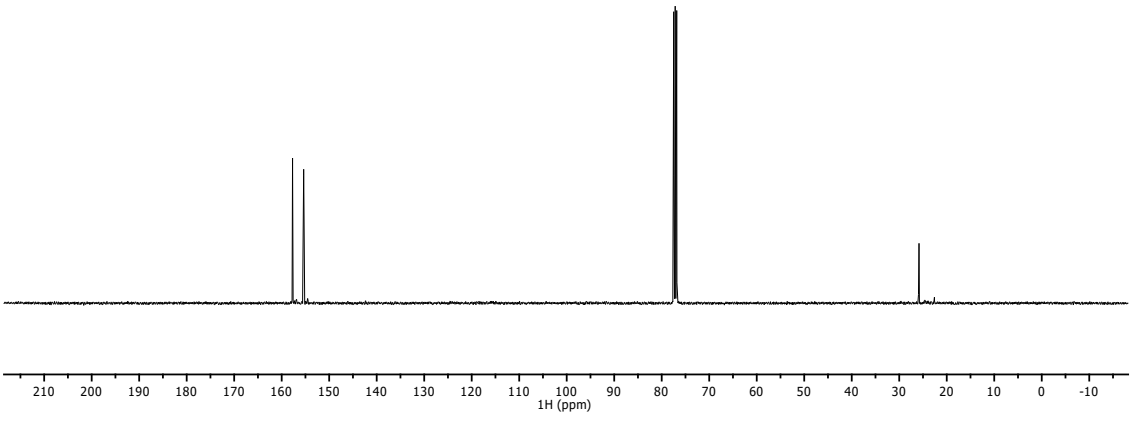
6


0.08
2.00
2.01
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1H (ppm)


157.72
155.37


NH2
D
13C
D
F

6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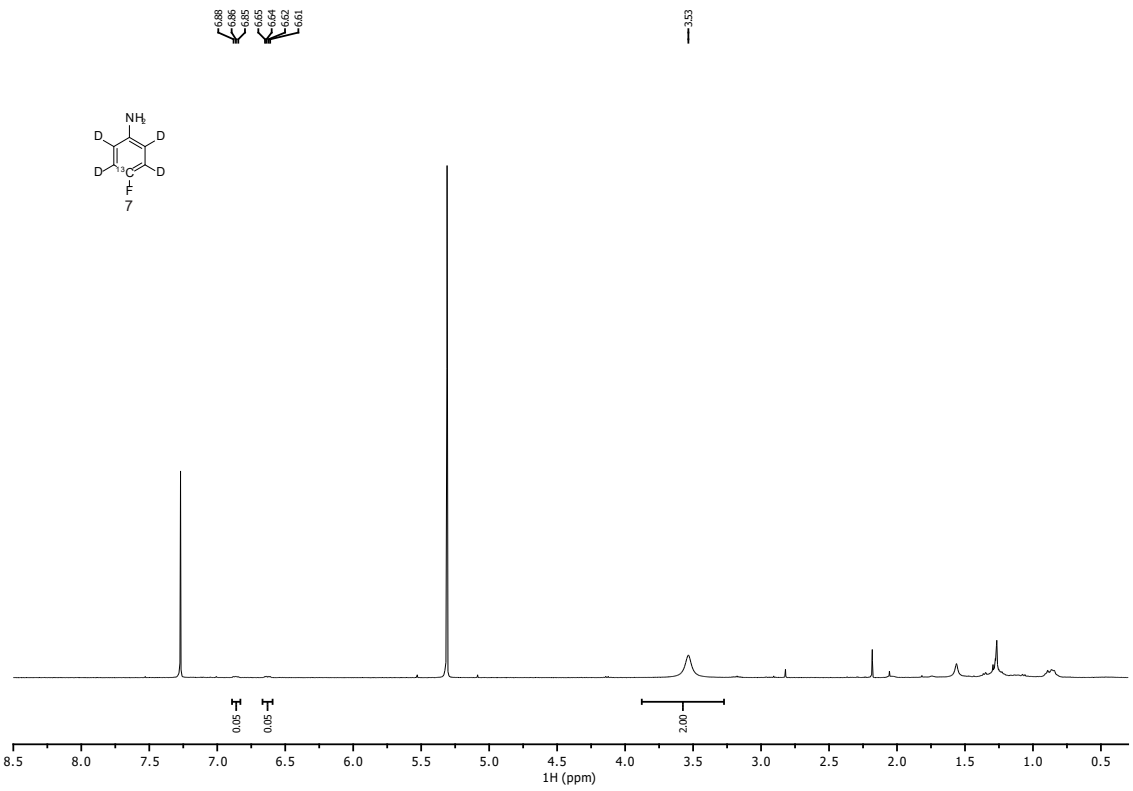
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
1H (ppm)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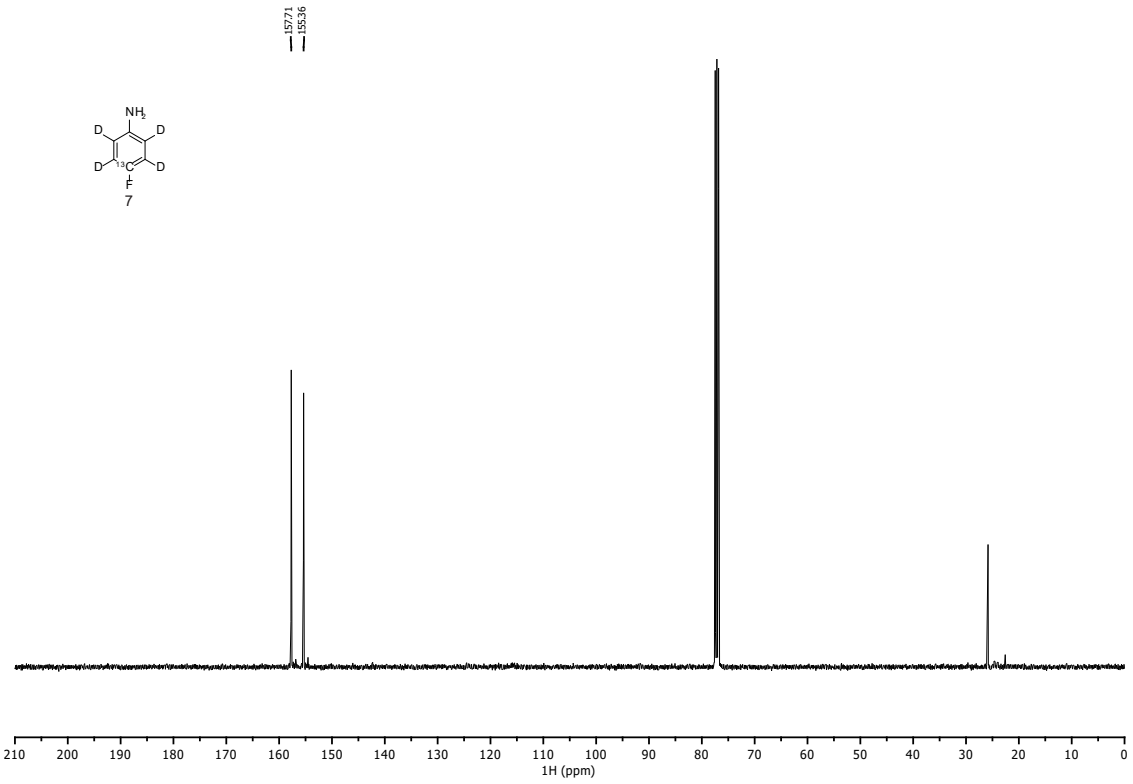
6.86
6.85
6.65
6.64
6.62
6.61
3.53
NH2
D
D
D
D
13C
F
7
0.05
0.05
2.00
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
1H (ppm)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155.36
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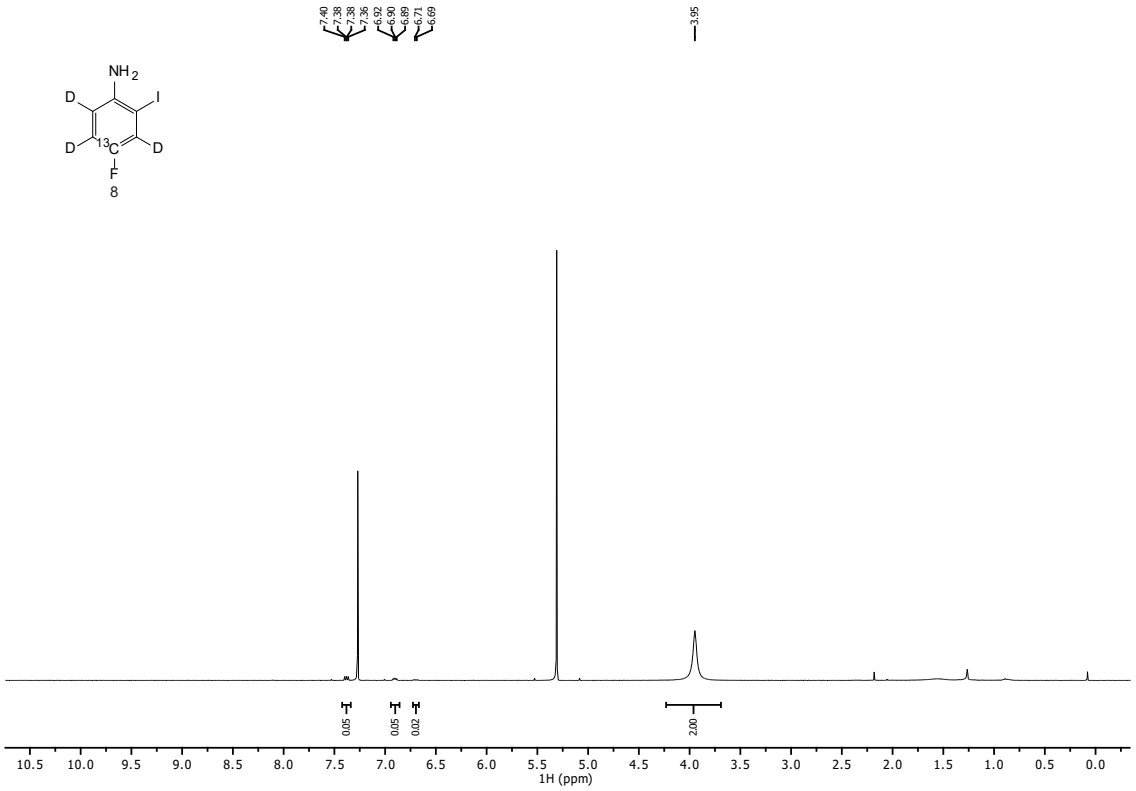
D
D
D
13C
F
7
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
1H (ppm)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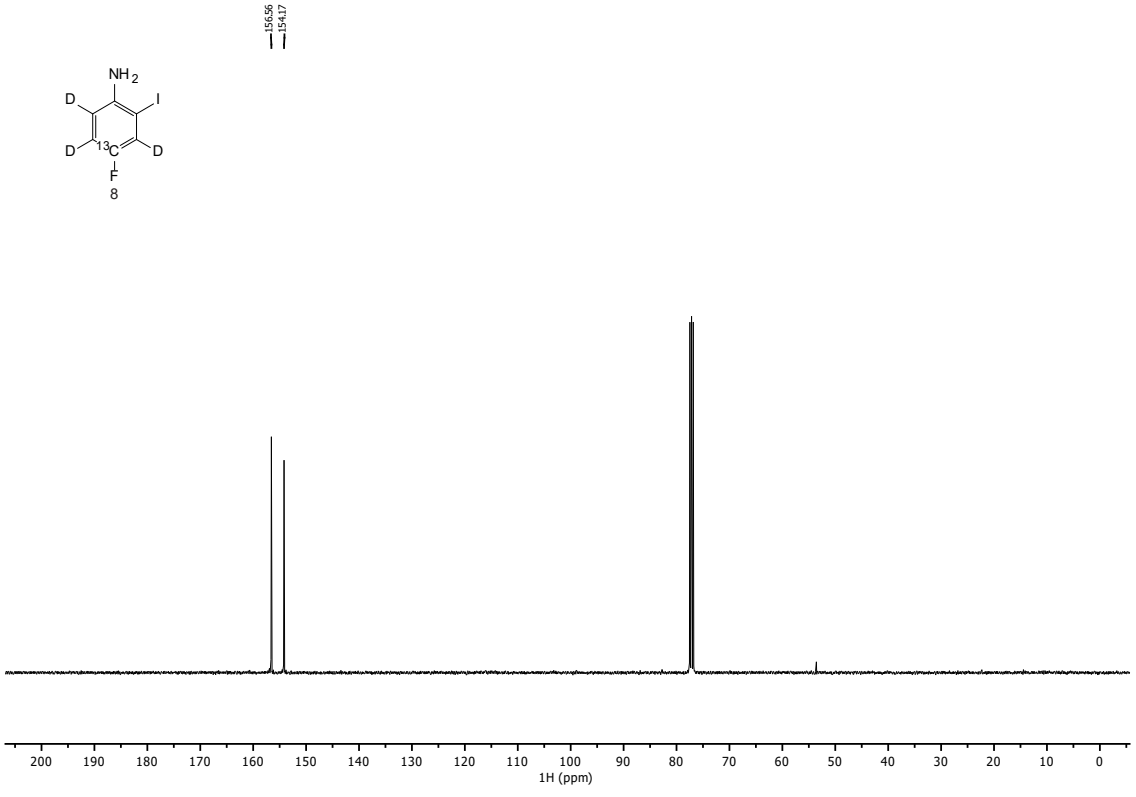
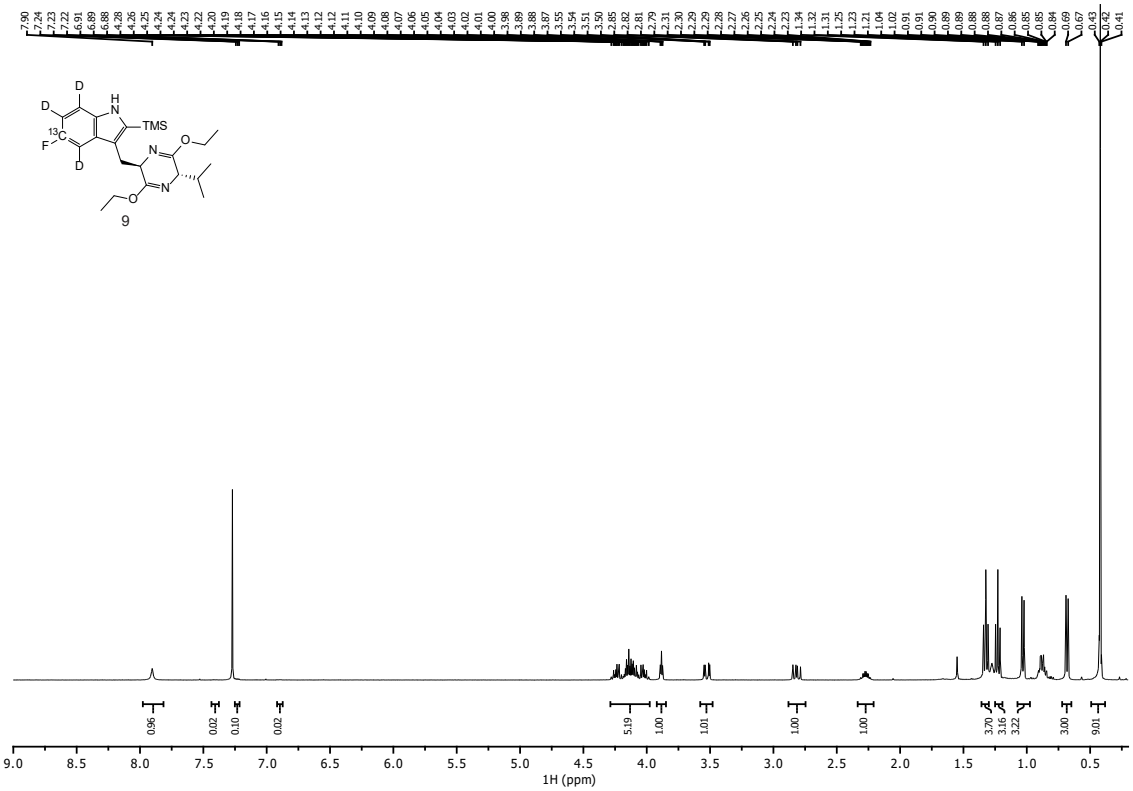
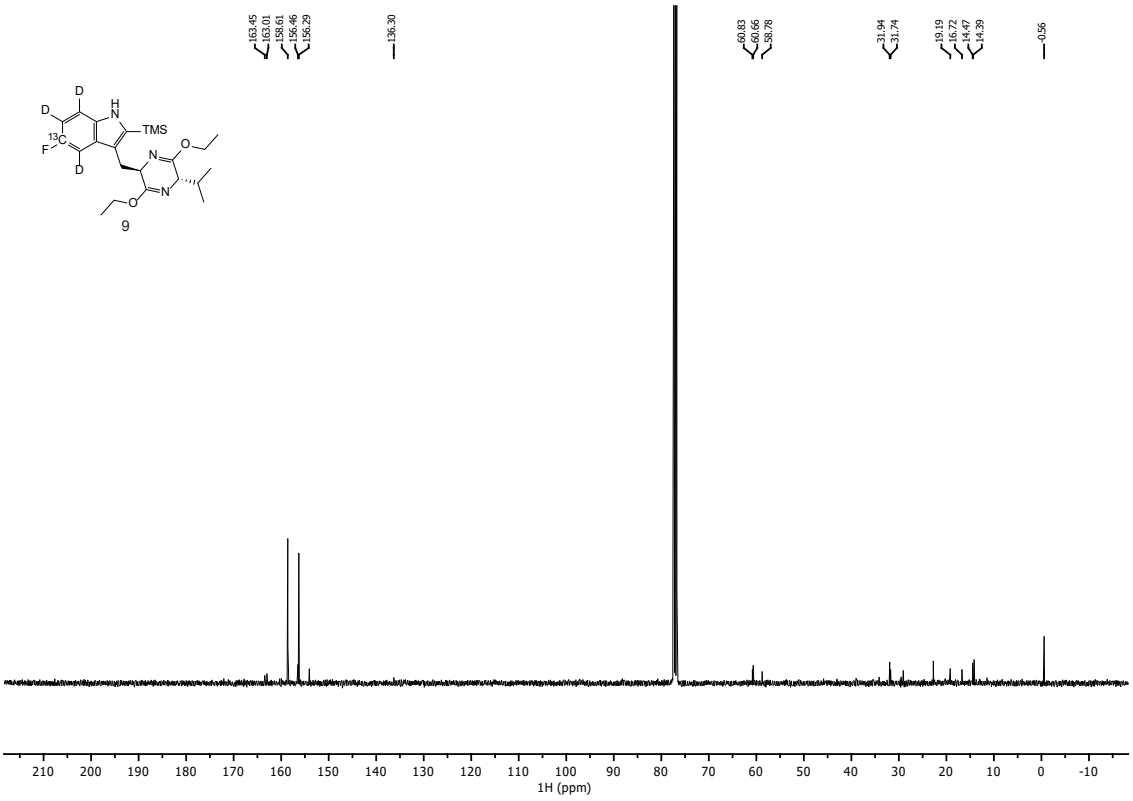
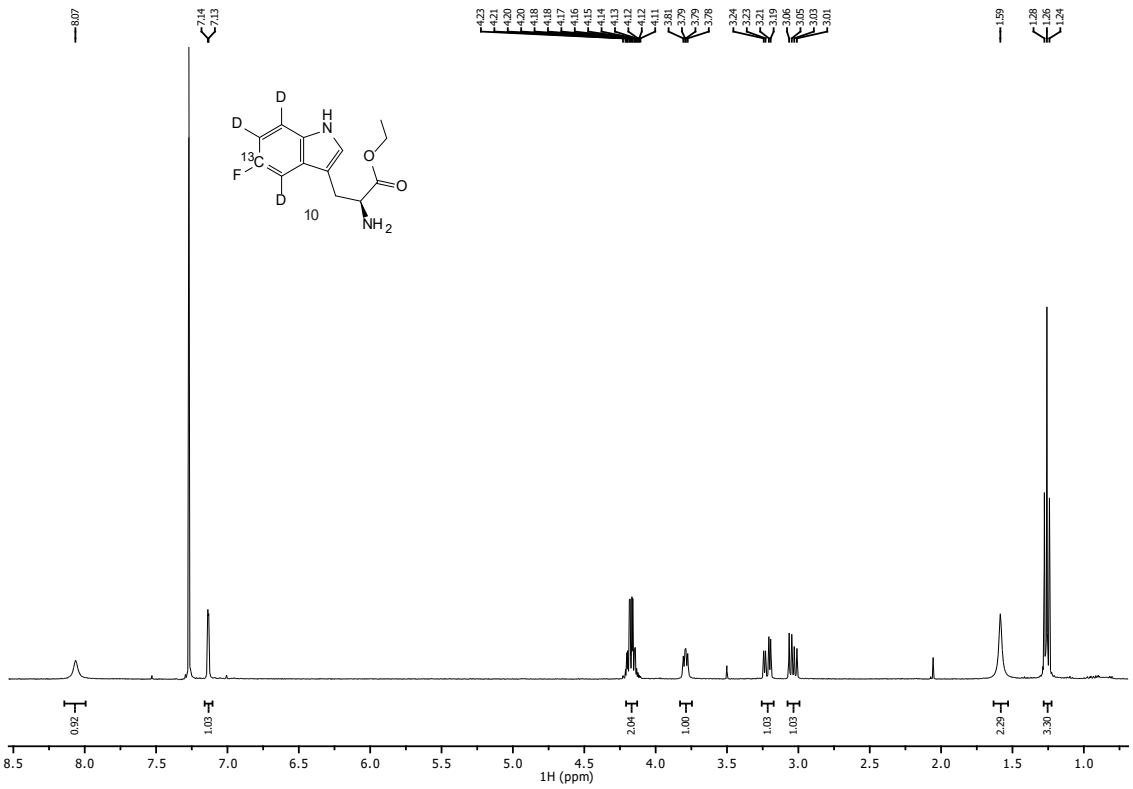
7.38
7.38
7.36
6.92
6.90
6.89
6.71
6.69
3.95
NH2
D
I
D
13C
D
F
8
0.05
0.05
0.02
2.00
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
1H (ppm)


156.56
154.17
NH2
D
I
D
13C
D
F
8
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
1H (ppm)


D
D
H
N
TMS
13C
F
D
N
O
N
O
9
1H (ppm)


D
D
H
N
TMS
13C
F
D
N
O
N
O
9
1H (ppm)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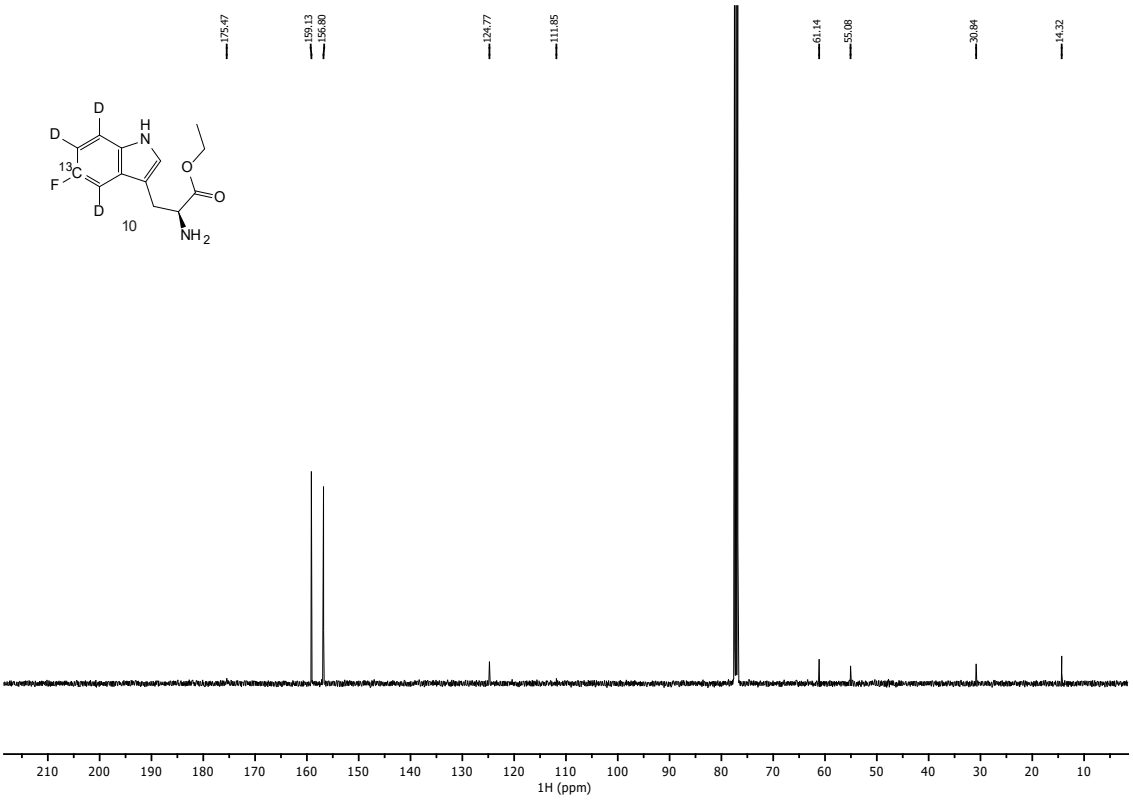
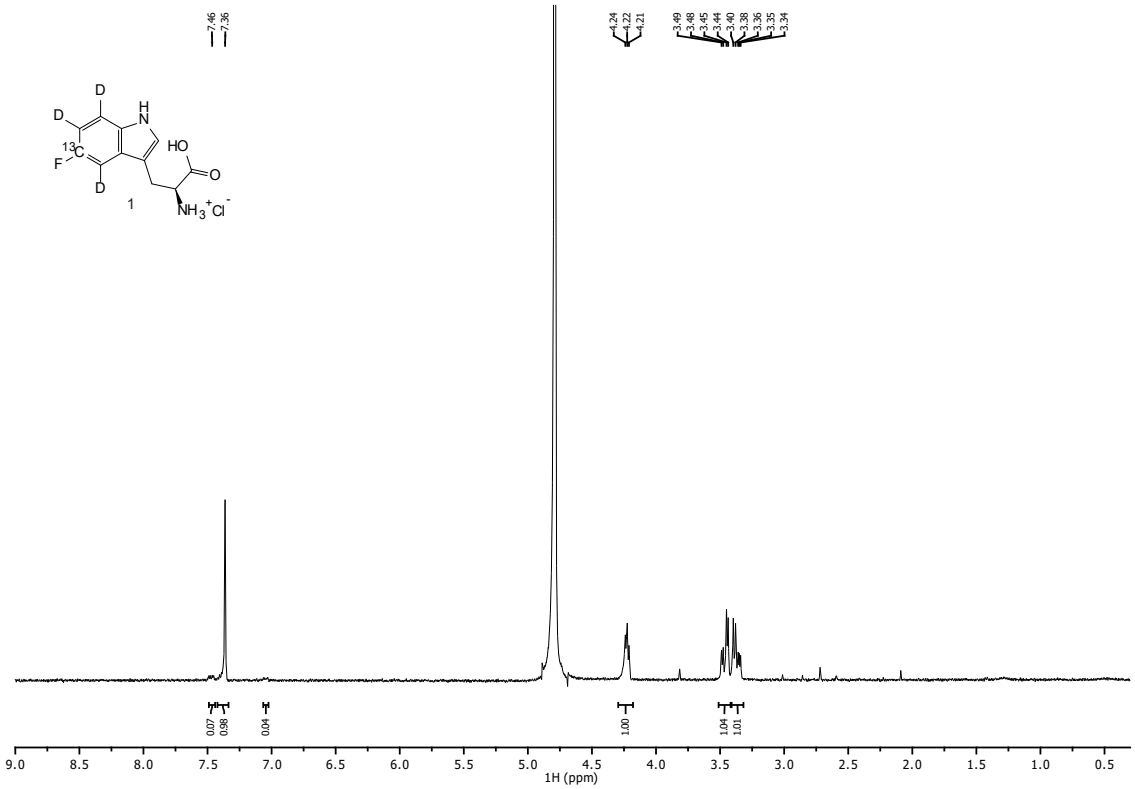
4.20
4.20
4.18
4.18
4.17
4.16
4.15
4.14
4.13
4.12
4.12
4.11
3.81
3.79
3.79
3.78
3.24
3.23
3.21
3.19
3.06
3.05
3.03
3.01
1.59
1.28
1.26
1.24
D
D
H
N
13C
F
D
O
O
10
NH2
0.92
1.03
2.04
1.00
1.03
1.03
2.29
3.30
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
1H (ppm)


175.47
159.13
156.80
124.77
111.85
61.14
55.08
30.84
14.32
D
D
H
N
13C
F
D
O
O
10
NH2
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
1H (ppm)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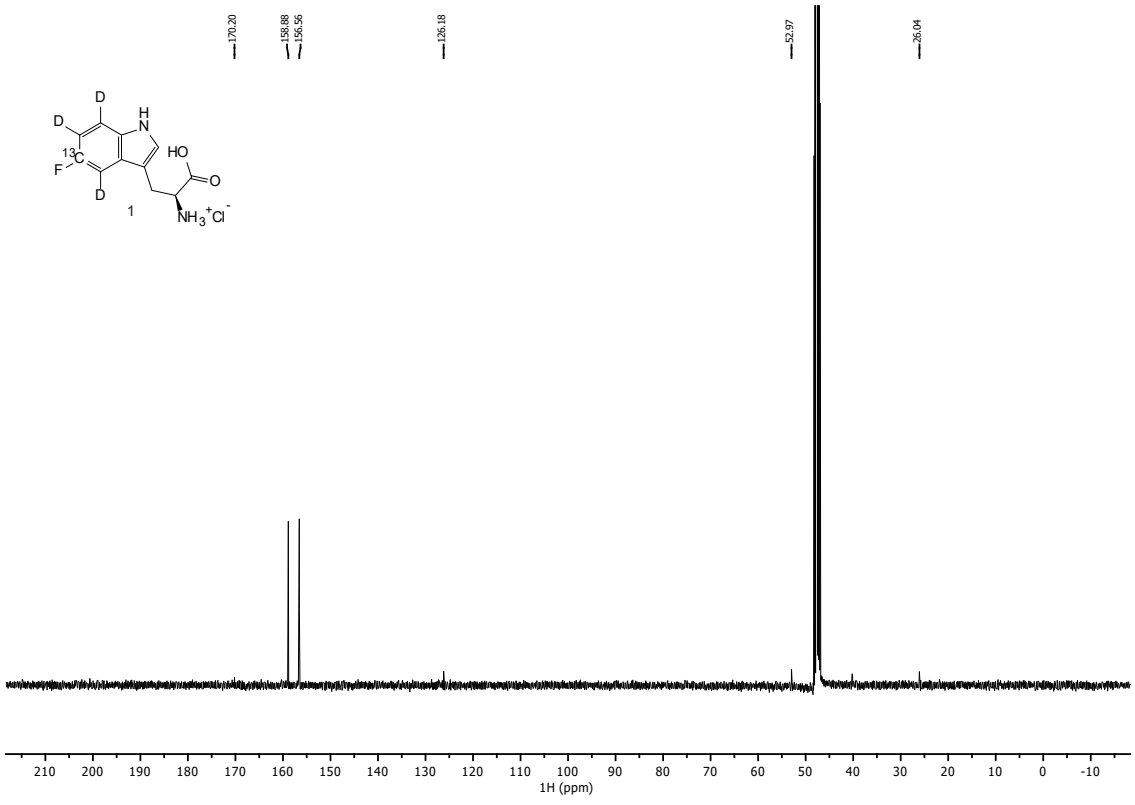
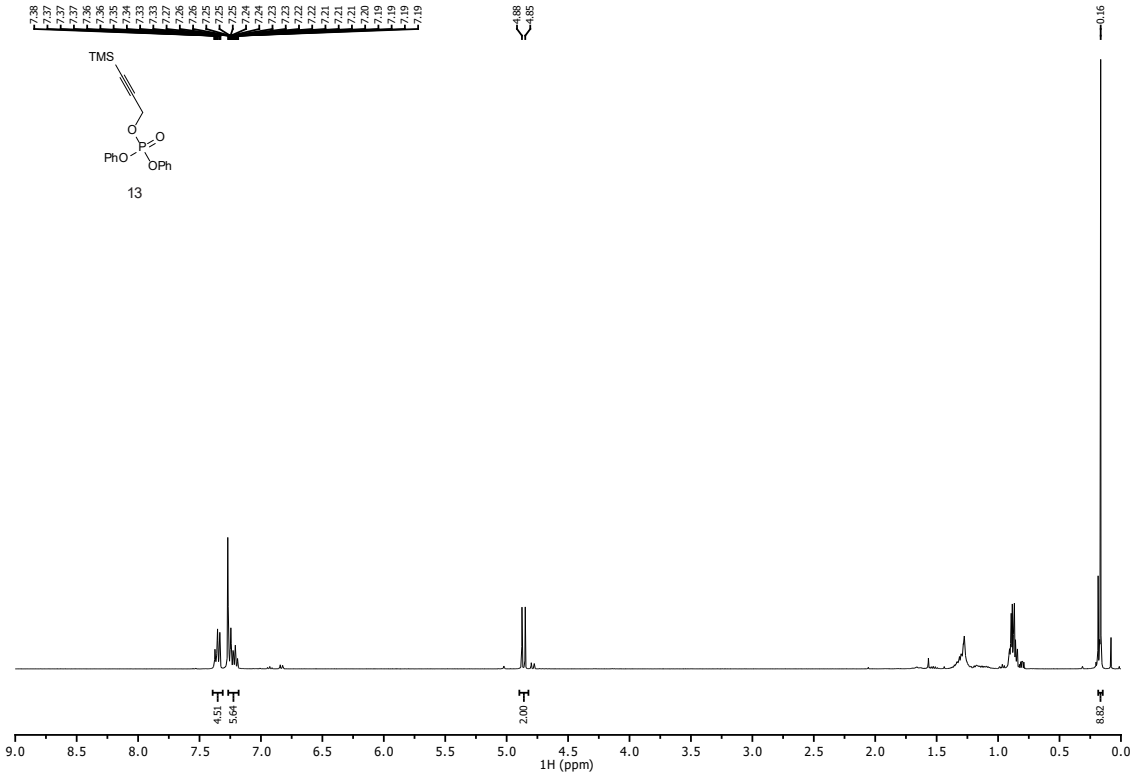
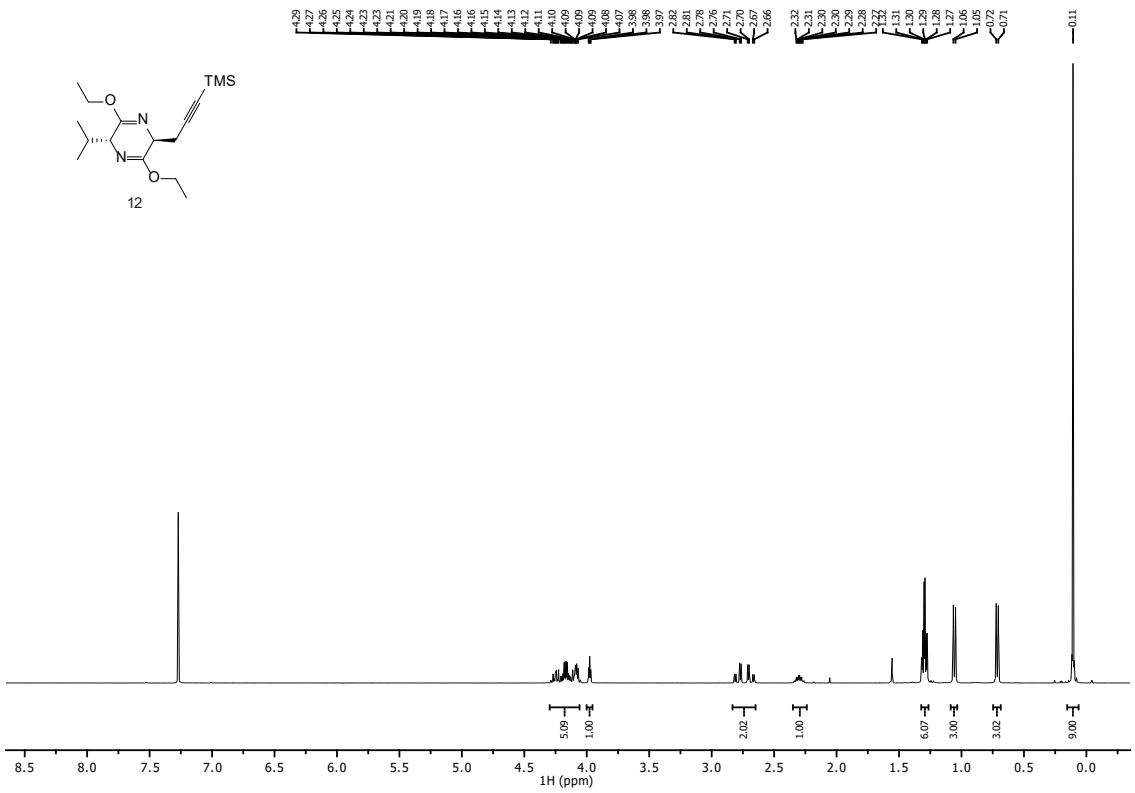
7.36
4.24
4.22
4.21
3.49
3.48
3.45
3.44
3.40
3.38
3.36
3.35
3.34
D
D
H
N
13C
F
HO
O
D
1
NH3+Cl-
0.07
0.98
0.04
1.00
1.04
1.01
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
1H (ppm)


170.20
158.88
156.56
126.18
52.97
26.04
D
D
H
N
13C
F
HO
O
D
1
NH3+Cl-
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
1H (ppm)


TMS
O
P
O
PhO
OPh
13
1H (ppm)


TMS
O
N
N
O
12
1H (ppm)

## 2. Expression of [$^{13}C$, $^{2}H_3$]-$^{19}F$ Trp CA2 and SOD1

Plasmids containing the coding sequences for carbonic anhydrase 2 (CA2, NP_000058.1) and superoxide dismutase 1 (SOD1, NP_000445.1) were previously obtained from the pHLsec vector by removal of its secretion sequence.[6] The plasmids were introduced in HEK293T cells (ATCC CTRL-3216) by transient transfection.[7] Cells were cultured in T75 flasks in Dulbecco's modified eagle medium (DMEM, Gibco). HEK293T were transfected with a mixture of 50 µg of polyethylenimine (Sigma Aldrich) and 25 µg of plasmid (2:1 ratio) after reaching 90-95% confluence. The transfected cells were cultured in DMEM with 2% v/v of fetal bovine serum (Life Technologies), 2 mM of glutamine (Life Technologies) and 100 µg/mL of penicillin/streptomycin (Life Technologies), in a sterile incubator at 37°C, 5% $CO_2$. After 24 h of incubation, a medium switch was performed with a custom-made DMEM, prepared according to the recipe of commercial DMEM but lacking L-tryptophan, and to which [$^{13}C$, $^{2}H_3$]-$^{19}F$ Trp was added at a final concentration of 80 µM. The transfected cells were harvested after 48 h of protein expression, resuspended in 150 µL of phosphate saline buffer (Life Technology), lysed by freezing and thawing cycles in liquid nitrogen, and centrifuged at 16000 g for 1 h at 4°C. The supernatant containing the soluble components was collected, supplemented with 10% $D_2O$, and transferred into a 3 mm NMR tube.

## 3. Purification of [$^{13}C$, $^{2}H_3$]-$^{19}F$ Trp CA2

[$^{13}C$, $^{2}H_3$]-$^{19}F$ Trp CA2 was purified by affinity chromatography following an existing strategy.[8] HEK293T cells were used for protein expression as described above. The soluble lysates from three T75 flasks of transfected cells were collected, pooled, and diluted in 3 mL of binding buffer (20 mM Tris buffer, pH 8). The solution was then loaded onto a 1 mL NiNTA column, previously equilibrated with binding buffer. After three washes with 1 mL of binding buffer, the protein was eluted in 3 mL steps of buffer with increasing concentration of imidazole. An SDS-PAGE of the solution samples confirmed that CA2 was eluted at high purity with 20 mM of imidazole. The buffer of the purified $^{13}C$-$^{19}F$ Trp CA2 was exchanged to 500 µL of 10 mM HEPES pH 6.8. The protein sample was supplemented with 10% $D_2O$, reaching a final concentration of 180 µM, and transferred in a 5 mm NMR tube.

## 4. NMR experiments

$^{13}C$-$^{19}F$ TROSY NMR spectra were recorded on a 14.1 T (600 MHz $^{1}H$, 564.6 MHz $^{19}F$) Bruker Avance NEO spectrometer equipped with a QCI-F cryoprobe. The $^{13}C$-start, $^{19}F$-detect TROSY pulse program from Boeszoermenyi et al. was employed.[9] Lysate samples were analyzed at 310 K with a series of ~3.75 h-long spectra recorded with 16 scans and 512 increments each, acquisition times of 69 ms ($^{19}F$) and 68 ms ($^{13}C$), and an interscan delay of 1.5 s. The spectra were processed in TopSpin 4.4.0 (Bruker) with sine squared window function and inspected to exclude protein degradation occurring over time. The first eight spectra were summed together. Purified [$^{13}C$, $^{2}H_3$]-$^{19}F$ Trp CA2 was analyzed at 298 K. A ~9.75 h-long $^{13}C$-$^{19}F$ TROSY spectrum was recorded with 32 scans and 512 increments, acquisition times of 69 ms ($^{19}F$) and 68 ms ($^{13}C$), and an interscan delay of 2 s, and processed as above. The chemical shifts were calibrated at each temperature with an external reference, trifluoroacetic acid for $^{19}F$ (set at -76.55 ppm), DSS for $^{13}C$ (indirectly referenced from $^{1}H$).

## 5. Supplementary figures

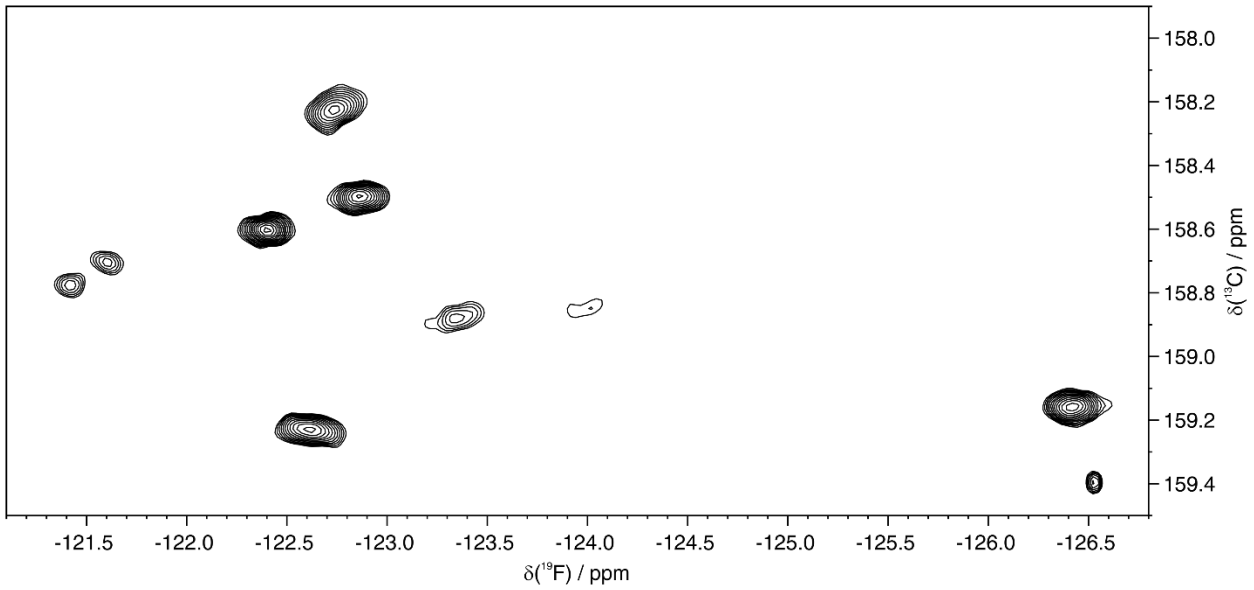



**Figure S1**: $^{13}C$-$^{19}F$ TROSY spectrum of purified [$^{13}C$, $^{2}H_3$]-$^{19}F$ Trp CA2. The signal splitting at -121.5 ppm is attributed to incomplete fluorotryptophan incorporation. The sharp signal at -126.5 ppm for $^{19}F$ and 159.4 ppm for $^{13}C$ is likely due to residual free fluorotryptophan that was initially bound to the protein and subsequently released into the solution.

## 6. References


1) R. J. Lichtenecker*, Org. Biomol. Chem*., 2014, **12**, 7551.
2) T. Fujimoto T. Ritter*, Org. Lett.,* 2015, **17**, 3, 544.
3) G. Toscano, J. Holzinger, B. Nagl, G. Kontaxis, H. Kählig, R. Konrat, R. J. Lichtenecker, *J Biomol* NMR, 2024, https://doi.org/10.1007/s10858-024-00440-z.
4) L. Gurskaya, L. Politanskaya, J. Wang, P. Ilyina, A. Volobueva, V. Zarubaev, *J. Fluor. Chem.,* 2024, **274**, 110240.
5) C. Ma, X. Liu, X. Li, J. Filippen-Anderson, S. Yu, J. M. Cook, *J. Org. Chem*, 2001, **66**, 4525.
6) a) L. Banci, L. Barbieri, I. Bertini, E. Luchinat, E. Secci, Y. Zhao, A. R. Aricescu, *Nat. Chem. Biol.*, 2013, **9**, 297; b) E. Luchinat, L. Barbieri, M. Cremonini, A. Nocentini, C. T. Supuran, L. Banci, *Angew. Chem. Int. Ed.*, 2020, **59**, 16, 6535.
7) L. Barbieri, E. Luchinat, L. Banci, *Nat. Protoc.*, 2016, **11**, 1001.
8) A. L. Banerjee, M. Swanson, S. Mallik, D. K. Srivastava, *Protein Expr. Purif.*, 2004, **37**, 2, 450.
9) A. Boeszoermenyi, S. Chhabra, A. Dubey, D. L. Radeva, N. T. Burdzhiev, C. D. Chanev, O. I. Petrov, V. M. Gelev, M. Zhang, C. Anklin, H. Kovacs, G. Wagner, I. Kuprov, K. Takeuchi, H. Arthanari, *Nature Methods*, 2019, **16**, 333.