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Supporting Information for

Chemoselective ^{18}F-incorporation into pyridyl acyltrifluoroborates for rapid radiolabelling of peptides and proteins at room temperature

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Table of Contents

2. Materials and Methods

2A. General Synthetic Methods

Unless otherwise stated, all reactions were carried out in oven-dried glassware sealed with rubber septa under an atmosphere of dry N_2 and were stirred with Teflon-coated magnetic stir bars. Thin layer chromatography (TLC) was performed on Merck TLC plates (0.25 mm) pre-coated with silica gel 60 F254 and visualised by UV quenching and staining with $KMnO_4$ or I_2. Flash column chromatography was performed under a forced-flow of air using Silicycle SiliaFlash F60 (40–63 μm particle size).

2B. Solvents, Reagents and Chemicals

All organic solvents (EtOH, MeOH, DMF, THF, CH_2Cl_2, EtOAc, hexanes, CH_3CN, DMSO) were used as supplied (ACS or HPLC grade) unless otherwise noted. THF was purified by distillation from sodium benzophenone ketyl prior to use. CH_2Cl_2 and Et_3N were purified by distillation from CaH_2. Anhydrous DMF and toluene were obtained by passage through two columns of anhydrous neutral A-2 alumina under an atmosphere of N_2 and then stored over activated 4 Å molecular sieves. MeOH and 1,4-dioxane were dried over activated 3 Å and 4 Å molecular sieves, respectively, and stored under an inert atmosphere of N_2. Anhydrous toluene was obtained from INERT PureSolv MD5solvent purification system under nitrogen (H_2O content < 10 ppm, Karl–Fischer titration). Deuterated solvents (D, 99.9%) were purchased from Cambridge Isotope Laboratories, Inc., except for $CDCl_3$ (D, 99.8%), which was obtained from Armar Chemicals. All reagents were purchased from ABCR, Acros, Combi-Blocks, Fluorochem, Fluka, Merck, Fisher-Scientific, TCI, Sigma Aldrich or STREM and used as received from the commercial suppliers, without further purification, unless stated otherwise. (Ethylthio-trifluoroborate)-methane dimethyliminium (KAT reagent, CAS: 1622923-36-7) was synthesised according to a previously established procedure by the Bode group.[1] T4 polynucleotide kinase, Phusion® High-Fidelity DNA Polymerase and DpnI were obtained from New England Biolabs (MA, USA). Oligonucleotides were synthesised by Microsynth AG (Balgach, Switzerland). Ampicillin sodium salt and lysozyme (22500 U/mg) were obtained from Axon Lab AG (Baden, Switzerland). DNase I was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Ni(II) – NTA Agarose was obtained from Qiagen GmbH (Hilden, Germany).

2C. LC-MS Analyses and HPLC

LC-MS analysis was performed on a Dionex UltiMate 3000 RSLC connected to a Surveyor MSQ Plus mass spectrometer; a reversed–phase RESTEK Pinnacle II C18 (4.6 x 50 mm) column was used, running a gradient of 5 to 100% CH_3CN in H_2O over 6.5 minutes and 100% CH_3CN for 2.5 minutes for analysis of small molecules. Peptides were purified by high performance liquid chromatography (HPLC)

on Jasco analytical and preparative instruments with dual pumps, mixer and degasser, a variable wavelength UV detector and a Rheodyne 7725i injector fitted with a 20 to 1000 µL sample loop. The mobile phase for analytical and preparative HPLC were Millipore-H_2O with 0.1% TFA (Buffer A) and HPLC grade CH_3CN with 0.1% TFA (Buffer B). The eluent was monitored simultaneously at 220 nm, 254 nm and 301 nm. Flow rates for analytical (4.6 x 250 mm) and preparative (20 x 250 mm) HPLC were 1 mL and 10 mL per minute, respectively.

2D. Mass Spectrometry

High-resolution mass spectra were obtained by the mass spectrometry service of the ETH Zürich Laboratorium für Organische Chemie on a Varian IonSpec FT-ICR (ESI), a Bruker Daltonics maXis ESI-QTOF spectrometer (ESI), a Bruker Daltonics SOLARIX spectrometer (MALDI), or a Bruker Daltonics UltraFlex II spectrometer (MALDI- TOF). For ESI (+MS) an enhanced quadratic calibration mode was used with the following reference mass peaks: 118.0863, 322.0481, 622.0290, 922.0098, 1221.9906, 1521.9715, 1821.9523, 2121.9332, 2421.9140, 2721.8948.

2E. NMR Spectroscopy

^{1}H NMR spectroscopy was performed on a Bruker AV-300, Bruker AV-400, Bruker AV-III-500, Bruker DRX-II-500or Bruker AV-III600 (600 MHz with cryoprobe) spectrometer and the recorded spectra were referenced to the residual solvent signal as internal standard (acetone-d_6: 2.05 ppm; $CDCl_3$: 7.26 ppm; DMSO-d_6: 2.50 ppm). ^{13}C NMR spectroscopy was performed on a Bruker DRX500 (126 MHz) or Bruker Avance III 600 (150 MHz) spectrometer and the recorded spectra were referenced to the solvent (acetone-d_6: 29.84 ppm; $CDCl_3$: 77.16 ppm; DMSO-d_6: 39.52 ppm).^{19}F NMR spectroscopy was performed on a Bruker DRX500 (470 MHz) spectrometer and the recorded spectra were referenced to an external standard of trifluoroacetic acid (–76.53 ppm). ^{11}B NMR spectra were recorded on a Bruker DRX500 (160 MHz) spectrometer and referenced to an external sample of $BF_3{\cdot}OEt_2$ (0 ppm). All measurements were carried out at ambient temperature (22 °C) and chemicals shifts are reported in ppm (δ). The data is reported as follows: chemical shift in ppm, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, sext = sextet, m = multiplet, dd = doublet of doublet, dt = doublet of triplet, brs = broad singlet), coupling constants (*J*) in Hz, and integration. In ^{19}F NMR and ^{11}B NMR, trifluoroborate multiplets are reported as the average of the observed signals. Service measurements were performed by the NMR service team of the Laboratorium für Organische Chemie at ETH Zürich.

2F. IR Spectroscopy

IR spectra were recorded on a Perkin Elmer Two FT-IR (UATR) as thin films. Absorption bands are given in wavenumbers (cm^{-1}).

2G. Melting points

Melting points were measured using a Büchi B-540 melting point apparatus using open glass capillaries and are uncorrected.

2H. UV-Vis Spectroscopy – Protein quantification

OD_{600} and protein concentration measurements were executed using a NanoDrop 2000c UV-Vis spectrophotometer. Protein concentrations for quantification after expression and purification were determined by the absorption at 280 nm using extinction coefficients calculated by ProtoParam (http://expasy.org/tools/protparam.html) based on the amino acid sequence of the protein.

2I. Fast Protein Liquid Chromatography (FPLC)

Protein purifications were performed on an ÄKTA pure chromatography system (GE Healthcare) using the UNICORN 6.3 Workstation system control software. All purifications were carried out at 4 °C. All buffers were freshly prepared, filtered and degassed immediately prior to use. Anion exchange chromatography purifications were performed using the strong ion exchange Mono Q 5/50 GL column (GE Healthcare). All protein purifications were monitored at wavelengths of 254 nm, 280 nm and 490 nm.

2J. Solid phase peptide synthesis (SPPS)

All peptide segments were synthesised on a CS Bio 136X peptide synthesiser using Fmoc SPPS chemistry. An inline UV detector was used for monitoring Fmoc deprotection. The following Fmoc amino acids with side-chain protection groups were used: Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH, and Fmoc-Val-OH. Fmoc deprotections were performed with 20% piperidine in DMF (2 × 8 minutes) and monitored by UV at 304 nm with a feedback loop to ensure complete Fmoc removal. Couplings were performed with Fmoc-amino acid (4.0 equiv to resin substitution), HCTU (3.9 equiv) and *i*Pr_2NEt (6.0 equiv) in DMF. After pre-activating for 3 minutes, the solution was transferred and allowed to react with the peptide on-resin for either 45 minutes or 75 minutes depending on the amino acid. After coupling, the resin was treated with 20% acetic anhydride in DMF for capping any unreacted free amine.

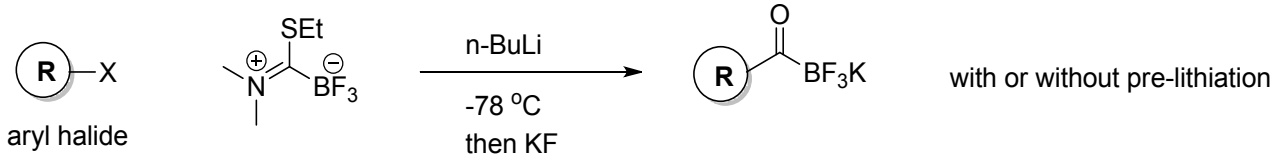
2K. Radio-HPLC

For reaction monitoring during evaluation of the different KAT precursors and determination of ^{18}F incorporation, a Waters Ultra-performance liquid chromatography (UPLC) system was used with an Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 µm, Waters) and an attached coincidence detector (FlowStar LBS13, Berthold). The mobile phase consisted of the following system (**system A**): aq NH_4HCO_3 10mM (solvent A), CH_3CN (solvent B); flow 0.6 mL/min; 0–0.5 min: 5% B, 0.5–2.5 min: 5–60% B, 2.5 – 2.8 min: 60% B, 2.8 – 2.9 min: 60 – 5% B, 2.9 – 3.0 min: 5% B; UV = 280/254 nm. Semi-preparative purification of radiolabelled material (**2d**) was performed on a Merck-Hitachi L6200A system equipped with Knauer variable wavelength detector and an Eberline radiation detector using a reverse phase column (Gemini, C18, 10 × 250 mm, 5 µm) using solvent **system B**: aq. KOAc 10 mM, adjusted to pH 4 by addition of AcOH (solvent A), CH_3CN (solvent B); flow 4 mL/min; 0 – 5 min: 5% B, 5 – 15 min: 5 – 27% B, 15 – 30 min: 27% B, 30 – 35 min: 27 – 40% B, 35 – 40 min 40% B; UV = 254 nm. Analytical radio-HPLC was performed on an Agilent 1100 system equipped with multi-UV wavelength and Raytest Gabi Star detectors and Gina Star software. The following systems were used; **system C**: column; Atlantis T3, 4.6×150 mm, 3 µm; solvent system; aq. NH_4OAc 3.75 mM + 0.05% AcOH (solvent A), CH_3CN (solvent B); flow 1 mL/min; 0 – 2 min: 5% B, 2 – 12 min: 5 – 60% B, 12 – 13 min: 60-5% B, 13 – 20 min: 5% B; UV = 254/280 nm; **system D**: column; LiChrospher 100RP-18, 4 mm × 250 mm, 5 µm); solvent system; H_2O (0.1% TFA) (solvent A), CH_3CN (0.1% TFA) (solvent B); flow 1 mL/min; 0 – 4 min: 5% B, 4 – 22 min: 5 – 90% B, 22 – 26 min: 90% B, 26 – 28 min: 90 – 5% B; 28 – 30 min: 5% B; UV = 254/280 nm; **system E**; column; Zorbax 300SB-C18, 4.6 × 150 mm, 5 µm; solvent system: H_2O (0.1% TFA) (solvent A), CH_3CN (0.1% TFA) (solvent B); flow 1 mL/min; 0 – 3 min: 10% B, 3 – 20 min: 10 – 95% B, 20 – 25 min: 95% B, 25 – 27 min: 95 – 10% B; 27 – 30 min: 10% B; UV = 254/280 nm. Molar activity for **[^{18}F]FPAT** (**2d**) was calculated by comparing the ultraviolet peak intensity of the final formulated product with calibration curves of the corresponding non-radioactive standard **[^{19}F]2d** of known concentrations.

3. Syntheses

3A. Syntheses of Potassium Acyltrifluoroborates (KATs) for ^{18}F-Labelling Studies

Scheme S1: Synthesis of radiolabelling precursors **1a-d** and reference compounds **[^{19}F]2a-d**



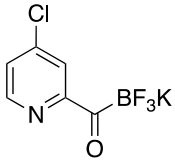
Precursors potassium trifluoro(4-nitrobenzoyl)borate (**1a**), potassium (6-bromopicolinoyl)trifluoroborate (**1b**) potassium (6-bromonicotinoyl)trifluoroborate (**1d**)[1] and reference compound potassium trifluoro(4-fluorobenzoyl)borate[2] **[^{19}F]2a** were synthesised according to previously established procedures by the Bode group.

General Procedure 1 – Synthesis of Potassium Acyltrifluroroborates (KATs) without Pre-Lithiation
Potassium acyltrifluoroborates (KATs) were synthesised according to a slight modification of a previously reported procedure by the Bode group.[1] A flame-dried, 10 mL round-bottom flask equipped with magnetic stir bar and septum under inert nitrogen atmosphere, was charged with pyridyl bromide (1.08 mmol, 1.00 equiv), (ethylthio-trifluoroborate)-methane dimethyliminium (200 mg, 1.08 mmol, 1.00 equiv) and a mixture of anhydrous toluene / THF (2:1, 4 mL). The mixture was cooled to -78 °C in a dry ice/acetone bath and *n*-butyllithium (1.6 M in hexane, 675 µL, 1.08 mmol, 1.00 equiv) was added dropwise over 30 minutes via syringe pump. After the mixture was stirred for 2 h at -78 °C, the residual *n*-butyllithium was quenched with acetone (80 µL, 1.08 mmol, 1.00 equiv). 10 minutes after the addition of acetone, an aqueous KF solution (6.5 M, 0.5 mL, 3.24 mmol, 3.00 equiv) was added at -78 °C. The flask was removed from the dry ice/acetone bath and the mixture was stirred for 1 h at RT. To the resulting heterogeneous mixture CH_2Cl_2 (5 mL) was added. The mixture was filtered and washed with additional CH_2Cl_2 (3 × 20 mL) and acetone (2 × 10 mL). The remaining filter cake was washed with DMF (50 mL) until the filtrate was colourless. The DMF was removed under reduced pressure at 50 – 60 °C to yield the desired KAT as a colourless or yellow solid.

General Procedure 2 – Synthesis of Potassium Acyltrifluroroborates (KATs) via Pre-Lithiation
Potassium acyltrifluoroborates (KATs) were synthesised according to a slight modification of a previously reported procedure by the Bode group.[1] A flame-dried 10 mL round-bottom flask equipped with magnetic stir bar and septum under inert nitrogen atmosphere, was charged with pyridyl bromide (1.08 mmol, 1.00

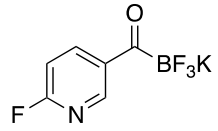
equiv) and a mixture of anhydrous toluene/THF (4:1, 4 mL). The mixture was cooled to -78 °C in a dry ice/acetone bath and *n*-butyllithium (1.6 M in hexane, 675 µL, 1.08 mmol, 1.00 equiv) was added dropwise over 30 minutes via syringe pump. After the mixture was stirred for 1 h at -78 °C, a solution of (ethylthio-trifluoroborate)-methane dimethyliminium (200 mg, 1.08 mmol, 1.00 equiv) in anhydrous THF (1.0 mL) was added dropwise over 30 minutes via syringe pump. After the mixture was stirred for 2 h at -78 °C, the residual *n*-butyllithium was quenched with acetone (80 µL, 1.08 mmol, 1.00 equiv). 10 minutes after the addition of acetone, an aqueous KF solution (6.5 M, 0.5 mL, 3.24 mmol, 3.00 equiv) was added at -78 °C. The flask was removed from the dry ice/acetone bath and the mixture was stirred for 1 h at RT. To the resulting heterogeneous mixture CH_2Cl_2 (5 mL) was added. The mixture was filtered and washed with additional CH_2Cl_2 (3 × 20 mL) and acetone (2 × 10 mL). The remaining filter cake was washed with DMF (50 mL) until the filtrate was colourless. The DMF was removed under reduced pressure at 50 – 60 °C to yield the desired KAT as a colourless or yellow solid.

Potassium (4-chloropicolinoyl)trifluoroborate 1c



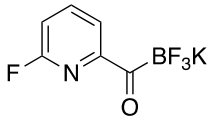
Prepared from 2-bromo-4-chloropyridine (208 mg) by General Procedure 1 and isolated as yellow solid (182 mg, 0.73 mmol, 68%). **M.p.** decomposition >175 °C. **IR** (neat, cm^{-1}) λ_{max} = 2988, 2902, 1658, 1567, 1551, 1227, 1075, 1037, 1000, 917, 837, 747, 697, 686, 628. **^{1}H NMR** (600 MHz, DMSO-d_6): δ 8.60 (dd, *J* = 5.2, 0.6 Hz, 1H), 7.85 (d, *J* = 2.0 Hz, 1H), 7.58 (dd, *J* = 5.2, 2.2 Hz, 1H). **^{13}C NMR** (151 MHz, DMSO-d_6): δ 234.3 − 230.0 (m), 159.1 (brs), 150.7, 143.3, 124.9, 122.8 (q, *J* = 2.9 Hz). **^{19}F NMR** (470 MHz, DMSO-d_6) δ -142.9 (dd, *J* = 55.5, 36.5 Hz). **^{11}B NMR** (160 MHz, DMSO-d_6) δ -1.28 (q, *J* = 47.9, 45.7 Hz). **HRMS**(ESI) calc'd for $C_6H_3BClF_3NO$ $[M - K]^-$ 207.9955, found 207.9956.

Potassium trifluoro(6-fluoronicotinoyl)borate [^{19}F]2d



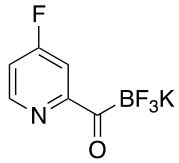
Prepared from 5-bromo-2-fluoropyridine (190 mg) by General Procedure 1 and isolated as pale-yellow solid (185 mg, 0.80 mmol, 74%). **M.p.** decomposition >190 °C. **IR** (neat, cm^{-1}) λ_{max} = 3173 (br), 1633, 1579, 1478, 1373, 1315, 1255, 1227, 1082, 1009, 991, 886, 845, 752, 692, 615. **^{1}H NMR** (600 MHz, acetone-d_6) δ 8.90 (d, *J* = 2.3 Hz, 1H), 8.42 (td, *J* = 8.4, 2.3 Hz, 1H), 7.06 (ddd, *J* = 8.4, 2.7, 0.7 Hz, 1H).**^{13}C NMR** (151 MHz, acetone-d_6) δ 233.9 – 231.2 (m), 166.1, 164.5, 150.5 (dq, *J* = 16.1, 3.1 Hz), 141.7 (d, *J* = 7.0 Hz), 135.8, 109.5 (d, *J* = 37.8 Hz). **^{19}F NMR** (470 MHz, acetone-d_6) δ -67.0 (d, 8.2 Hz, 1H), -145.7 (dd, *J* = 100.6, 49.4 Hz, 3H). **^{11}B NMR** (160 MHz, acetone-d_6) δ -1.01 (q, *J* = 50.6 Hz). **HRMS**(ESI) calc'd for $C_6H_3BF_4NO$ $[M - K]^-$ 192.0250, found 192.0254.

Potassium trifluoro(6-fluoropicolinoyl)borate [^{19}F]2b



Prepared from 2-bromo-6-fluoropyridine (190 mg) by General Procedure 2 and isolated as yellow solid (180 mg, 0.78 mmol, 72%). **M.p.** decomposition >180 °C. **IR** (neat, cm^{-1}) λ_{max} = 1660, 1595, 1570, 1449, 1308, 1270, 1259, 1106, 1034, 990, 955, 844, 819, 741, 711, 633. **^{1}H NMR** (600 MHz, DMSO-*d*$_6$) δ 8.07 (td, *J* = 8.3, 7.3 Hz, 1H), 7.89 (dd, *J* = 7.3, 2.8 Hz, 1H), 7.21 (ddd, *J* = 8.1, 3.1, 0.7 Hz, 1H). **^{13}C NMR** (151 MHz, DMSO-*d*$_6$) δ 232.8 – 229.7 (m), 162.5 (d, *J* = 236.0 Hz), 155.8 (brs), 142.5 (d, *J* = 7.3 Hz), 121.7 (p, *J* = 3.0 Hz), 111.1 (d, *J* = 38.7 Hz). **^{19}F NMR** (470 MHz, DMSO-*d*$_6$): δ -67.4 (s, 1F), -142.9 (dd, *J* = 98.7, 45.1 Hz, 3F). **^{11}B NMR** (160 MHz, DMSO-*d*$_6$): δ -1.31 (q, *J* = 51.1, 50.3 Hz). **HRMS**(ESI) calc'd for $C_6H_3BF_4NO$ [M − K]$^-$ 192.0250, found 192.0245.

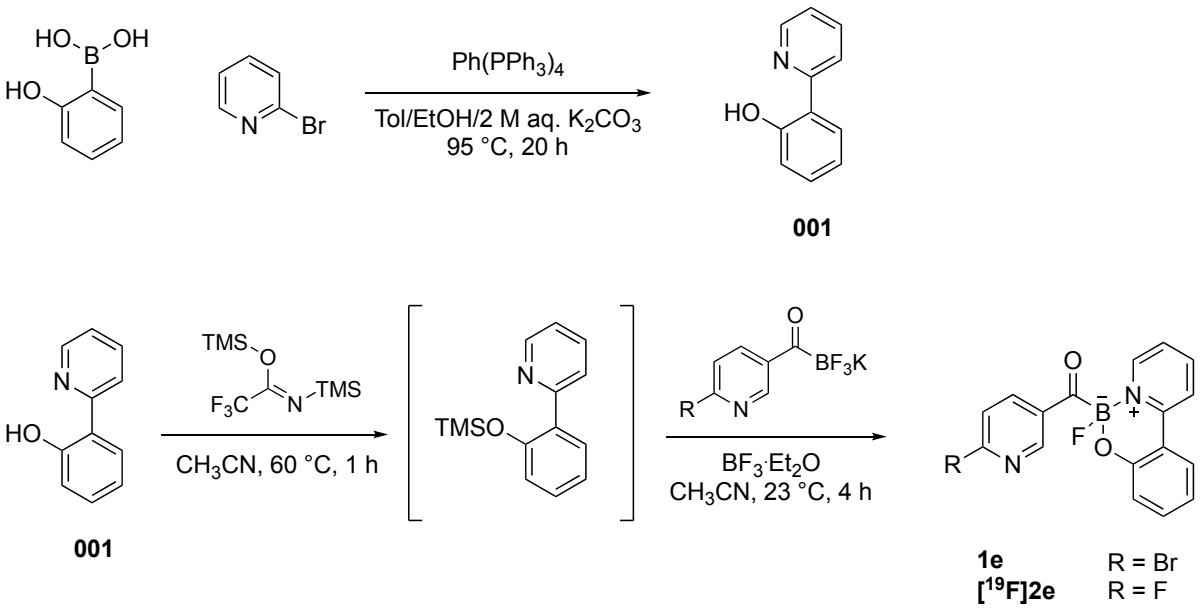
Potassium trifluoro(4-fluoropicolinoyl)borate [^{19}F]2c



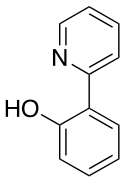
Prepared from 2-bromo-4-fluoropyridine (190 mg) by a slight modification of General Procedure 2. After General Procedure 2, the isolated pale-yellow solid was resuspended in THF and a solution of concentrated aqueous KHF_2 (0.5 mL) was added. The mixture was stirred for 2 h at RT. The solvents were removed under reduced pressure and the H_2O residue was lyophilised. The crude material was washed with DMF (3 × 10 mL). The combined DMF filtrates were evaporated to dryness under reduced pressure at 50 – 60 °C to yield the desired product as yellow solid (130 mg, 0.56 mmol, 52%). **M.p.** decomposition >180 °C. **IR** (neat, cm^{-1}) λ_{max} = 1662, 1577, 1178, 1033, 997, 953, 822, 752, 630. **^{1}H NMR** (500 MHz, DMSO-*d*$_6$) δ 8.64 (dd, *J* = 8.8, 5.5 Hz, 1H), 7.58 (dd, *J* = 10.1, 2.6 Hz, 1H), 7.34 (ddd, *J* = 8.8, 5.5, 2.7 Hz, 1H). **^{13}C NMR** (151 MHz, DMSO-*d*$_6$) δ 234.0 − 230.2 (m), 168.5 (d, *J* = 260.2 Hz), 161.4, 151.8 (d, *J* = 5.9 Hz), 112.4 (d, *J* = 16.0 Hz), 109.8 (dq, *J* = 15.8, 2.8 Hz). **^{19}F NMR** (470 MHz, DMSO-*d*$_6$) δ -103.8 (s, 1F), -142.8 (dd, *J* = 53.8, 44.6 Hz, 3F). **^{11}B NMR** (160 MHz, DMSO-*d*$_6$) δ -1.29 (q, *J* = 51.1, 50.5 Hz). **HRMS**(ESI) calc'd for $C_6H_3BF_4NO$ [M − K]$^-$ 192.0250, found 192.0253.

3B. Synthesis of Monofluoroborates for ^{18}F-Labelling Studies

Scheme S2: Synthesis of radiolabelling precursor **1e** and reference compound **[^{19}F]2e**



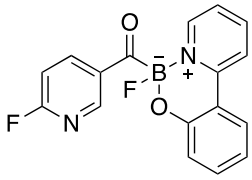
2-(2-hydroxyphenyl)pyridine (001)



A Schlenk flask equipped with a stir bar was evacuated, degassed with N_2 and charged with 2-bromopyridine (706 mg, 4.47 mmol, 1.0 equiv), $Pd(PPh_3)_4$ (103 mg, 0.09 mmol, 0.02 equiv), toluene (20 mL) and an aqueous 2 M solution of potassium carbonate (4.45 mL, 8.94 mmol, 2.0 equiv). The solution was stirred at 23 °C for 15 minutes before addition of 2-hydroxyphenylboronic acid (0.817 g, 6.71 mmol, 1.5 equiv) in EtOH (11 mL). The mixture was heated to 85 °C for 20 h. After cooling to RT, H_2O was added and the product was extracted using EtOAc (3 × 100 mL). The combined organic layers were washed with brine (50 mL) and H_2O (50 mL) and filtered over a short plug of Celite. The filtrate was dried over $MgSO_4$ and the volatiles were removed under reduced pressure. The crude product was purified by column chromatography (EtOAc/hexanes 1:10) to afford the desired product as a yellow solid (630 mg, 3.68 mmol) in 82% yield. The spectral data of **001** was in agreement with that reported in the literature.[1] **^{1}H NMR** (300 MHz, $CDCl_3$) δ 14.48 (s, 1H), 8.44 (ddd, *J* = 5.0, 1.8, 1.0 Hz, 1H), 7.90 – 7.69 (m, 3H), 7.32 (ddd, *J* = 8.2, 7.2, 1.7 Hz, 1H), 7.17 (ddd, *J* = 7.4, 5.0, 1.2 Hz, 1H), 7.07 (dd, *J* = 8.3, 1.3 Hz, 1H), 6.91 (ddd, *J* = 8.0, 7.2, 1.3 Hz, 1H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 160.11, 157.96, 145.92, 137.86, 131.59, 126.21, 121.60, 119.14, 118.90, 118.87, 118.71.

Having synthesised **001**, precursor **1e** was prepared according to published procedures.[3]

2-fluoro-5-monofluoroboronate [^{19}F]2e



To a flame-dried 10 mL Schlenk flask equipped with a magnetic stirbar was added 2-(2-hydroxyphenyl)pyridine **001** (171 mg, 1.00 mmol, 1.0 equiv). Anhydrous CH_3CN (0.68 mL) and *N,O*-bis(trimethylsilyl)trifluoroacetamide (320 µL, 1.20 mmol, 1.2 equiv) were added and the mixture was stirred for 1 h at 60 °C. After cooling to RT, all volatiles were removed carefully under vacuum and the residual pale-yellow oil was further dried for 2 h at 60 °C to give the TMS-capped ligand, which was used in the next step without further purification. A flame-dried 50 mL Schlenk flask equipped with a magnetic stir bar was charged with **[^{19}F]2d** (1.00 mmol, 1.0 equiv) synthesised above. A solution of the TMS-capped ligand in anhydrous CH_3CN (6.0 + 2.0 + 2.0 mL) was added. To this suspension $BF_3{\cdot}OEt_2$ (123 µL, 1.00 mmol, 1.0 equiv) was added dropwise. The mixture became homogeneous and was stirred for 4 h at 23 °C. The reaction mixture was carefully evaporated (temperature of water bath less than 30 °C). The crude material was directly purified by silica gel column chromatography (EtOAc/hexanes 1:5) to afford the desired product as a yellow solid (156 mg, 0.48 mmol, 48%). R_f 0.32 (EtOAc/hexanes 1:1). **M.p.** 177.6 – 178.2 °C. **IR** (neat, cm^{-1}) λ_{max} = 1639, 1622, 1607, 1578, 1560, 1500, 1482, 1456, 1433, 1367, 1292, 1228, 1170, 1140, 1112, 1075, 1054, 1018, 961, 843, 788, 756, 740, 620. **^{1}H NMR** (500 MHz, $CDCl_3$) δ 9.14 (d, *J* = 1.6 Hz, 1H), 8.50 (dd, *J* = 5.9, 0.9 Hz, 0H), 8.47 (td, *J* = 8.2, 2.4 Hz, 1H), 8.21 (ddd, *J* = 8.4, 7.5, 1.7 Hz, 1H), 8.11 (d, *J* = 8.6 Hz, 1H), 7.76 (dd, *J* = 8.1, 1.6 Hz, 1H), 7.60 (ddd, *J* = 7.3, 5.9, 1.3 Hz, 1H), 7.39 (ddd, *J* = 8.3, 7.2, 1.6 Hz, 1H), 7.04 (dd, *J* = 8.5, 0.9 Hz, 1H), 7.00 – 6.96 (m, 2H). **^{13}C NMR** (151 MHz, $CDCl_3$) δ 227.5 – 225.1 (m), 165.0 (d, *J* = 243.9 Hz), 154.7 (d, *J* = 5.2 Hz), 150.3 (dd, *J* = 15.9, 3.5 Hz), 149.8, 142.5, 142.4 (d, *J* = 6.3 Hz), 141.0 (d, *J* = 9.0 Hz), 134.8, 133.3, 125.4, 122.9, 120.9, 120.3, 120.3, 116.4, 109.3 (d, *J* = 37.2 Hz). **^{19}F NMR** (470 MHz, $CDCl_3$) δ -62.6 (dd, *J* = 7.8, 2.9 Hz), -159.6 (d, *J* = 70.6 Hz). **^{11}B NMR** (160 MHz, $CDCl_3$) δ 1.84 (d, *J* = 48.1 Hz). **HRMS** (ESI) calc'd for $C_{17}H_{11}BF_2N_2NaO_2$ $[M + Na]^+$ 347.0777, found 347.0783.

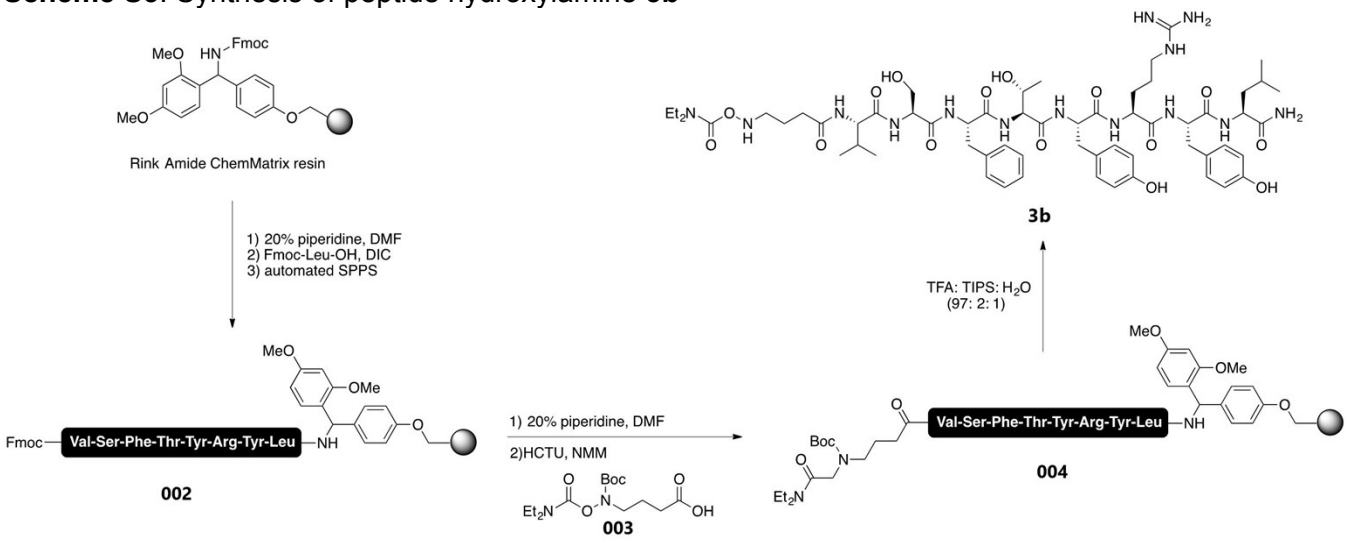
3C. Synthesis of Hydroxylamines 3a-c for Ligation Studies with [^{18}F]FPAT

Model hydroxylamine 3a

N-ethyl-N-(((phenethylamino)oxy)carbonyl)ethanamine **3a** was synthesised according to published procedures.[1]

Peptide hydroxylamine 3b

Scheme S3: Synthesis of peptide hydroxylamine **3b**



Peptide hydroxylamine **3b** was synthesised on Rink Amide ChemMatrix resin with a loading capacity of 0.44 mmol/g. The synthesis was performed on a 0.5 mmol scale (1.14 g of resin) with an initial Fmoc-deprotection using 20% piperidine in DMF following the SPPS procedure described in the General Methods section. This was followed with the treatment of Fmoc-Leu-OH (1.77g, 5.00 mmol, 10.0 equiv to resin) and *N,N'*-diisopropylcarbodiimide (0.39 mL, 2.5 mmol, 5.0 equiv to resin) in CH_2Cl_2/DMF followed by agitation at RT for 36 h. The synthesis was continued through the use of automated Fmoc-SPPS up to Val, using the procedure described in the general methods section. At this point, **002** was liberated of its *N*-terminal Fmoc group with 20% piperidine in DMF and subsequently coupled to *N*-Boc protected carboxylic acid hydroxylamine **003**[1] (636 mg, 2.0 mmol, 4.0 equiv to resin) using HCTU (828 mg, 2.0 mmol, 4.0 equiv to resin) and NMM (440 µL, 4.0 mmol, 8.0 equiv to resin) in DMF for 3 h yielding **004**. The resin supporting peptide **004** was treated with TFA:TIPS:H_2O (97:2:1) for 3 h and filtered to remove the solid support. Volatiles from the filtrate were subsequently removed under vacuum. The residue was triturated with methyl *tert*-butyl ether (MTBE), centrifuged and the solvent carefully decanted (repeated 3 cycles) to obtain the crude peptide. Purification was performed by preparative HPLC using Shiseido Capcell Pak C18 column (20 × 250 mm) eluting with 10% CH_3CN (0.1% TFA) for 10 minutes,

followed by a gradient elution of 10 to 90 % CH_3CN (0.1% TFA) in 20 minutes. The product peak eluting at t_R = 22.2 minutes was collected and lyophilised to obtain 99 mg of pure **3b** (0.08 mmol, 16% for synthesis from starting resin, cleavage and purification steps). Analytical HPLC (Shiseido C18 (5 µm, 120 Å pore size, 4.6 mm I.D. x 250 mm) column with a gradient of 10 to 90% CH_3CN with 0.1% TFA in 20 minutes) and HRMS were used to confirm the purity and exact mass of the product. **HRMS** (ESI) calc'd for $C_{60}H_{91}N_{14}O_{15}$ $[M + H]^+$: 1278.6788, found: 1278.6783.

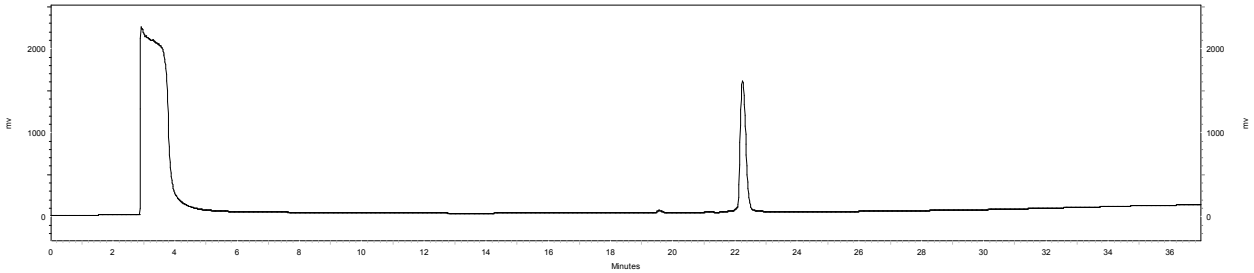


Figure F1. Analytical HPLC trace of purified **3b**

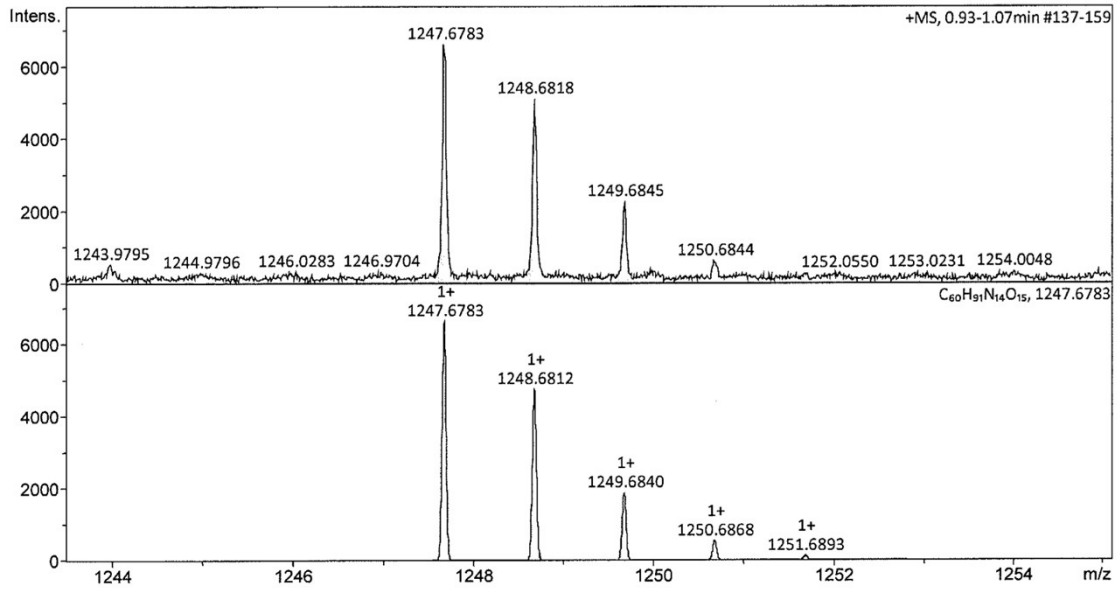
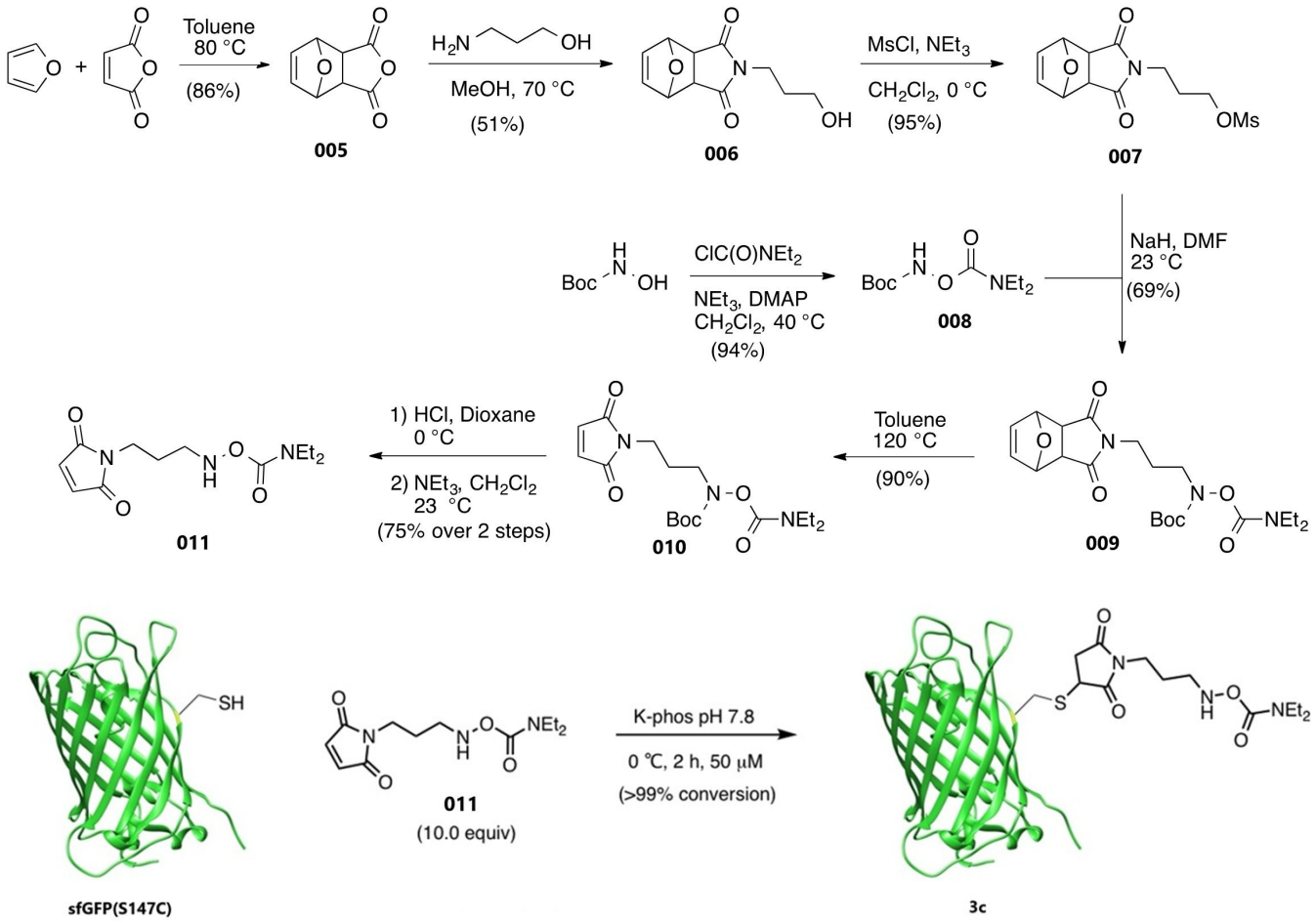


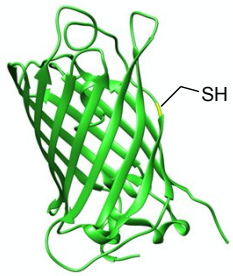
Figure F2. HRMS (ESI) of purified **3b**

Protein sfGFP(S147C) hydroxylamine 3c

Scheme S4: Synthesis of sfGFP(S147C) hydroxylamine **3c**



Protein expression and purification of sfGFP(S147C)



Molecular subcloning:

The plasmid, pBAD24-sfGFPx1 was a gift from Sankar Adhya and Fransisco Malagon[4] (Addgene plasmid #51558). Following an overnight liquid culture inoculation in lysogeny broth (LB) – Miller (10 mL) (Ampicillin 100 µg/mL), the plasmid was isolated from DH5α cells using a GeneJET Plasmid Miniprep Kit

(Thermo Fisher) following the manufacturer's instructions. Site-directed mutagenesis for the S147C mutation was carried out following the QuikChange II Site-Directed Mutagenesis protocol (Agilent). The following primers were used to for the S147C mutation[5]:
Forward: 5' – CATAAGCTGGAATACAATTTTAACTGCCACAATGTTTACATCACCGCC – 3'
Reverse: 5' – GGCGGTGATGTAAACATTGTGGCAGTTAAAATTGTATTCCAGCTTATG – 3'

A 3' – GSGHHHHHH tag was inserted using Overhang PCR with the following primers:
Forward: 5' – **TCATCACCACCAC**TGATGACCC – 3'
Reverse: 5' – **TGATGACCAGAACC**TTTGTACAGTACAGTTCATCCATACCATG – 3'

The resulting sequence of pBAD24–sfGFP(S147C)–His_6 was confirmed by DNA Sanger sequencing performed by MicroSynth AG (Balgach, Switzerland).

Protein expression and purification:
A dense overnight culture of *E. coli* strain DH10B (Thermo Fisher) transformed with pBAD24–sfGFP(S147C)–His_6 was used to inoculate 1.0 L of LB-Miller medium supplemented with 100 µg/mL of ampicillin in 2.0 L beveled Erlenmeyer flasks. The culture was incubated at 37 °C, 100 rpm until OD_{600} reached 0.6 – 0.7, at which point protein expression was induced by the addition of 0.2% (w/v) L-(+)-arabinose. After an additional 21 h of culturing, the cells were harvested by centrifugation at 5000 rpm and 4 °C for 45 minutes. The cell pellet was washed with 20 mM sodium phosphate buffer pH 7.4 (100 mL), re-suspended in 20 mM sodium phosphate, 500 mM NaCl, 35 mM imidazole, pH 7.4 (15 mL) and stored at -80 °C.
Following thawing on ice, the suspension was treated with DTT (1 mM), lysozyme (1 mg/mL) and a spatula tip of DNase I and incubated on ice for 1 h. The cells were then lysed by sonication (5 x 1 minutes at 0 °C) and the solution cleared by centrifugation at 14000 rpm and 4 °C for 30 minutes. The supernatant was filtered via passage through a 0.2 µm membrane filtered and loaded onto 4 mL of Ni(II)-NTA agarose resin that had been equilibrated with 20 mM sodium phosphate, 500 mM NaCl, 35 mM imidazole, 1 mM DTT, pH 7.4. Following washing with the same buffer (50 mL), the protein was eluted with 15 mL of the equilibration buffer containing 500 mM imidazole. The eluent containing **5** was dialyzed 3 times against 2.0 L of 20 mM Tris HCl, 1 mM DTT, pH 8.5 at 4 °C. The dialyzed protein solution was further purified by anion exchange chromatography (Mono Q 5/50 GL) with buffer A (20 mM Tris HCl, 1 mM DTT, pH 8.5) and a gradient of buffer B (20 mM Tris HCl, 1.0 M NaCl, 1 mM DTT). The purified protein **012** was concentrated and exchanged into degassed 20 mM Tris HCl, 1 mM DTT, pH 7.4 buffer through spin diafiltration (Amicon® Ultra – 4, 10 kDa MWCO) to a final concentration of 500 µM. The resulting solution containing sfGFP(S147C) may be stored for up to one month on ice at 0 °C, without appreciable,

irreversible oxidative decomposition. The expression yield was shown to be approximately 15 mg/1.0 L culture. An aliquot of sfGFP(S147C) (0.01 µmol) was passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with MilliQ H_2O, prior to mass determination (ESI).

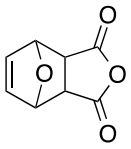
Calculated mass: 27826.1 Da (27846.1 Da for sequence -20 Da for GFP chromophore formation)
Observed mass: 27826.0 Da

Protein sequence:

MRKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICTTGKLPVPWPTLVTT
LTYGVQCFARYPDHMKQHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEGDTLVNRIELKGIDFK
EDGNILGHKLEYNFN**C**HNVYITADKQKNGIKANFKIRHNVEDGSVQLADHYQQNTPI
GDGPVLLPDNHYLSTQSVLSKDPNEKRDHMVLLEFVTAAGITHGMDELYK**GSGHHHHHH**

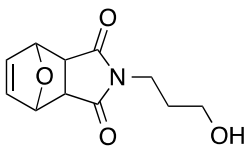
Synthesis of Maleimide-hydroxylamine (011)

3a,4,7,7a-tetrahydro-4,7-epoxyisobenzofuran-1,3-dione (005)



Compound **005** was prepared as previously described in the literature with slight modifications.[6] Maleic anhydride (40.0 g, 408 mmol) was suspended in anhydrous toluene (200 mL), which was followed by the addition of furan (30 mL, 43 mmol). The resulting mixture was stirred at 80 °C for 1 h, at which point the solution became homogeneous. The solution was then cooled to RT, and stirred for an additional 24 h. During the time, the product **002** precipitated from the reaction solution, which was subsequently isolated by vacuum filtration, washed with diethyl ether and dried *in vacuo*. This afforded **002** as a white solid (58.36 g, 86% yield). The product was found to be 96% exo by ^{1}H NMR and was used without further purification. The spectral data of **005** was in agreement with that reported in the literature.[6] Major (exo) isomer characterised: **^{1}H NMR** (400 MHz, DMSO-d_6) δ 6.58 (s, 2H), 5.35 (s, 2H), 3.31 (s, 2H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 171.5, 136.8, 81.6, 49.1.

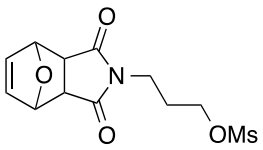
2-(3-hydroxypropyl)-3a,4,7,7a-tetrahydro-1*H*-4,7-epoxyisoindole-1,3(2*H*)-dione (006)



Compound **006** was prepared as previously described in the literature with slight modifications.[7] To a stirred suspension of **002** (10.00 g, 60.2 mmol, 1.0 equiv) in anhydrous MeOH (20 mL) was added 3-amino-1-propanol (4.5 mL, 60.5 mmol, 1.0 equiv) dropwise over 20 minutes. The resulting yellow homogeneous solution

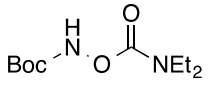
was then fitted with a reflux condenser and stirred at 70 °C for 24 h. The solution was then cooled to RT and concentrated *in vacuo* to yield an orange viscous mixture, which was then suspended in CH_2Cl_2 (300 mL) and washed with H_2O (3 x 100 mL). The combined aqueous phases were back-extracted with CH_2Cl_2 (4 × 50 mL) and the combined organic phases were dried over Na_2SO_4, filtered and concentrated under reduced pressured with 40 g of Celite. The crude material (dry-loaded onto Celite) was purified by flash chromatography, eluting with 10% MeOH in $CHCl_3$ which afforded the product **006** as a white, crystalline solid (6.88 g, 51% yield). The spectral data **006** was in agreement with that reported in the literature.[7] **^{1}H NMR** (400 MHz, $CDCl_3$) δ 6.51 (s, 2H), 5.26 (s, 2H), 3.67 – 3.59 (m, 2H), 3.54 – 3.48 (m, 2H), 2.86 (s, 2H), 1.75 (dt, *J* = 11.5, 6.1 Hz, 2H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 177.1, 136.6, 81.1, 58.7, 47.6, 35.3, 30.4.

3-(1,3-dioxo-1,3,3a,4,7,7a-hexahydro-2*H*-4,7-epoxyisoindol-2-yl)propyl methanesulfonate (007)



Compound **007** was prepared as previously described in the literature with slight modifications.[7] Alcohol **006** (3.35 g, 15.0 mmol, 1.0 equiv) was dissolved in anhydrous CH_2Cl_2 (80 mL) and subsequently cooled to 0 °C in an ice bath with stirring. Methanesulfonyl chloride (1.4 mL, 18.0 mmol, 1.2 equiv) was then added, followed by the drop-wise addition of NEt_3 (4.2 mL, 30.0 mmol, 2.0 equiv) over 30 minutes. The resulting solution was stirred at 0 °C for 30 minutes, at which point H_2O (50 mL) was added and warmed to RT. The two phases were separated and the aqueous phase was extracted with CH_2Cl_2 (3 × 50 mL). The combined organic phases were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude material was purified by flash chromatography, eluting with 10% acetone in $CHCl_3$, which afforded the product **007** as an off-white solid (4.29 g, 95% yield). The spectral data of **007** was in agreement with that reported in the literature.[7] **^{1}H NMR** (400 MHz, $CDCl_3$) δ 6.51 (t, *J* = 0.9 Hz, 2H), 5.26 (t, *J* = 1.0 Hz, 2H), 4.17 (t, *J* = 6.0 Hz, 2H), 3.63 (t, *J* = 6.6 Hz, 2H), 3.02 (s, 3H), 2.86 (s, 2H), 2.04 (p, *J* = 6.3 Hz, 2H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 176.3, 136.6, 81.1, 67.0, 47.6, 37.4, 35.4, 27.2.

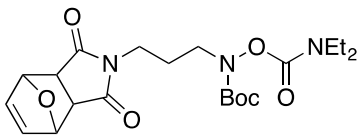
***N*-*tert*-Butoxycarbonyl-*O*-diethylcarbamoylhydroxylamine (008)**



Compound **008** was prepared as previously described in the literature with slight modifications.[8] To a solution of *N*-*tert*-butoxylcarbonylhydroxylamine (6.10 g, 45.8 mmol, 1.0 equiv) and Et_3N (6.30 mL, 45.8 mmol, 1.0 equiv) in CH_2Cl_2 (70 mL) was added slowly *N*,*N*-diethylcarbamoylchloride (4.60 mL, 35.2 mmol, 0.8 equiv) over 5 minutes and then DMAP (430 mg, 3.52 mmol) at RT. The resulting mixture was fitted with a reflux condenser and stirred at

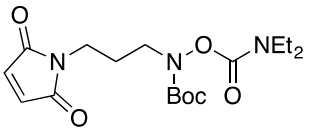
40 °C for 40 h or until TLC indicated full conversion of *N*,*N*-diethylcarbamoylchloride (20% EtOAc in hexanes). After cooling to RT, the mixture was diluted with CH_2Cl_2 (200 mL) and washed with 1 M aq. HCl (200 mL). The aqueous phase was extracted with CH_2Cl_2 (3 × 200 mL). The combined organic phases were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude material was purified by flash chromatography using a gradient elution of 10% to 20% EtOAc in hexanes. This afforded the product **008** as colourless oil, which slowly crystallised into a white solid (7.29 g, 94% yield). The spectral data of **008** was in agreement with that reported in the literature.[8] **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.78 (s, 1H), 3.34 (q, *J* = 7.2 Hz, 4H), 1.48 (s, 9H), 1.28-1.11 (m, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 156.7, 155.7, 82.9, 43.1, 41.6, 28.2, 14.1, 13.4. **HRMS** (ESI) calc'd for $C_{10}H_{20}N_2NaO_4$ $[M + Na]^+$: 255.1315, found: 255.1317.

tert-Butyl ((diethylcarbamoyl)oxy)(3-(1,3-dioxo-1,3,3a,4,7,7a-hexahydro-2H-4,7-epoxyiso-indol-2-yl)propyl)carbamate (009)



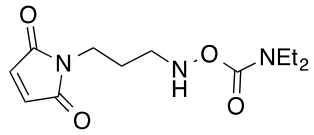
Sodium hydride (0.66 g, 16.5 mmol, 1.10 equiv) was suspended in anhydrous DMF (20 mL) and cooled to 0 °C in an ice bath. A solution of hydroxylamine **008** (3.66 g, 15.8 mmol, 1.05 equiv) in DMF (15 mL) was added to the stirred suspension of NaH over 5 minutes. The resulting mixture was stirred at 0 °C for 30 minutes, at which point a solution of mesylate **007** (4.70 g, 15.0 mmol, 1.00 equiv) in DMF (15 mL) was added. The reaction solution was warmed to RT and stirred overnight for 16 h. The solution was cooled back down to 0 °C and the reaction was quenched with sat. aq. NH_4Cl (50 mL). The resulting suspension was subsequently warmed to RT and diluted with H_2O (50 mL) and EtOAc (300 mL). The aqueous phase was separated and the organic phase was washed with H_2O (50 mL) and brine (4 × 50 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude material was purified by flash chromatography using a gradient elution of 50% to 60% EtOAc in hexanes. This afforded the product **009** as a colourless viscous oil (4.55 g, 69% yield). **IR** (KBr pellet, cm^{-1}) λ_{max} = 2976, 2929, 1770, 1698, 1587, 1473, 1409, 1383, 1281, 1223, 1162, 1051, 985. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 6.49 (t, *J* = 1.0 Hz, 2H), 5.24 (t, *J* = 1.0 Hz, 2H), 3.62 (bs, 2H), 3.60 – 3.51 (m, 2H), 3.30 (q, *J* = 7.1 Hz, 4H), 2.82 (s, 2H), 1.87 (p, *J* = 7.0 Hz, 2H), 1.45 (s, 9H), 1.16 (bs, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 176.2, 155.0, 154.2, 136.6, 82.0, 81.0, 48.1, 47.5, 43.1, 41.7, 36.9, 28.3, 25.5, 14.3, 13.5. **HRMS** (ESI) calc'd for $C_{21}H_{32}N_3O_7$ $[M + H]^+$: 438.2235, found: 438.2232.

***tert*-Butyl ((diethylcarbamoyl)oxy)(3-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)propylcarbamate (010)**



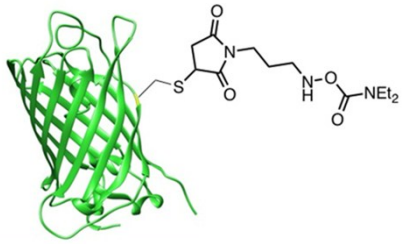
A solution of hydroxylamine **009** (4.53 g, 10.4 mmol) in anhydrous toluene (80 mL) in a single-neck round-bottomed flask was fitted with a glass tube that was sealed at the top with a rubber septum that was pierced with an outlet needle. The solution was stirred at 120 °C for 6 h, then subsequently cooled to RT and concentrated under reduced pressure. The crude material was purified by flash chromatography using a gradient elution of 20% to 40% EtOAc in hexanes. This afforded the product **010** as a pale-yellow viscous oil (3.46 g, 90% yield). **IR** (KBr pellet, cm^{-1}) λ_{max} = 2971, 2932, 2869, 1768, 1702, 1585, 1475, 1405, 1381, 1282, 1220, 1159, 1045, 965. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 6.67 (s, 2H), 3.71 – 3.54 (m, 4H), 3.31 (q, *J* = 7.1 Hz, 4H), 1.91 (p, *J* = 7.0 Hz, 2H), 1.45 (s, 9H), 1.18 (bs, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 170.7, 155.1, 154.2, 134.2, 82.0, 48.0, 43.1, 41.7, 35.8, 28.3, 26.3, 14.3, 13.5. **HRMS** (ESI) calc'd for $C_{17}H_{27}N_3NaO_6$ $[M + Na]^+$: 392.1792, found: 392.1793.

1-(3-(((diethylcarbamoyl)oxy)amino)propyl)-1*H*-pyrrole-2,5-dione (011)



Maleimide **010** (3.19 g, 8.6 mmol, 1.0 equiv) was dissolved in anhydrous 1,4-dioxane (10 mL) was subsequently cooled to 0 °C in an ice bath with stirring. 4.0 M HCl in dioxane (32.0 mL, 128 mmol, 14.9 equiv) was then added and the resulting mixture was stirred at 0 °C for 5 h. The solution was then warmed to RT and then N_2 was carefully bubbled through the stirred solution for 2 h to remove excess HCl. The solution was then concentrated under reduced pressure, dissolved in 50 mL of CH_2Cl_2 and subsequently concentrated again under reduced pressure. This process of dilution and concentration with CH_2Cl_2 was repeated two additional times. The crude residue was then dissolved in anhydrous CH_2Cl_2 (40 mL) and to this solution was added NEt_3 (4.2 mL, 30 mmol, 3.5 equiv). This resulting mixture was stirred at RT for 1 h and subsequently diluted with CH_2Cl_2 (50 mL). This solution was then washed with 10% aq. citric acid (50 mL), H_2O (50 mL) and brine (50 mL). The collected organic phase was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude material was purified by flash chromatography using a gradient elution of 30% to 50% EtOAc in hexanes. This afforded the product **011** as a yellow oil (1.73 g, 75% yield). **IR** (KBr pellet, cm^{-1}) λ_{max} = 3239, 3097, 2975, 2937, 2877, 1769, 1699, 1586, 1478, 1410, 1380, 1276, 1223, 1161, 1046, 960, 829. **^{1}H NMR** (400 MHz, DMSO-d_6) δ 7.62 (t, *J* = 5.4 Hz, 1H), 7.00 (s, 2H), 3.46 (t, *J* = 7.0 Hz, 2H), 3.20 (q, *J* = 7.1 Hz, 4H), 2.89 – 2.77 (m, 2H), 1.67 (p, *J* = 7.0 Hz, 2H), 1.04 (t, *J* = 7.1 Hz, 6H). **^{13}C NMR** (101 MHz, DMSO) δ 171.1, 155.7, 134.5, 49.3, 41.1, 35.2, 25.9, 13.6. **HRMS** (ESI) calc'd for $C_{12}H_{19}N_3NaO_4$ $[M + Na]^+$: 292.1268, found: 292.1266.

sfGFP(S147C) – hydroxylamine (3c)



An aliquot of sfGFP(S147C) (0.025 µmol) was treated with DTT (25.0 µL, 500 equiv, 0.5 M in H_2O) and diluted to a concentration of 200 µM with Millipore H_2O. The resulting solution was vortexed quickly and incubated at RT for 1 h. Excess DTT was subsequently removed through three cycles of spin diafiltration (Amicon® Ultra – 4, 10 kDa MWCO) with a freshly degassed (argon balloon) 0.1 M potassium phosphate, 1 mM EDTA, pH 7.8 buffer at 4 °C. The resulting solution containing reduced sfGFP(S147C) (devoid of DTT) was diluted (50 µM) with additional pH 7.8 buffer and to it was added maleimide hydroxylamine **011** (25.0 µL, 10.0 equiv, 10 mM in DMF). The solution was vortexed quickly and incubated on ice at 0 °C for 2 h. The reaction mixture was passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with Millipore H_2O for mass analysis. ESI-MS indicated full conversion to the corresponding hydroxylamine conjugate **3c**.

Calculated mass: 28095.4 Da, observed mass: 28095.2 Da.

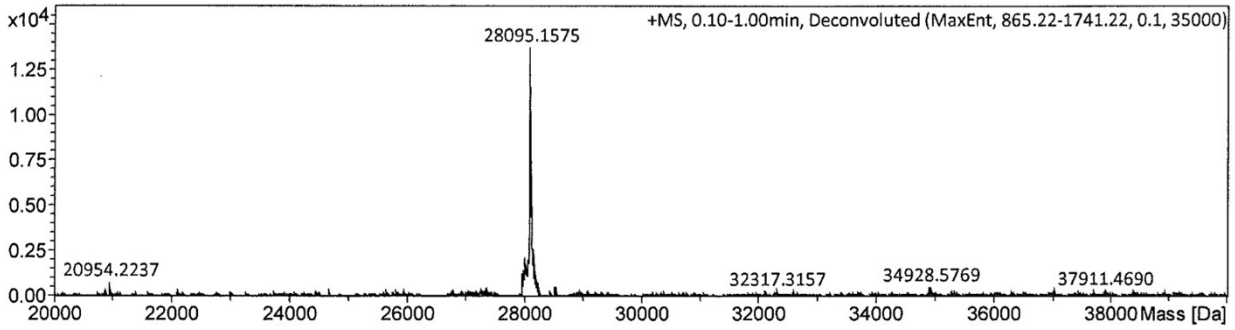
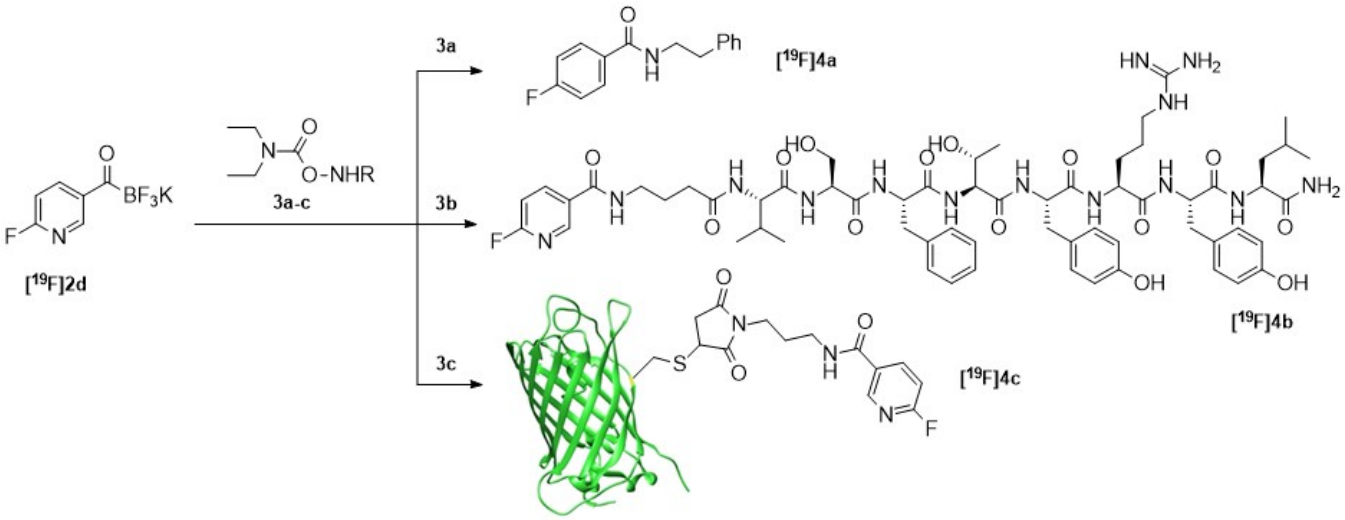


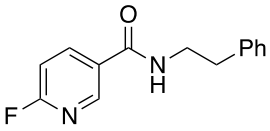
Figure F3. Deconvoluted mass spectrum (ESI) of bioconjugate **3c**

Syntheses of references [^{19}F]4a-c

Scheme S5: Synthesis of references **[^{19}F]4a-c**

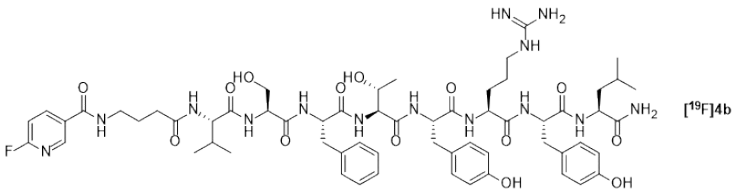


***N*-(2-Phenethyl)-2-fluoro-3-picolinamide [^{19}F]4a**



To a solution of **[^{19}F]2d** (20.0 mg, 0.09 mmol, 1.00 equiv) and **3a** (21.5 mg, 0.09 mmol, 1.05 equiv) in *t*-BuOH/H_2O (1:1, 2.0 mL), 1 drop of oxalic acid (0.1 M) was added. The reaction was stirred at 23 °C for 2 h. After full conversion was indicated by TLC, EtOAc was added and the mixture was extracted with H_2O, dried over Na_2SO_4 and concentrated under reduced pressure. The crude material was purified by flash column chromatography (EtOAc/hexanes 1:3) to yield the desired product as a pale-yellow oil (19.0 mg, 0.06 mmol, 91%). R_f 0.41 (EtOAc/hexanes 1:1). **M.p.** 97.5 – 98.3 °C. **IR** (neat, cm^{-1}) λ_{max} = 3292, 3063, 3028, 2924, 2853, 1634, 1594, 1540, 1475, 1880, 1322, 1295, 1259, 1025, 881, 845, 776, 743, 696, 625, 599. **^{1}H NMR** (600 MHz, $CDCl_3$) δ 8.47 (dt, *J* = 2.6, 0.8 Hz, 1H), 8.17 (ddd, *J* = 8.5, 7.5, 2.5 Hz, 1H), 7.36 – 7.32 (m, 2H), 7.28 – 7.22 (m, 3H), 6.99 (ddd, *J* = 8.5, 2.9, 0.7 Hz, 1H), 6.07 (brs, 1H), 3.74 (td, *J* = 6.9, 5.8 Hz, 2H), 2.95 (t, *J* = 6.9 Hz, 2H). **^{13}C NMR** (151 MHz, $CDCl_3$) δ 165.9, 164.53, 164.3, 146.5 (d, *J* = 16.0 Hz), 140.9 (d, *J* = 8.8 Hz), 138.6, 129.0 (d, *J* = 15.0 Hz), 128.8 (d, *J* = 4.6 Hz), 127.0, 109.9 (d, *J* = 37.4 Hz), 41.3, 35.7. **^{19}F NMR** (470 MHz, $CDCl_3$): δ -63.4 (d, *J* = 4.5 Hz). **HRMS** (ESI) calc'd for $C_{14}H_{14}FN_2O$ $[M + H]^+$ 245.1085, found 245.1091.

Reference peptide [^{19}F]4b



Peptide hydroxylamine **3b** (4.5 mg, 0.0035 mmol, 1.0 equiv) was dissolved in a 1:1 *t*-BuOH:0.1 M aq. oxalic acid mixture (3.5 mL) at RT by sonication. To the solution was added KAT **[^{19}F]2d** (4.0 mg, 0.017 mmol, 5.0 equiv) and the resulting reaction was stirred for 2 h at RT. Peptide **[^{19}F]4b** was then directly purified by preparative HPLC using Shiseido Capcell Pak C18 column (20 × 250 mm) eluting with 5% CH_3CN (0.1% TFA) for 10 minutes, followed by a gradient elution of 5 to 95 % CH_3CN (0.1% TFA) in 34 min (flow rate = 10 mL/min). The product peak eluting at t_R = 28.4 min was collected and lyophilised to obtain 2.1 mg of pure **[^{19}F]4b** (1.7 µmol, 48% isolated yield). Analytical HPLC (Shiseido C18 (5 µm, 120 Å pore size, 4.6 mm I.D. x 250 mm) column with a gradient of 10 to 90% CH_3CN with 0.1% TFA in 20 min) and HRMS were used to confirm the purity and exact mass of the product. m/z calc'd for $C_{61}H_{84}FN_{14}O_{14}$ $[M + H]^+$ 1255.6270, found: 1255.6275.

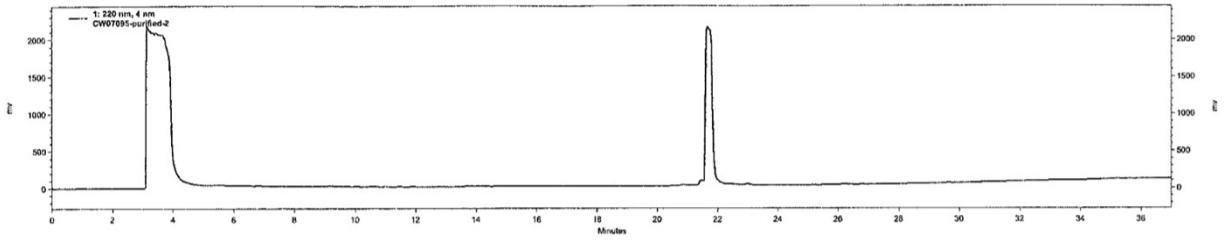


Figure F4. Analytical HPLC trace of purified **[^{19}F]4b**

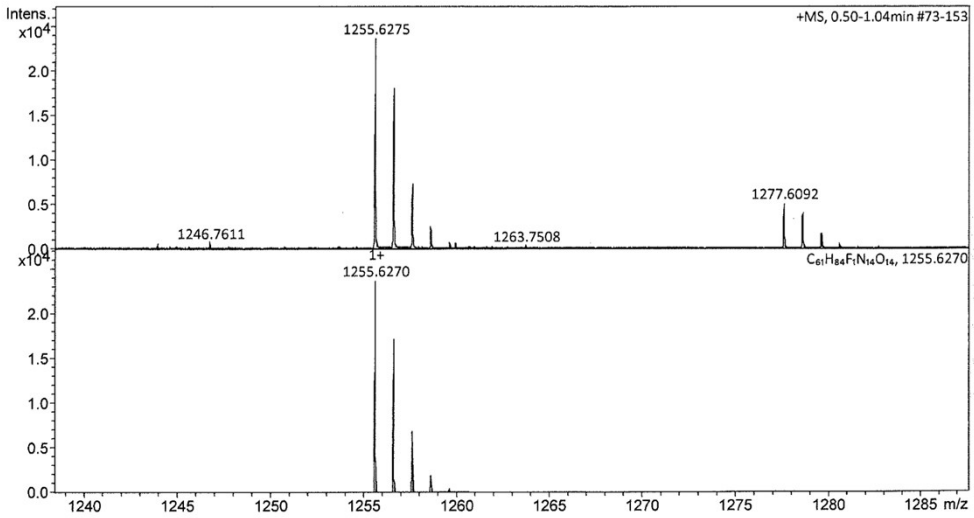
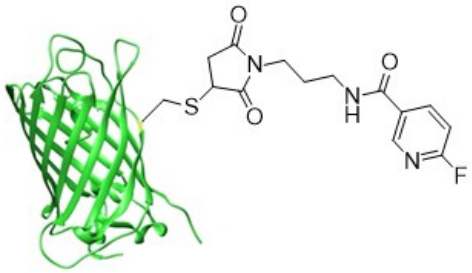


Figure F5. HRMS(ESI) of purified **[^{19}F]4b**

Reference sfGFP(S147C) [^{19}F]4c



Freshly prepared hydroxylamine bioconjugate **3c** (0.015 μmol) was exchanged into a 0.1 M K phos, 5% DMF, pH 6.0 buffer through three cycles of spin diafiltration (Amicon® Ultra – 4, 10 kDa MWCO) at 4 °C. The solution was adjusted to a concentration of 35 μM with additional pH 6.0 buffer and to it was added KAT **[^{19}F]2d** (30.0 μL, 20.0 equiv, 10 mM in DMF). The reaction was then vortexed quickly and allowed to gently stir overnight for 12 h at RT. The reaction mixture was passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with Millipore H_2O for mass analysis. ESI-MS indicated full conversion to the corresponding hydroxylamine conjugate **[^{19}F]4c**. Calculated mass: 28103.3 Da, observed mass: 28103.1 Da.

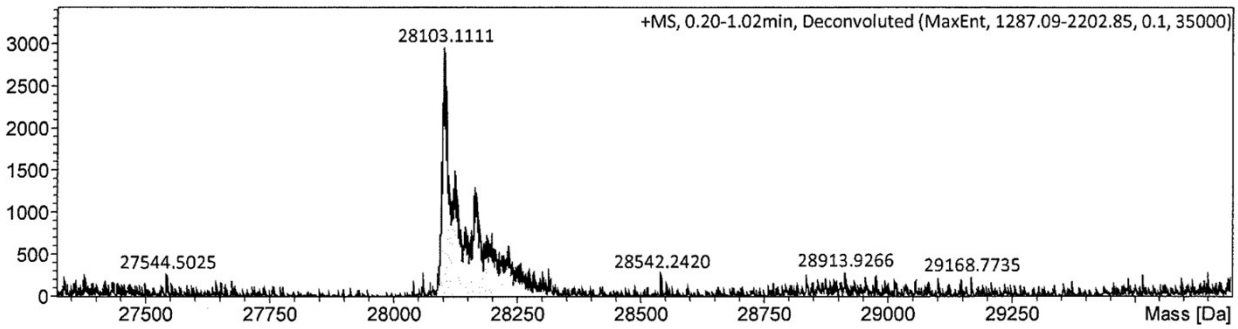
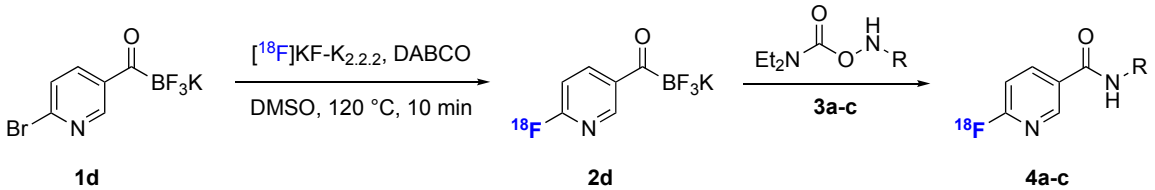


Figure F6. Deconvoluted mass spectrum (ESI) of bioconjugate **[^{19}F]4c**

4. Radiosyntheses

General Scheme



General notes for radiochemical conversion (RCC) determination

The evaluation of the different precursors **1a-e** for the best ^{18}F-incorporation and the subsequent optimization efforts for **1d** were assessed with UPLC, using system A as mobile phase (described in section 2K). According to Ory et al.,[9] mobile phases with a pH higher than 5 for silica-based C18 columns cause no [^{18}F]fluoride retention and therefore, in our case, there should not be an overestimation of the radiochemical conversion. Nevertheless, in order to make sure that the **specific** mobile phase and column do not cause [^{18}F]fluoride retention, its recovery was determined using system A conditions with or without the UPLC column in the flow. Briefly, after injection of the aqueous [^{18}F]fluoride solution, elution was started applying conditions from system A and using a bypass or the specified column in consecutive runs (n=4). The % efficiency of the recovery was calculated by comparing the AUCs of the fluoride peaks between each bypass and column run. Indeed the recovery was 95.1%±0.5 (n=4) which proves that there is no [^{18}F]fluoride retention with the conditions used. A typical UPLC radiochromatogram during optimization efforts for **1d** is shown in section 7. The identity of **2d** was always confirmed with a subsequent HPLC co-injection with **[^{19}F]2d**.

Optimised radiosynthesis for [^{18}F]FPAT (2d)

[^{18}F]fluoride was obtained via the ^{18}O(p,n)^{18}F reaction using 98% enriched ^{18}O-water. $^{18}F^-$ was trapped on a light QMA carbonate cartridge (Waters). Kryptofix $K_{2.2.2}/K_2CO_3$ solution (10 mg of Kryptofix $K_{2.2.2}$, 1.2 mg of K_2CO_3 in CH_3CN (1.4 mL) and H_2O (0.6 mL)) was used for the elution of $^{18}F^-$ from the cartridge. The solvents were evaporated at 110 °C under vacuum with a slight inflow of N_2. **No drying step was performed**. A solution of DABCO (4.5 mg, 0.04 mmol) in DMSO (0.15 mL) was added, followed by **1d** (1.0 mg, 3.4 µmol) in DMSO (0.15 mL). The reaction mixture was heated at 120 °C for 10 min. After cooling down, the crude reaction was diluted with H_2O (4 mL) and transferred via cannula to a vial containing 30 mL of H_2O. The reaction vial was washed with 4 mL of CH_3CN/H_2O (1:1) and was

transferred to the dilution vial. The solution was passed through an HLB Plus cartridge (preconditioned with 5 mL CH_3CN, 5 mL MeOH and 5 mL H_2O). The cartridge was washed with H_2O (2 mL) and radioactivity was eluted with CH_3CN (1 mL). After dilution with HPLC buffer (10 mM, pH 4, 2 mL), the product was purified by semi-prep HPLC using HPLC system B with the product **[^{18}F]FPAT** (**2d**) eluting between 18-21 min. 9-10 mg of sodium ascorbate or (tris(2-carboxyethyl)phosphine (TCEP) was added to the HPLC purified fraction and the HPLC solvent was removed at 90 °C under vacuum with a slight inflow of N_2 gas (approximately 10 min). The concentrated activity was used for further manipulations. Average radiochemical yield of **2d** was 58% ± 2.5 (n=3, 0.3 – 5 GBq of product), decay corrected from end of bombardment (EOB). Quality control of **[^{18}F]FPAT** was performed with analytical radio-HPLC using **system D** and its identity was confirmed by co-injection with the corresponding non-radioactive standard **[^{19}F]2d**; t_R = 10.4 min Radiochemical purity was greater than 95% and average molar radioactivity was 52.8 ± 7.1 (n=3). Average synthesis time was 55 min from EOB.

Radiosynthesis of model compound 4a

The concentrated radioactivity of [^{18}F]FPAT(**2d**) was reconstituted in *t*-BuOH (0.3 mL) and oxalic acid 0.1 M (0.3 mL). Model oxime **3a** was then added dissolved in *t*-BuOH (0.2 mL). To evaluate temperature dependency, **3a** (2.0 mg, 8.5 µmol, 10 mM) was added and the reaction mixture was performed at either 80 °C or 25 °C for 15 min. In both cases, quantitative consumption of labelled intermediate **2d** was observed (radio-UPLC, **system A**) providing cleanly **4a**. To assess concentration dependency, the reaction was repeated at 25 °C with either 0.2 mg (0.85 µmol, 1.0 mM) or 0.02 mg (0.085 µmol, 0.1 mM) of oxime **3a**. In the first case, consumption of the labelled intermediate was complete within 3 min while the second reaction needed 30 min for the conversion to be complete. In all cases, the identity of **4a** was confirmed by co-injection with the corresponding non-radioactive standard **[^{19}F]4a**; HPLC system C, t_R = 13.8 min.

Radiosynthesis of labelled peptide 4b

The concentrated activity of [^{18}F]FPAT(**2d**) was reconstituted in DMSO (50 µL) and 0.1 M aq. oxalic acid (150 µL) and the peptide-precursor **3b** was added as a solution in 10% aq. DMSO (0.2 mL) and the reaction was stirred at 25 °C for 15 min. For 0.4 mg (800 µM) and 0.2 mg (400 µM) of peptide the conversion to product **4b** was quantitative, while for 0.1 mg (200 µM) the conversion to **4b** was 20%. The identity of **4b** was confirmed by co-injection with the corresponding non-radioactive standard **[^{19}F]4b**; HPLC system D, t_R = 16.4 min.

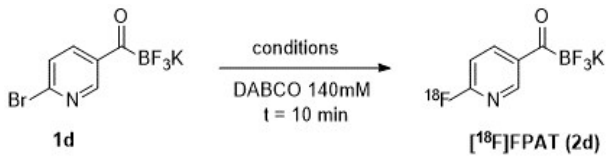
Radiosynthesis of labelled GFP 4c

The GFP precursor **3c** (MW = 28078) was provided as a 4.1 mg/mL solution (146 μM) in K-phos buffer 0.1 M, pH 6.0. The purified intermediate [^{18}F]FPAT (**2d**) was reconstituted in 10% DMSO K-phos buffer 0.1 M, pH 6.0. An aliquot was taken (50-100 MBq) and added to a solution of GFP protein precursor, followed by the addition of 1 drop of 0.1 M aq. oxalic acid and the reaction was stirred for 15 min at 25 °C. Conversion to product was checked with HPLC at that time point.

Two experiments were performed at different concentrations of precursor. For reaction A (20 μM), precursor (0.2 mg, 50 μL) was mixed with [^{18}F]FPAT (250 μL, 75 MBq) to give product **4c** with 80% conversion. For reaction B (70 μM), precursor **3c** (0.4 mg, 100 μL) was mixed with [^{18}F]FPAT (100 μL, 100 MBq) to afford a quantitative conversion to the labelled protein **4c**. The identity of **4c** was confirmed by co-injection with the corresponding non-radioactive standard **[^{19}F]4c**; HPLC system E, t_R = 13.3 min.

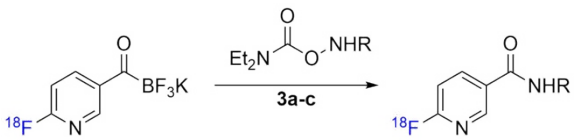
Results overview

A. optimisation efforts of [^{18}F]FPAT



Entry	1d (µmol)	Temperature	Solvent	base	azeotropic drying	RCC	NOTES
1	10	150	dry DMSO	Cs_2CO_3	YES	44.7% ± 6.2, n=4	initial conditions
2	3.4	120	dry DMSO	Cs_2CO_3	YES	43.1% ± 5.8, n=4	-
3	3.4	120	DMSO	Cs_2CO_3	NO	71.1% ± 12.5, n=11	-
4	3.4	120	DMSO	NBu_4HCO_3	NO	90.5% ± 5.2, n=6	difficult semiprep purification
5	3.4	120	DMSO	K_2CO_3	NO	84.2% ± 1.2, n=5	optimal conditions

B. Conjugation of [18F]FPAT with substrates 3a-c



Entry	Hydroxylamine	Product	Solvent	Amount / μmol	Conc. / mM	Time / min	Conv.
1	**3a**	**4a**	^{t}BuOH/0.1 M aq. oxalic acid (1:1)	8.50	10	3	quant.[a]
2	**3a**	**4a**	^{t}BuOH/0.1 M aq. oxalic acid (1:1)	0.85	1.0	3	quant.[a]
3	**3a**	**4a**	^{t}BuOH/0.1 M aq. oxalic acid (1:1)	0.085	0.1	15	87%[a]
4	**3b**	**4b**	25% DMSO in 0.1 M aq. oxalic acid/ 10% DMSO (1:1)	0.32	0.8	15	quant.[b]
5	**3b**	**4b**	25% DMSO in 0.1 M aq. oxalic acid/ 10% DMSO (1:1)	0.16	0.4	15	quant.[b]
6	**3b**	**4b**	25% DMSO in 0.1 M aq. oxalic acid/ 10% DMSO (1:1)	0.08	0.2	15	20%[b]
7	**3c**	**4c**	10% DMSO in 0.1 M K phos buffer, pH 6[c]	0.14	0.07	15	quant.[b]
8	**3c**	**4c**	10% DMSO in 0.1 M K phos buffer, pH 6[c]	0.007	0.02	15	80%[b]

[a] determined by γ-UPLC; [b] determined γ-HPLC, [c] a drop of 0.1 M aq. oxalic acid added

5. *In vivo* PET imaging

Animal care and subsequent experiments were in accordance with Swiss Animal Welfare legislation and were allowed by the Veterinary Office of the Canton Zurich, Switzerland. A C57BL/6 male mouse was purchased from Charles River (Sulzfeld, Germany). The mouse (21.4 g, 6 weeks old) was anesthetised using isoflurane and underwent a dynamic scan inside a PET/CT scanner (Super Argus, Sedecal, Madrid, Spain) upon tail-vein injection of 11.21 MBq (scan time 1-91 min post-injection) of [^{18}F]FPAT. For anatomical alignment of the mouse, computed tomography (CT) was performed following the PET scan. The data obtained from these scans were reconstructed in user-defined time frames with a voxel size of 0.3875 × 0.3875 × 0.775 mm^3 as previously outlined.[10] The image analysis was carried out using PMOD v3.9 (PMOD Technologies, Zurich, Switzerland). The colour bar shown indicates the standardised uptake value (SUV) which is defined as the decay-corrected tissue radioactivity normalised to the injected radioactivity and body weight.

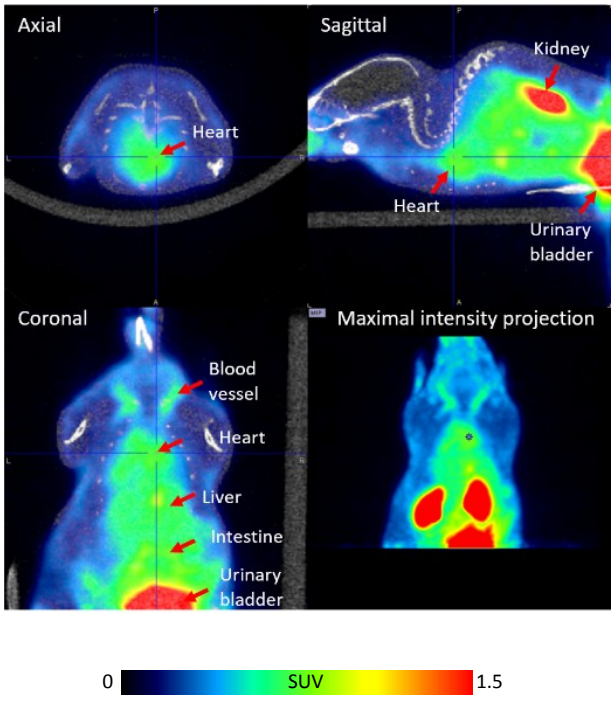
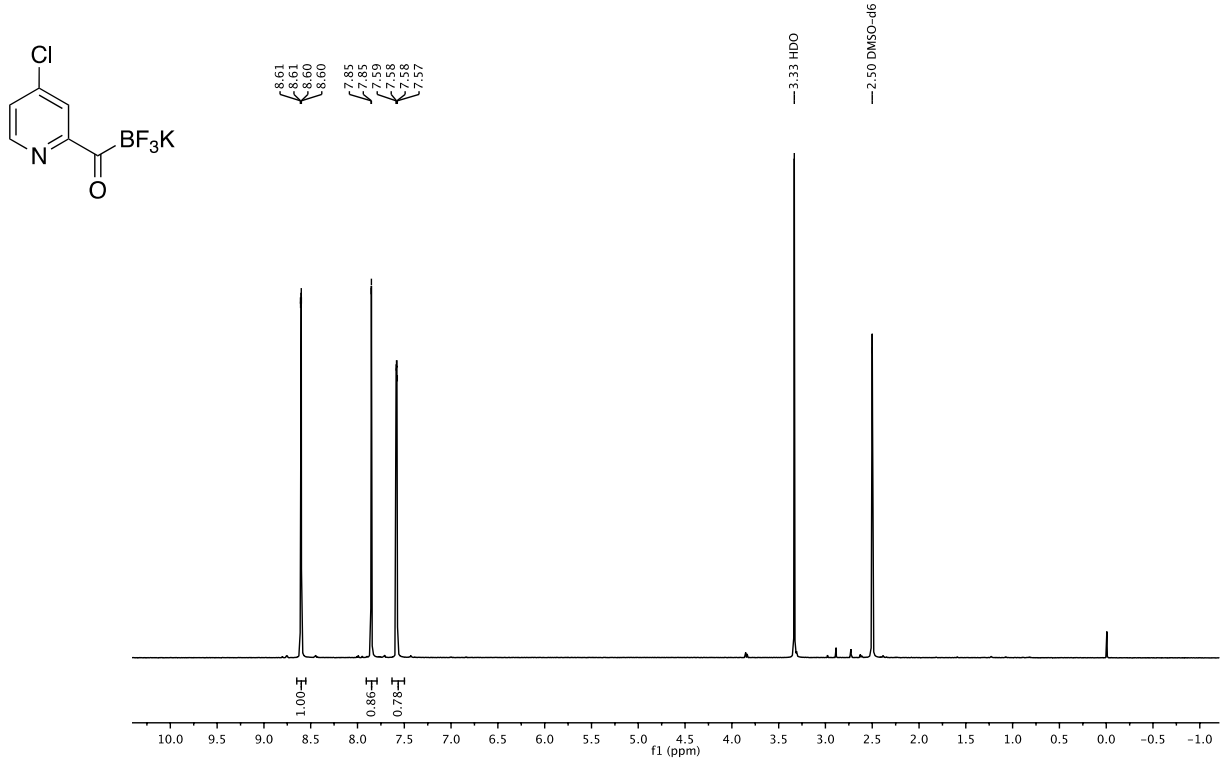


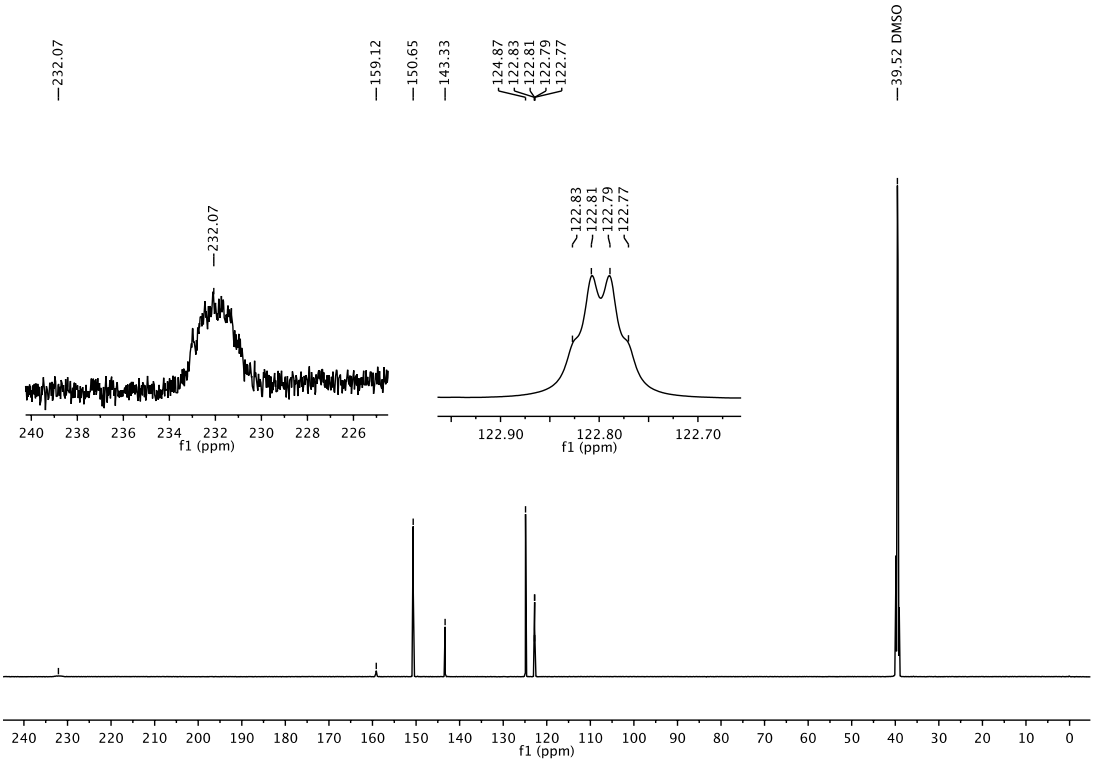
Figure F7. PET/CT images of [^{18}F]FPAT averaged from 1 to 91 min post-tracer injection.

6. NMR spectra

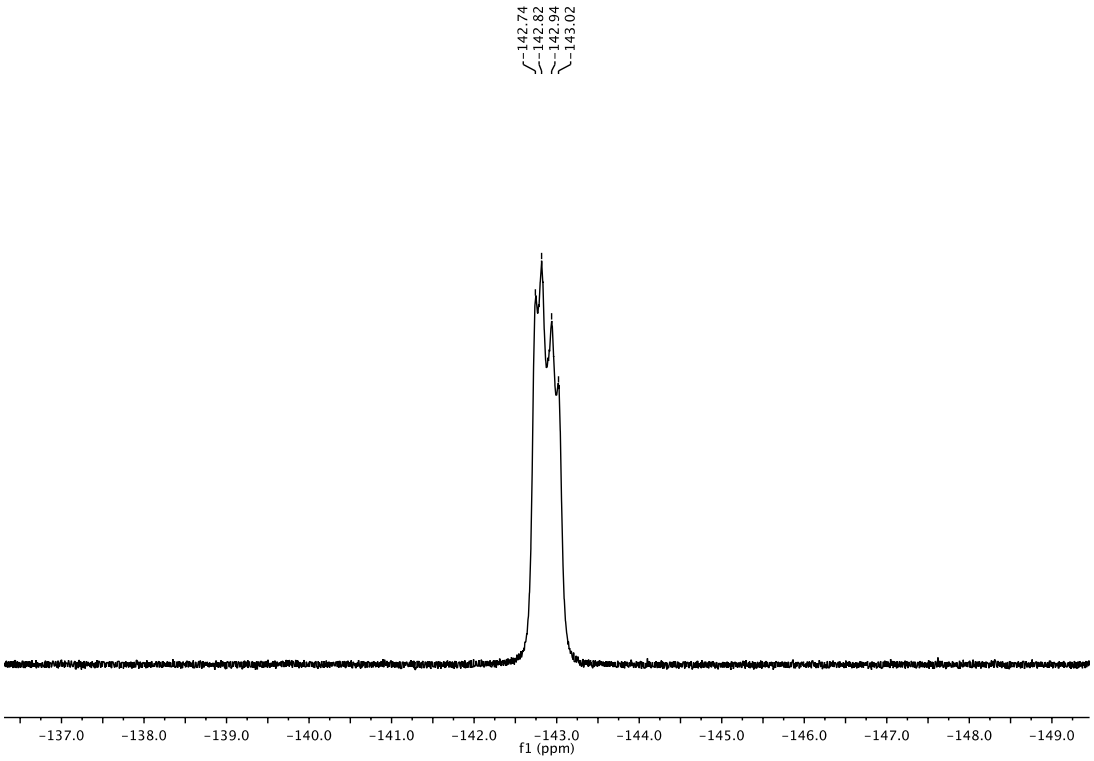
Compound 1c ^{1}H NMR (600 MHz, DMSO-d_6)



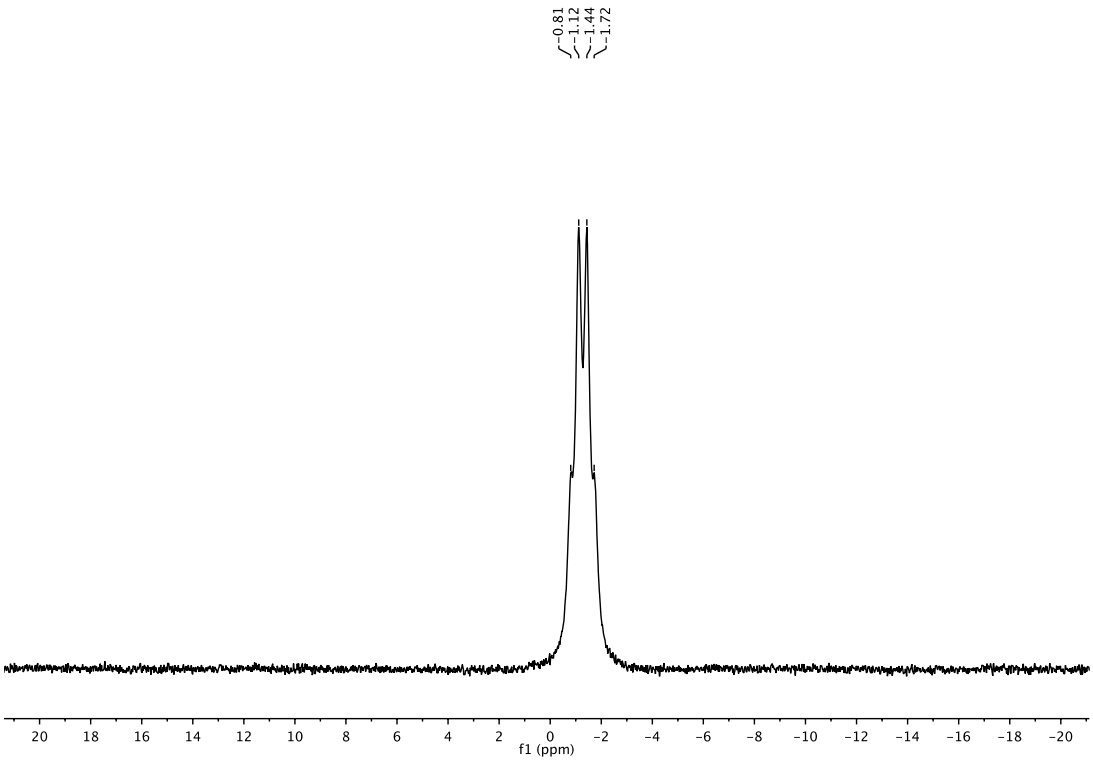
Compound 1c ^{13}C NMR (150 MHz, DMSO-d_6)



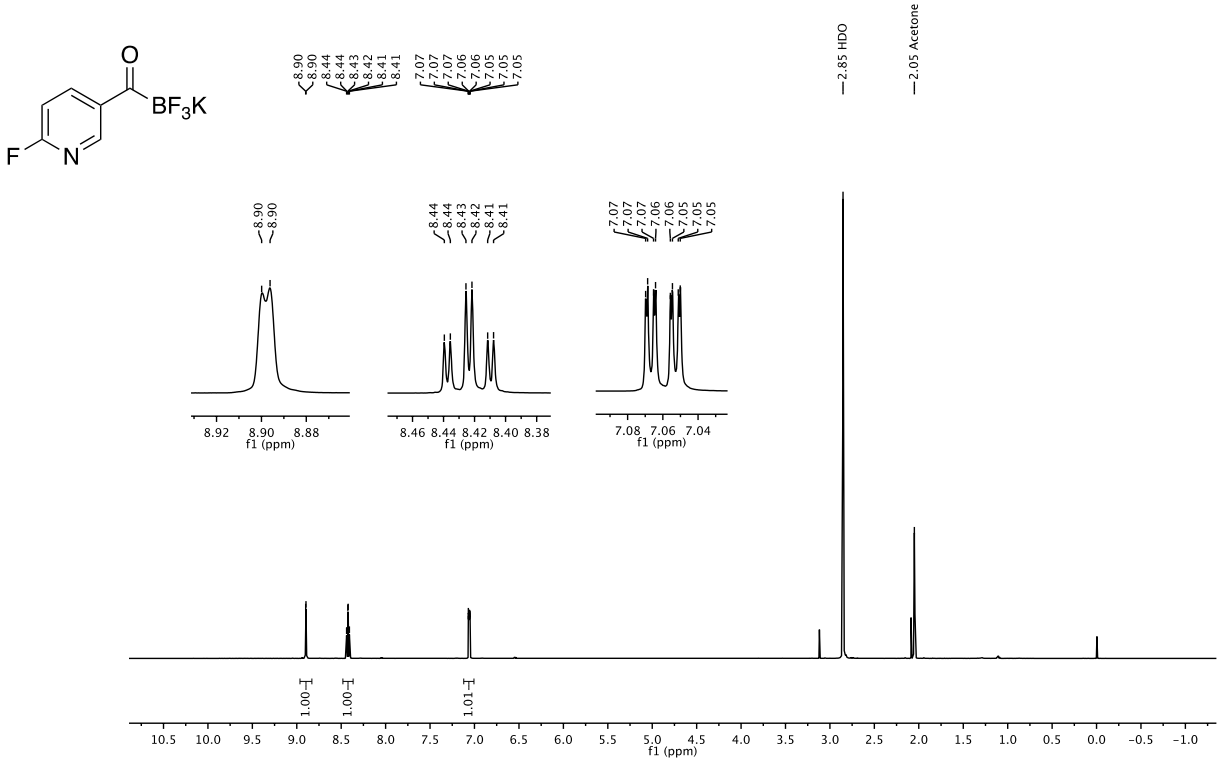
Compound 1c ^{19}F NMR (470 MHz, DMSO-d_6)



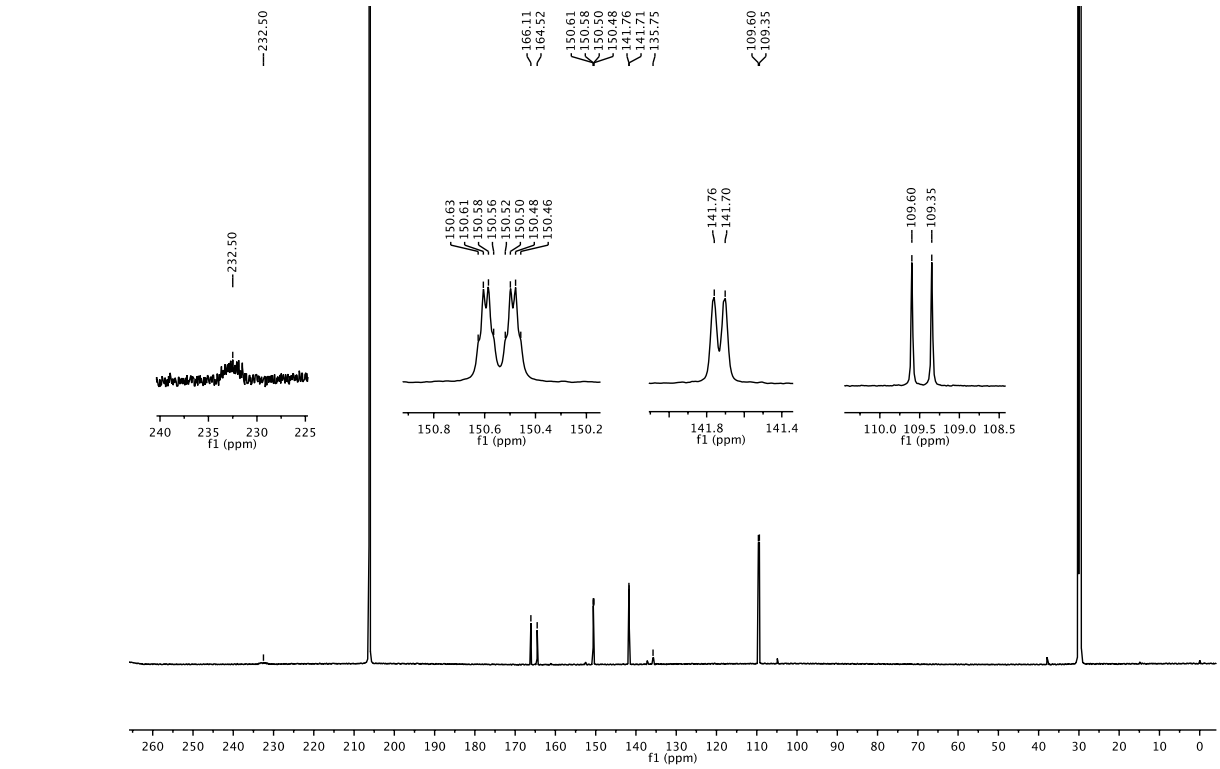
Compound 1c ^{11}B NMR (160 MHz, DMSO-d_6)



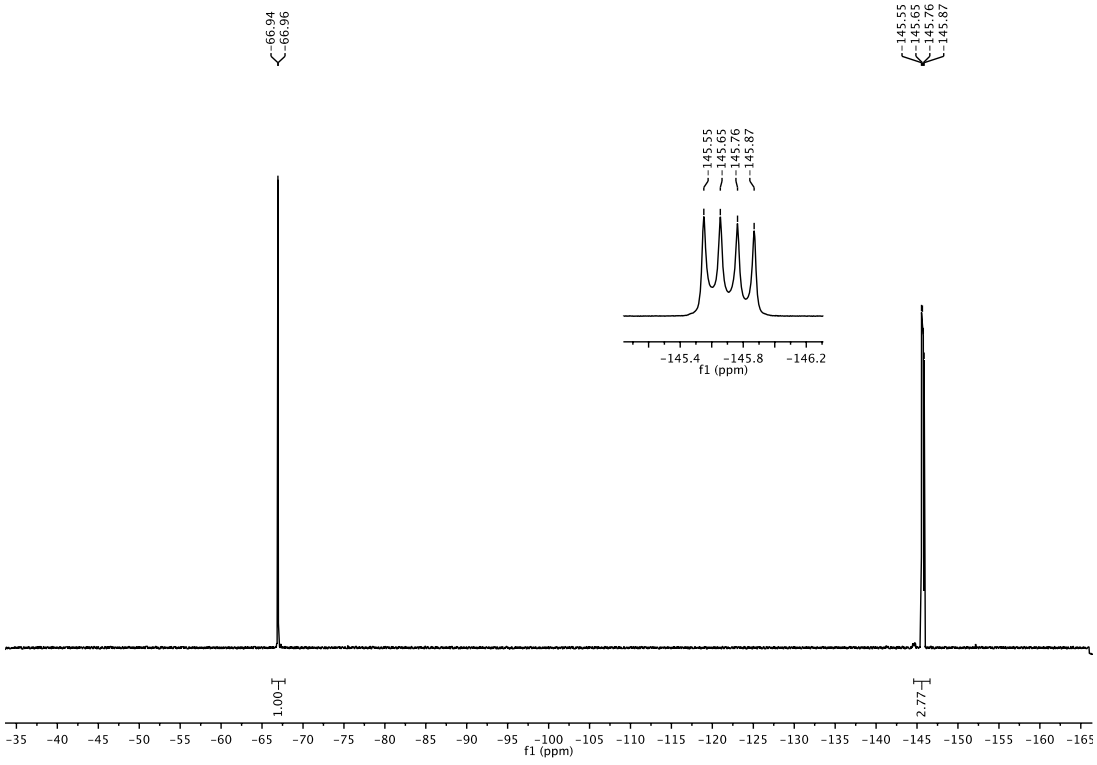
Compound [^{19}F]2d ^{1}H NMR (600 MHz, acetone-d_6)



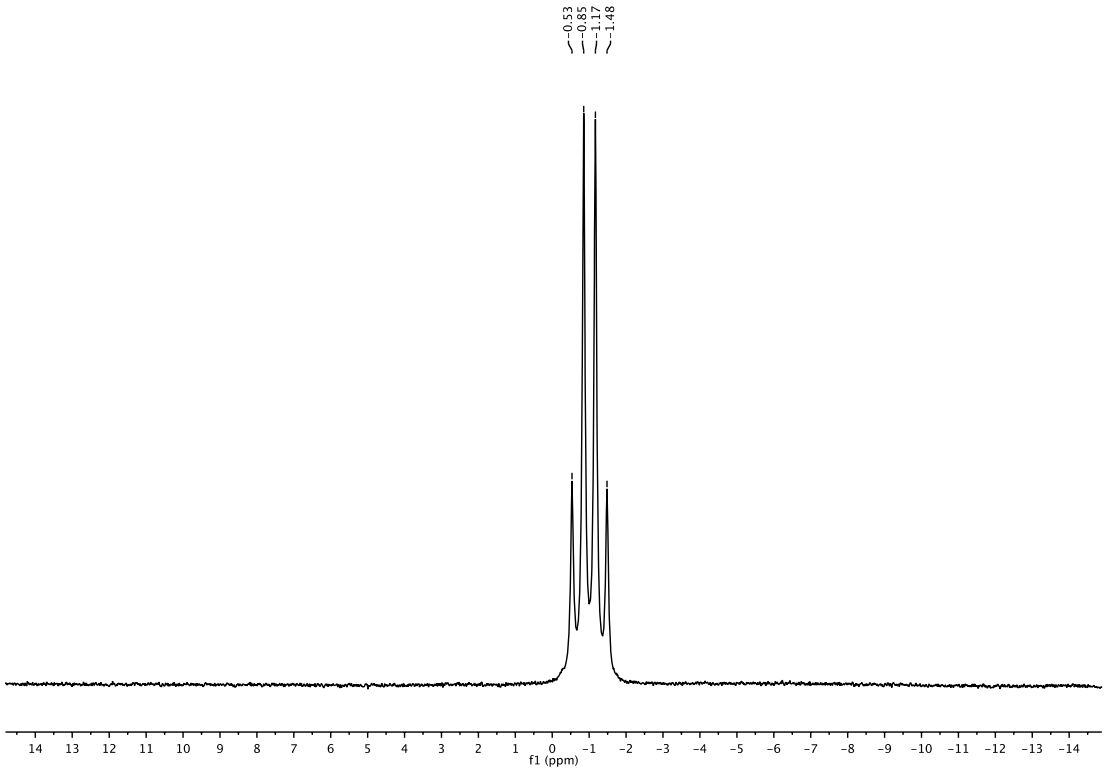
Compound [^{19}F]2d ^{13}C NMR (150 MHz, acetone-d_6)



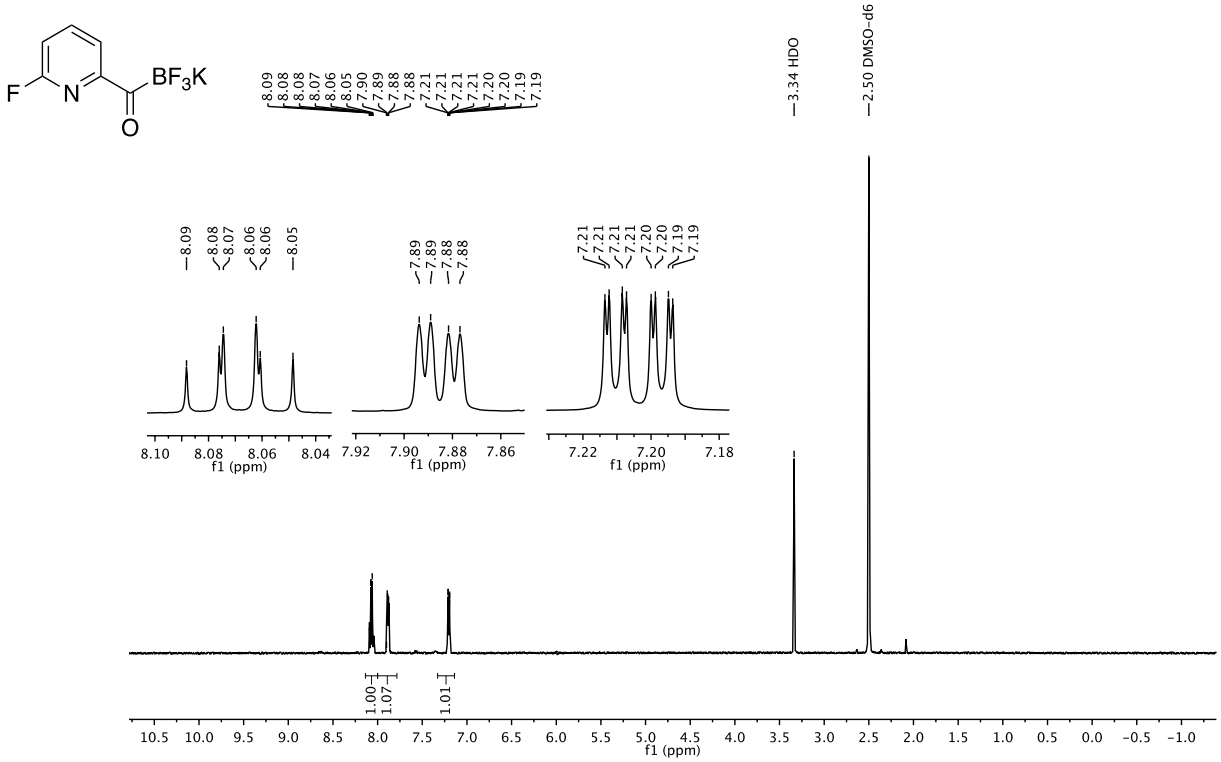
Compound [^{19}F]2d ^{19}F NMR (470 MHz, acetone-d_6)



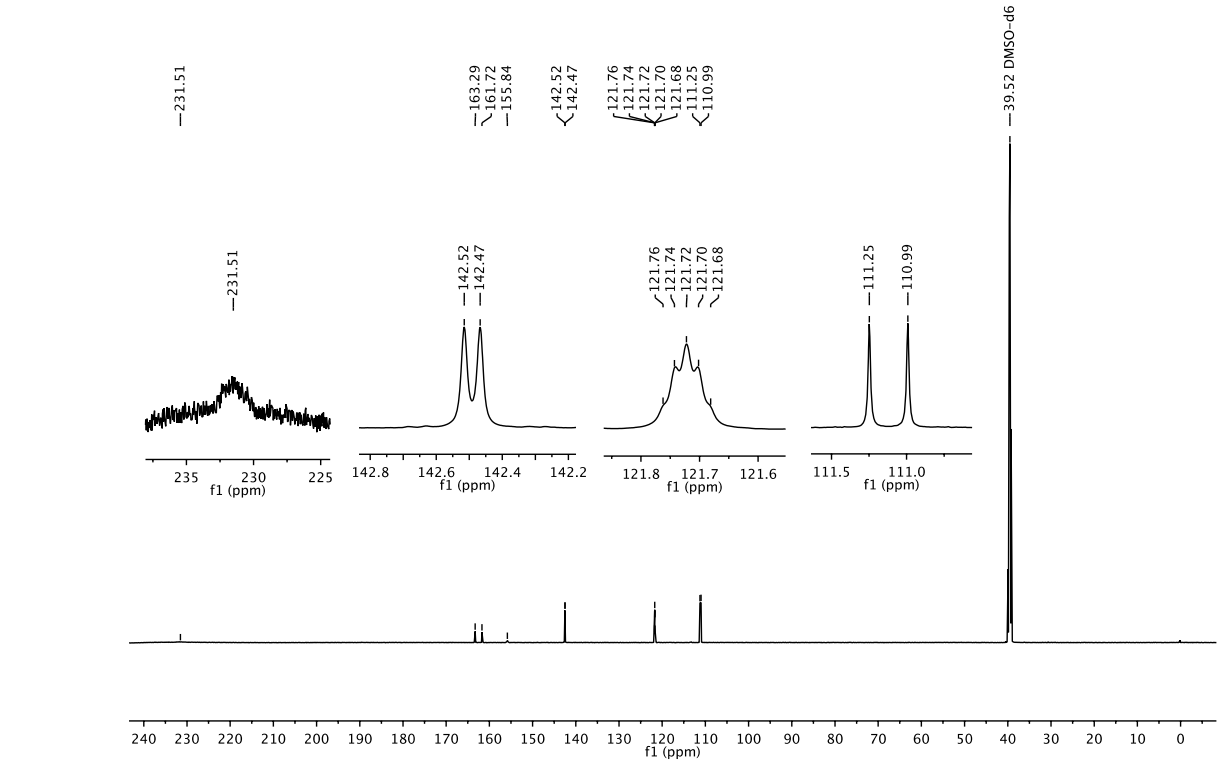
Compound [^{19}F]2d ^{11}B NMR (160 MHz, acetone-d_6)



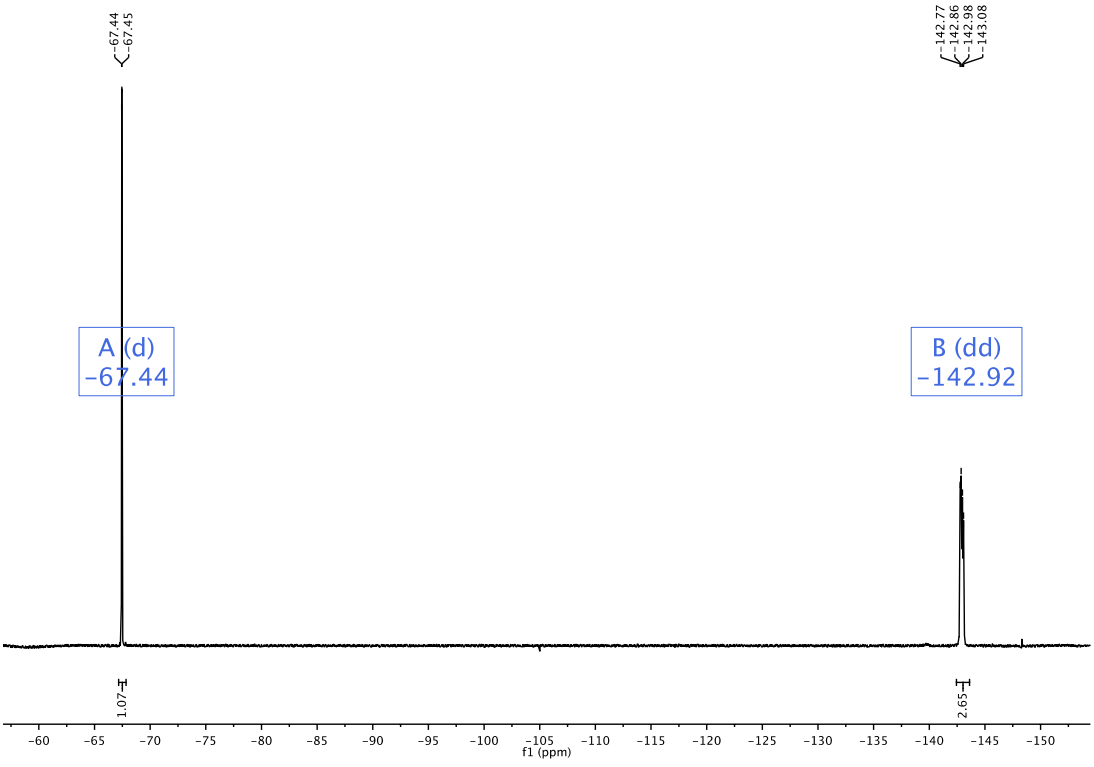
Compound [^{19}F]2b ^{1}H NMR (600 MHz, DMSO-d_6)



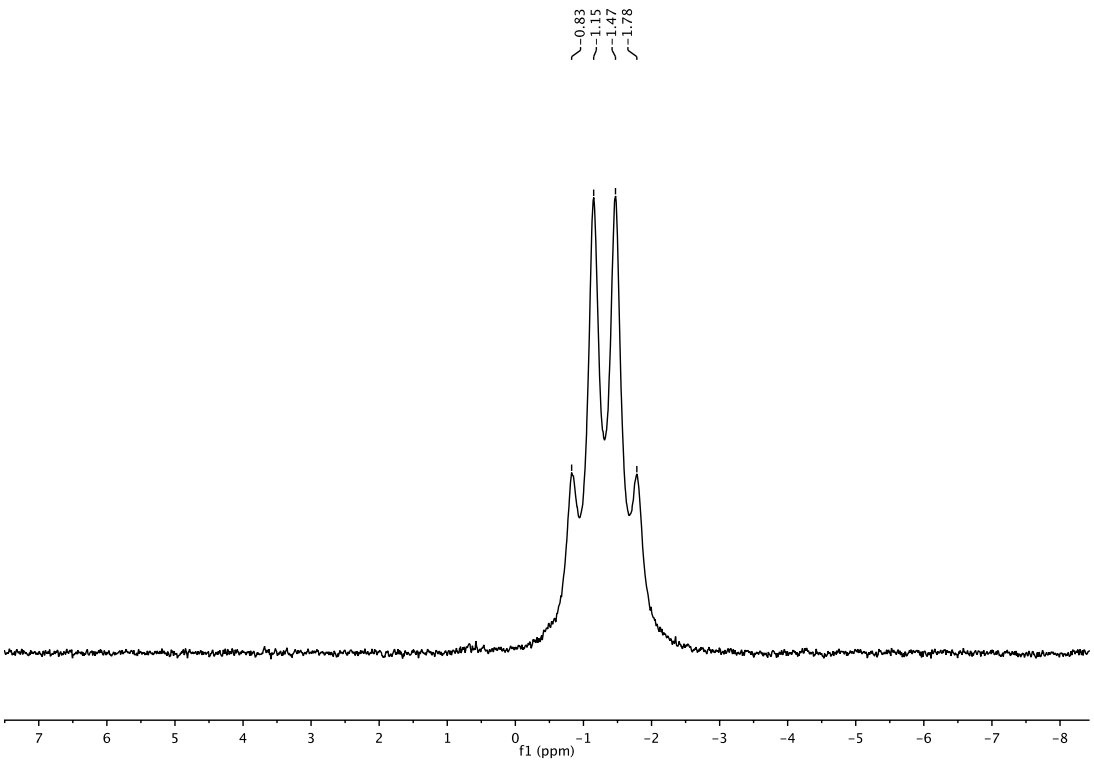
Compound [^{19}F]2b ^{13}C NMR (150 MHz, DMSO-d_6)



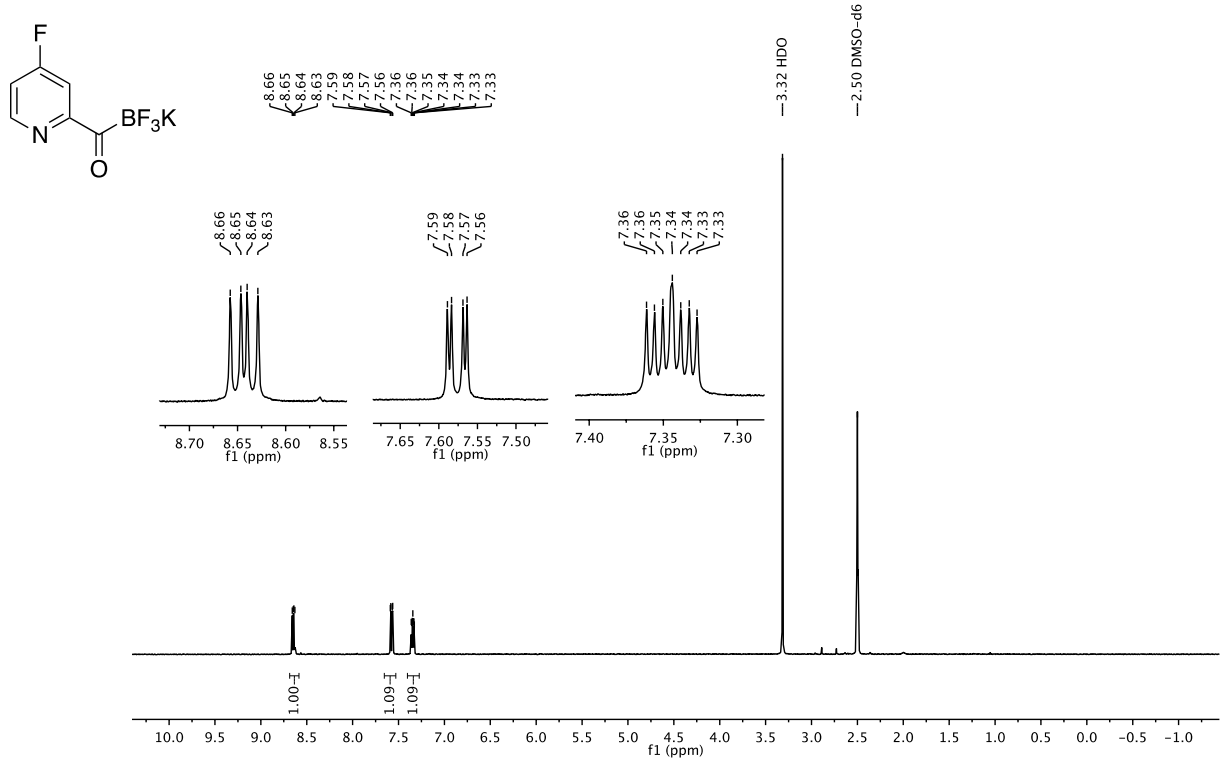
Compound [^{19}F]2b ^{19}F NMR (470 MHz, DMSO-d_6)



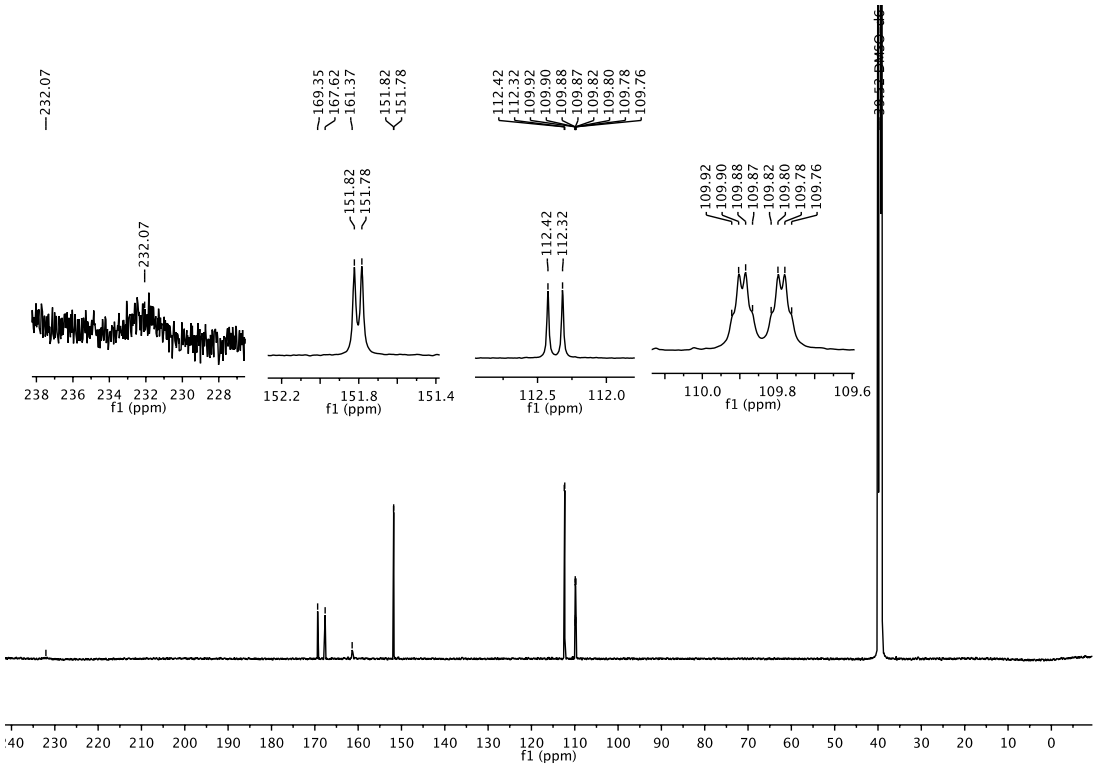
Compound [^{19}F]2b ^{11}B NMR (160 MHz, DMSO-d_6)



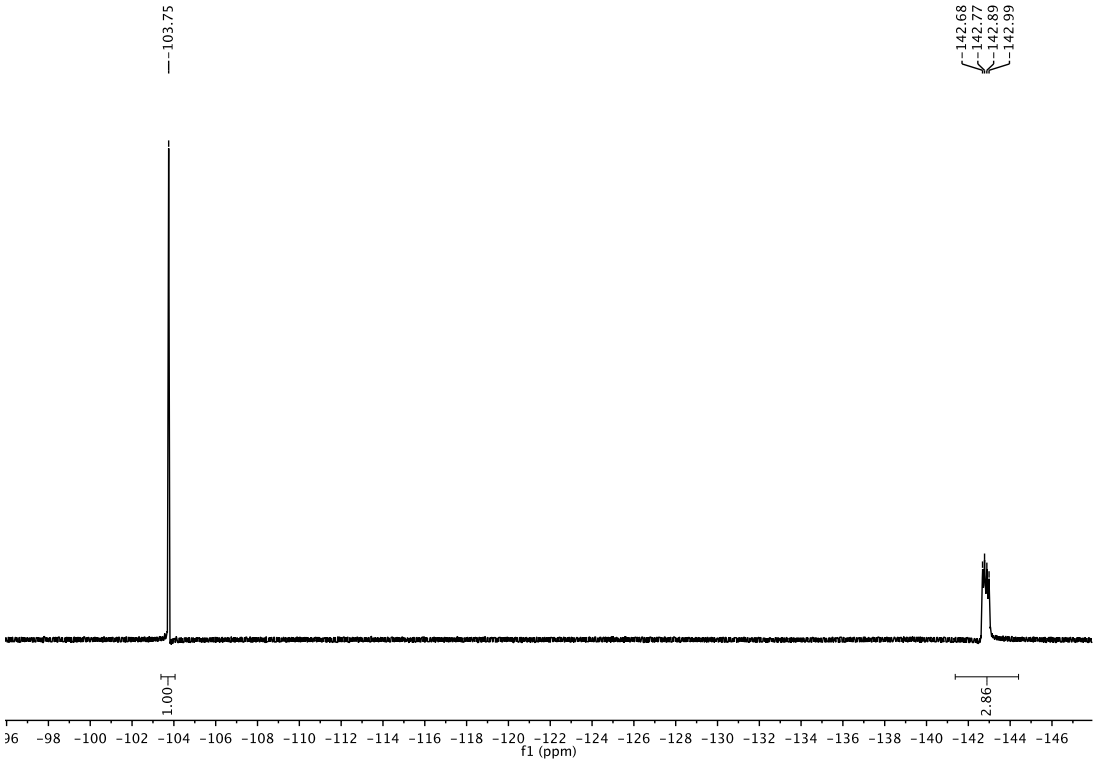
Compound [^{19}F]2c ^{1}H NMR (600 MHz, DMSO-d_6)



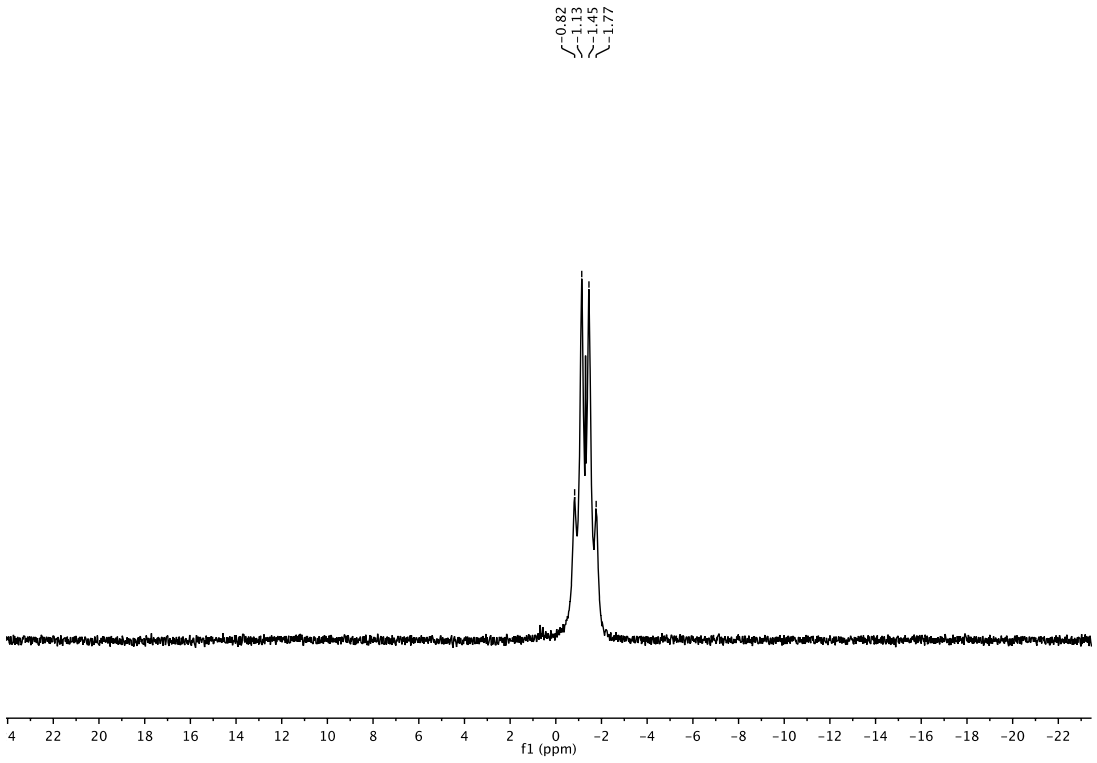
Compound [^{19}F]2c ^{13}C NMR (150 MHz, DMSO-d_6)



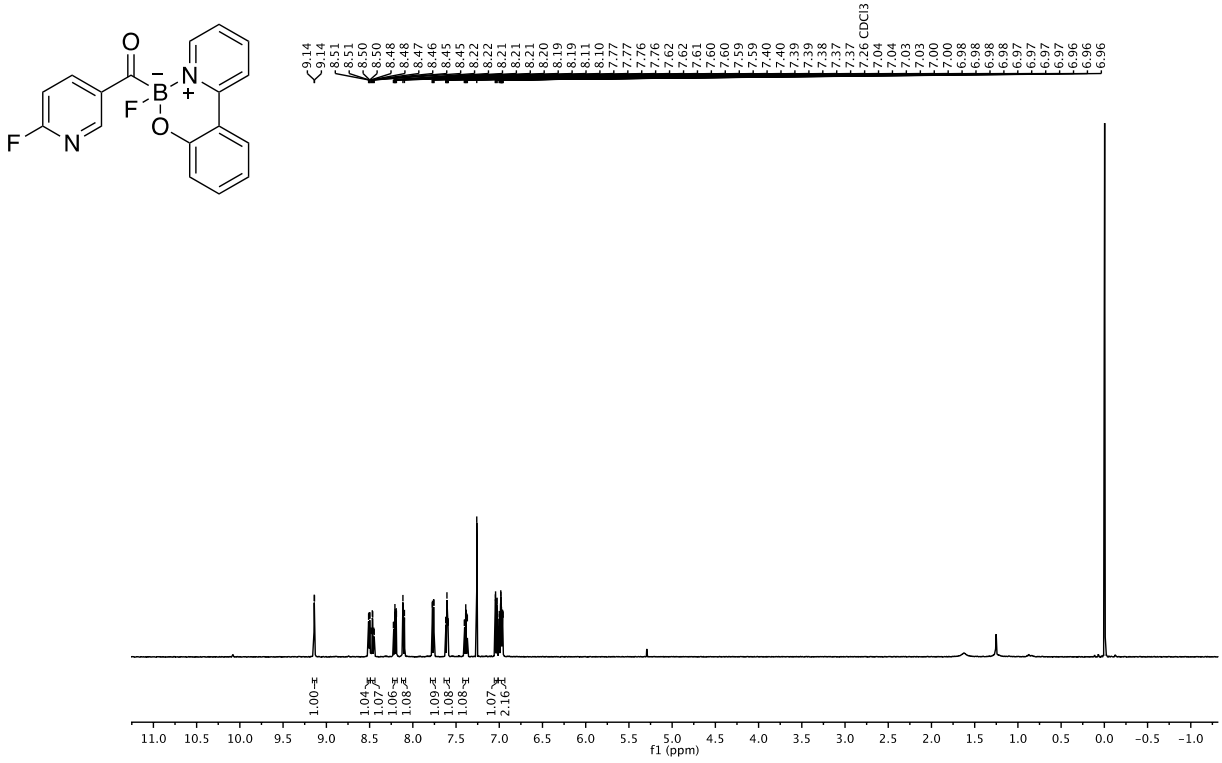
Compound [^{19}F]2c ^{19}F NMR (470 MHz, DMSO-d_6)



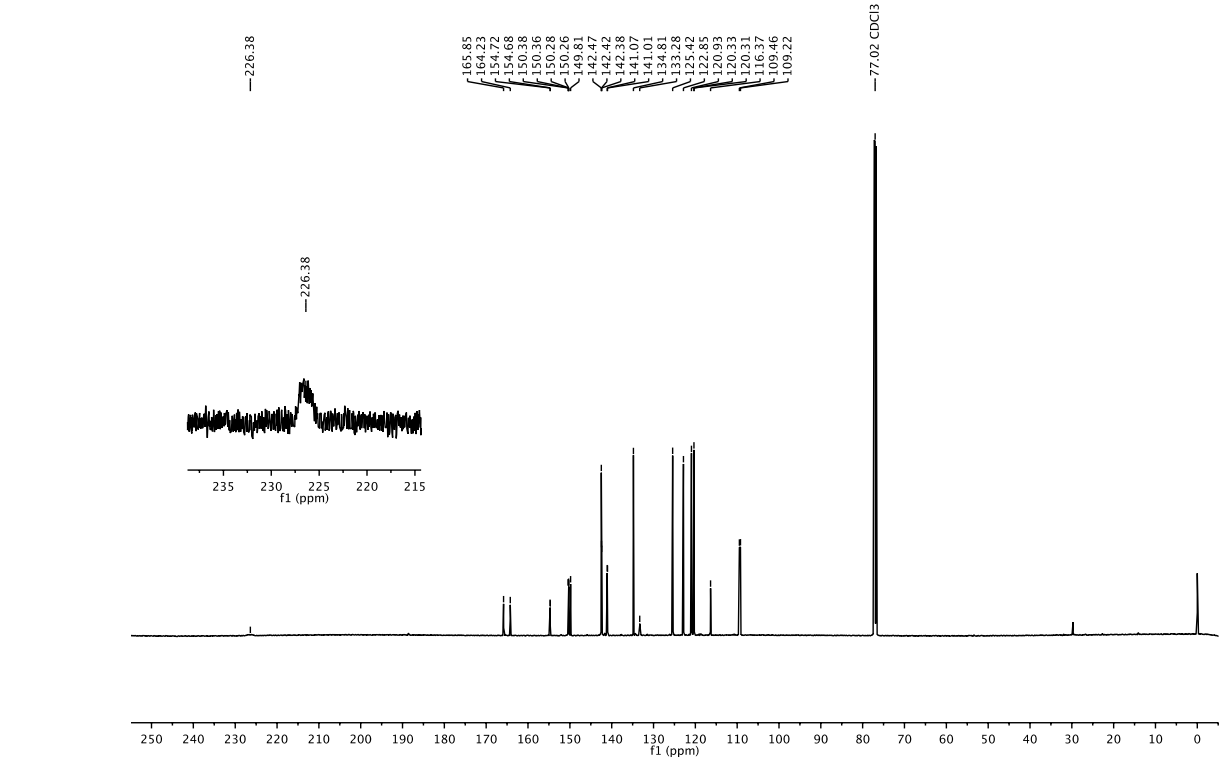
Compound [^{19}F]2c ^{11}B NMR (160 MHz, DMSO-d_6)



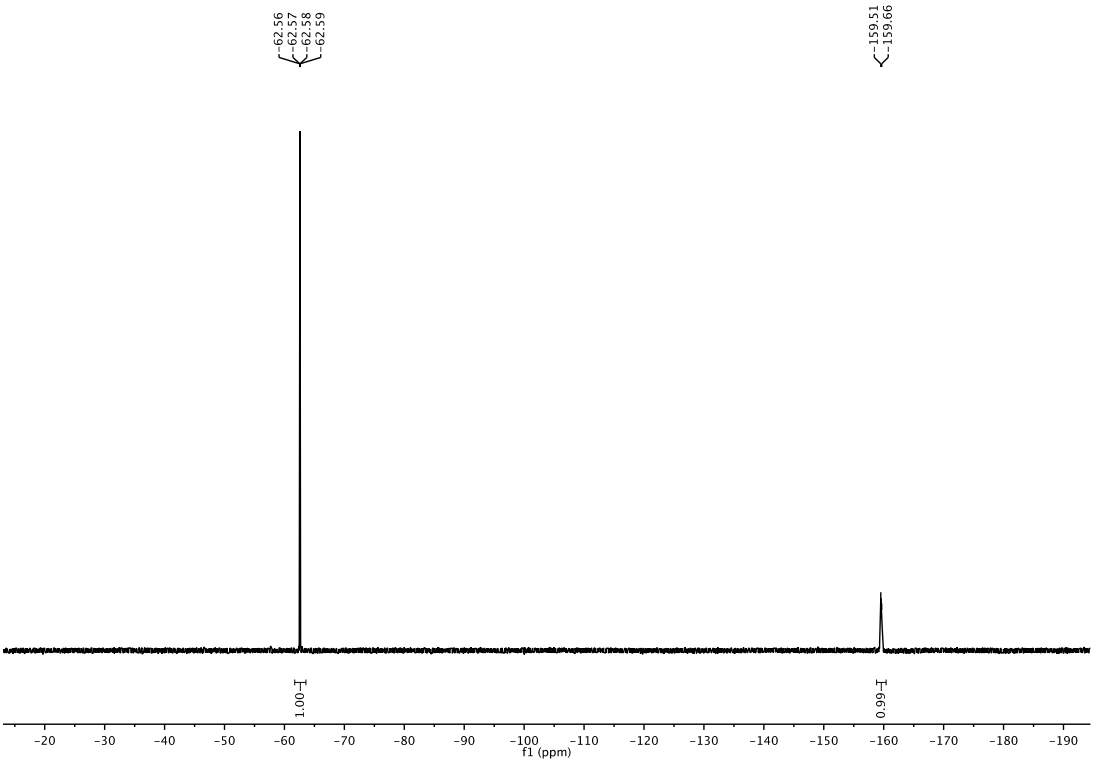
Compound [^{19}F]2e ^{1}H NMR (600 MHz, $CDCl_3$)



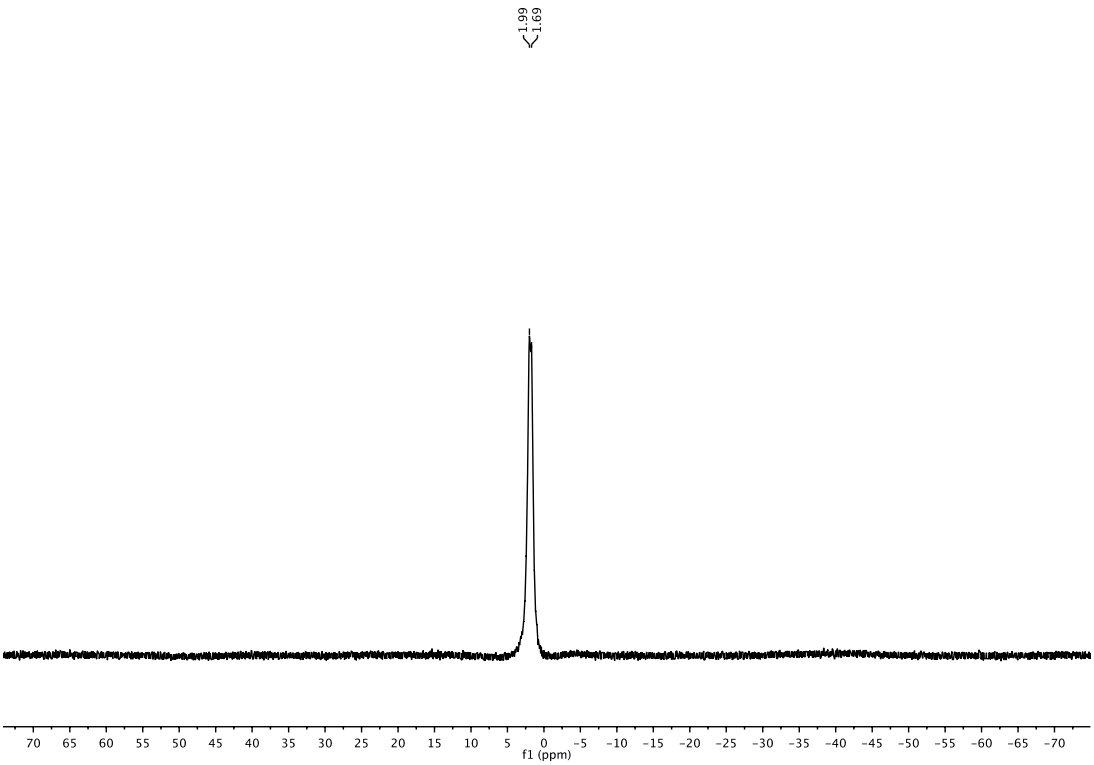
Compound [^{19}F]2e ^{13}C NMR (150 MHz, $CDCl_3$)



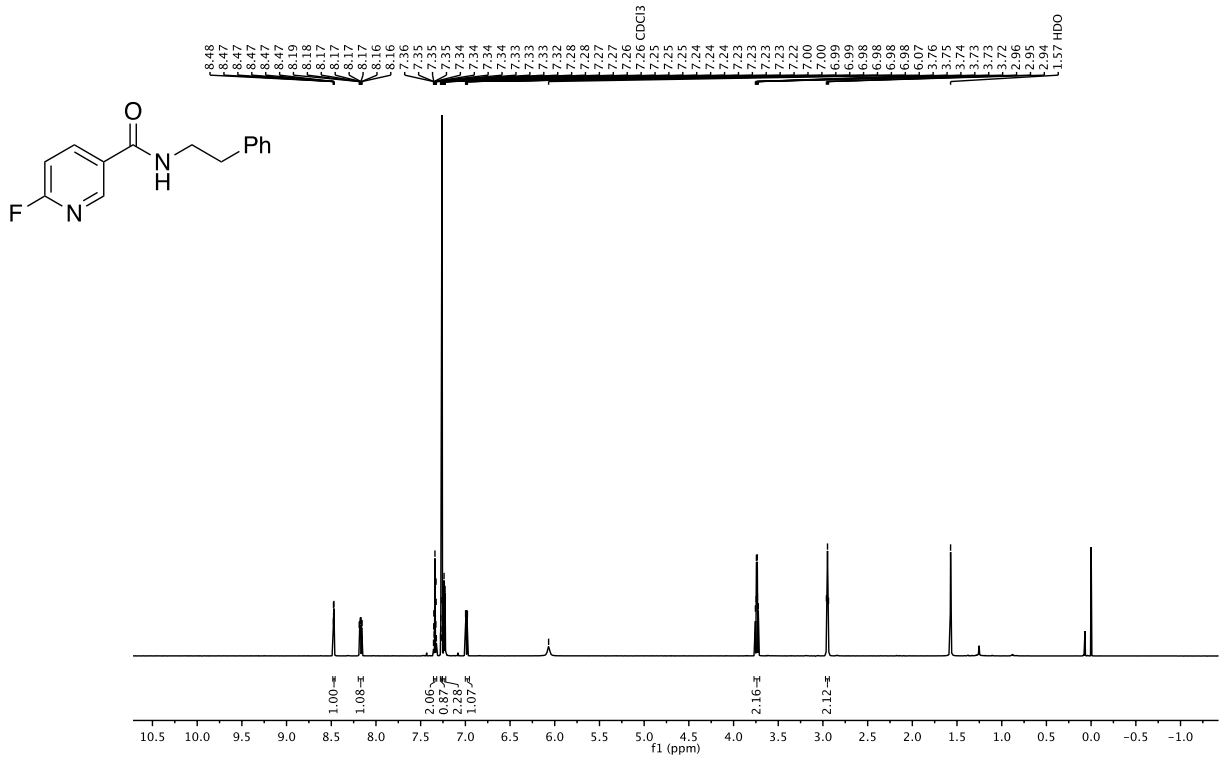
Compound [^{19}F]2e ^{19}F NMR (470 MHz, $CDCl_3$)



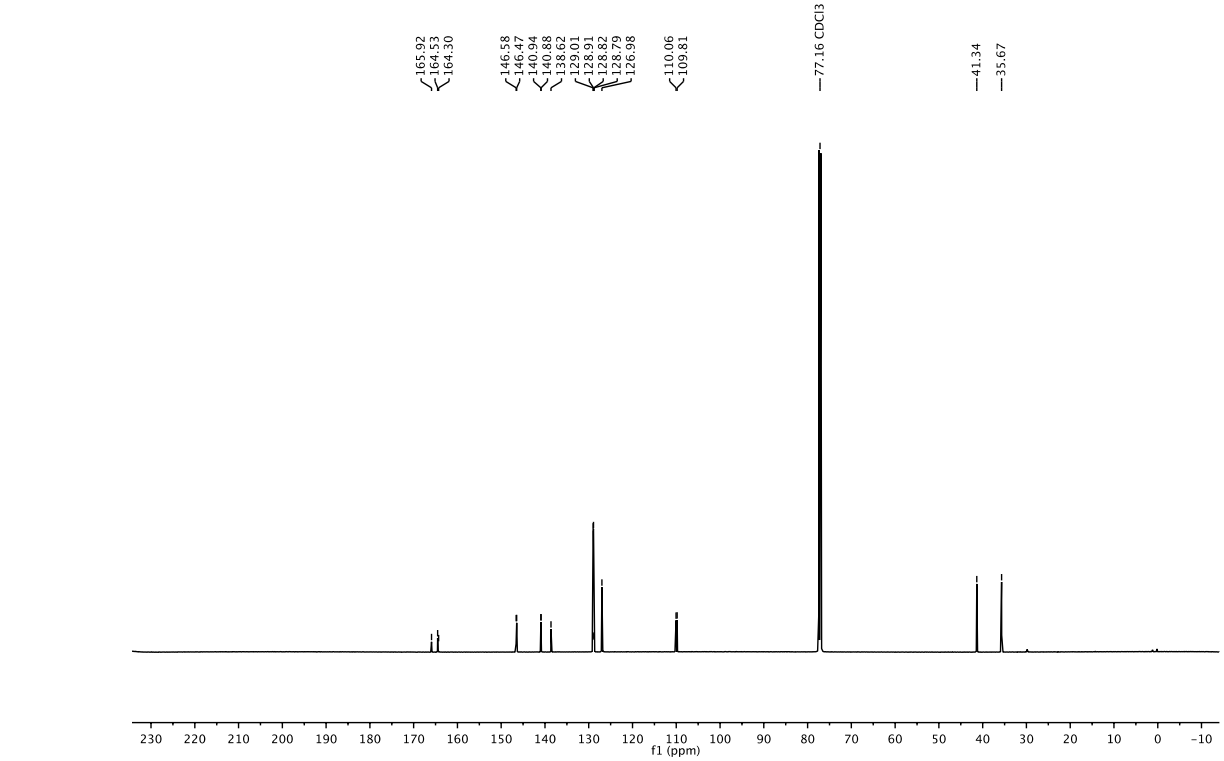
Compound [^{19}F]2e ^{11}B NMR (160 MHz, $CDCl_3$)



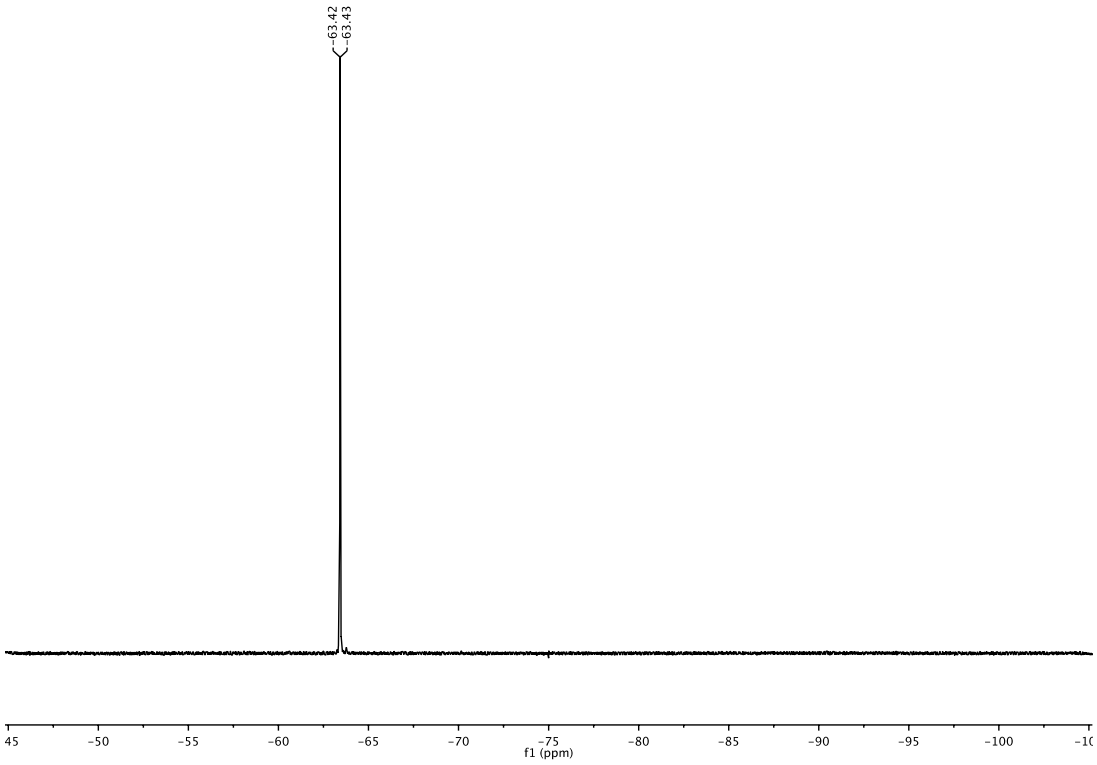
Compound [^{19}F]4a ^{1}H NMR (600 MHz, $CDCl_3$)



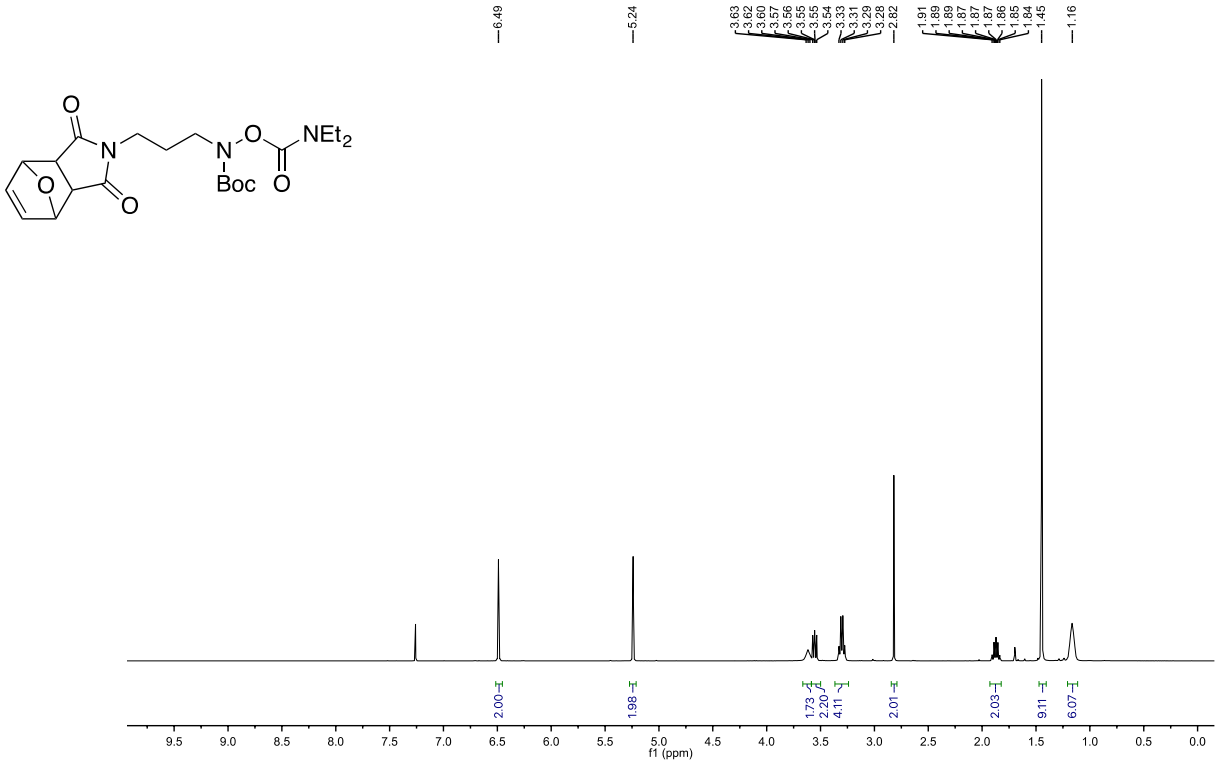
Compound [^{19}F]4a ^{13}C NMR (150 MHz, $CDCl_3$)



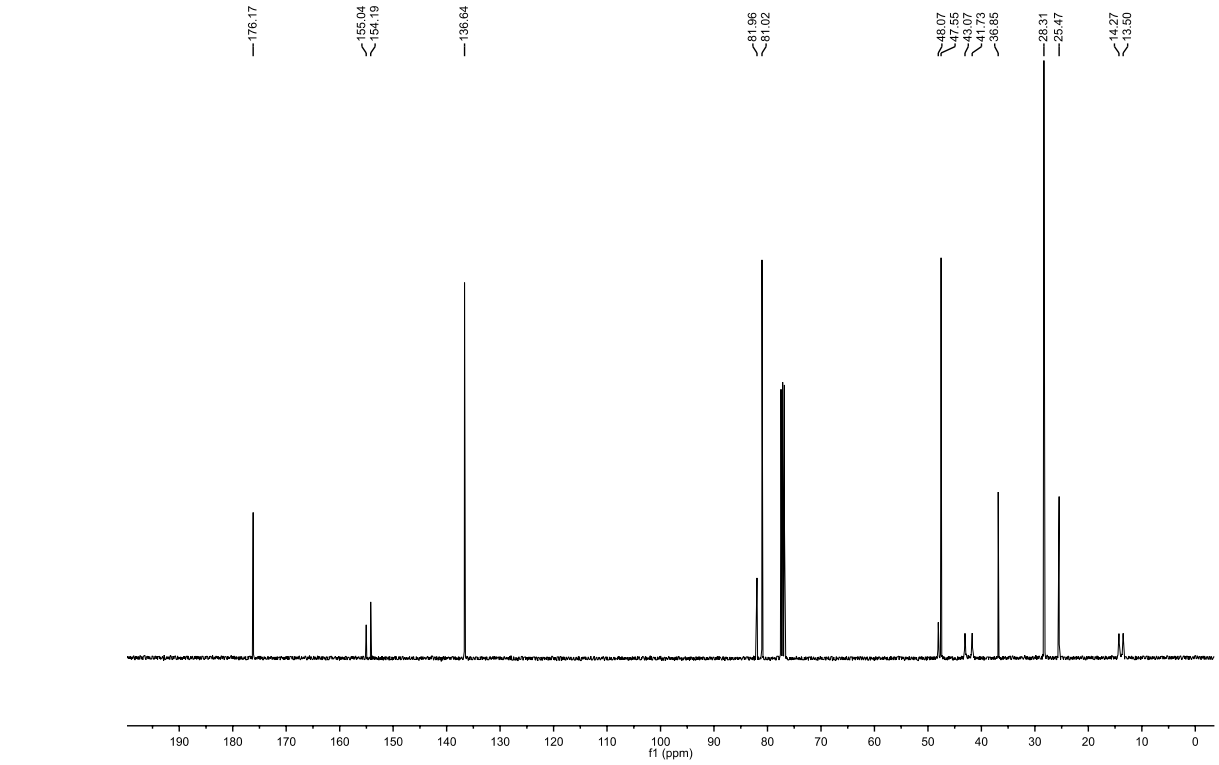
Compound [^{19}F]4a ^{19}F NMR (470 MHz, $CDCl_3$)



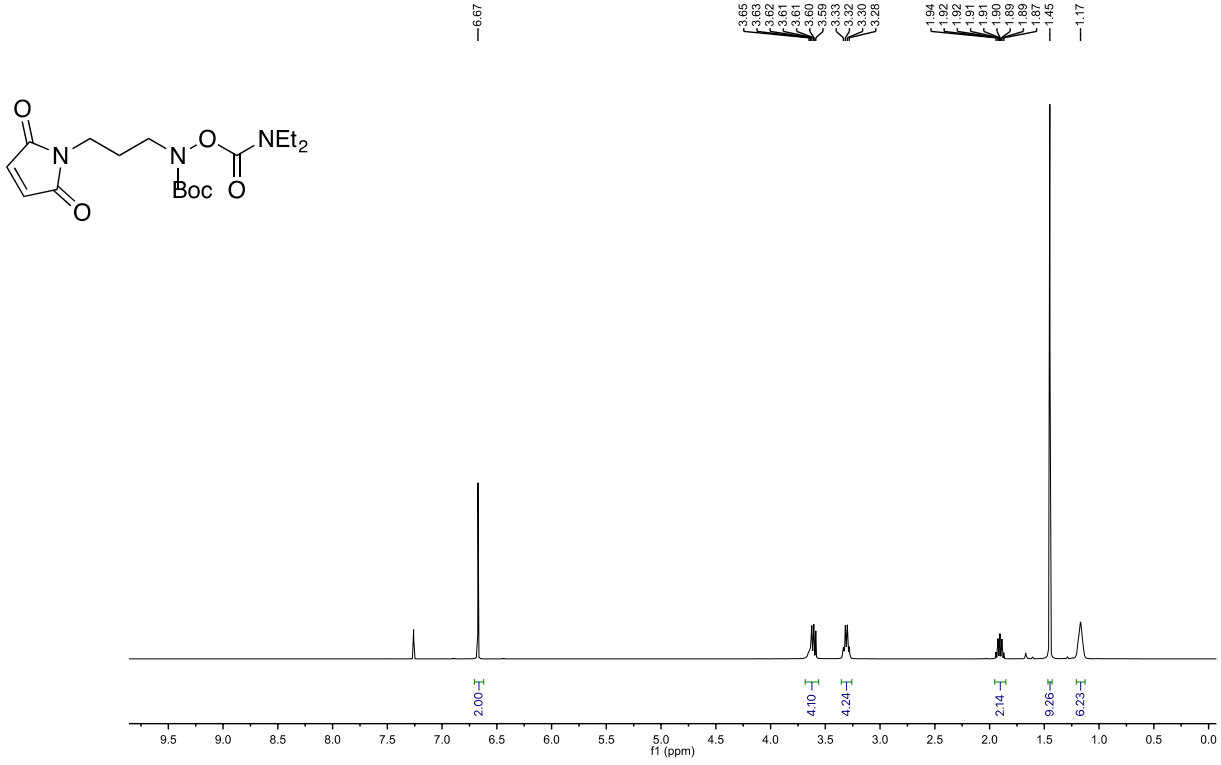
Compound 009 ^{1}H NMR (400 MHz, $CDCl_3$)



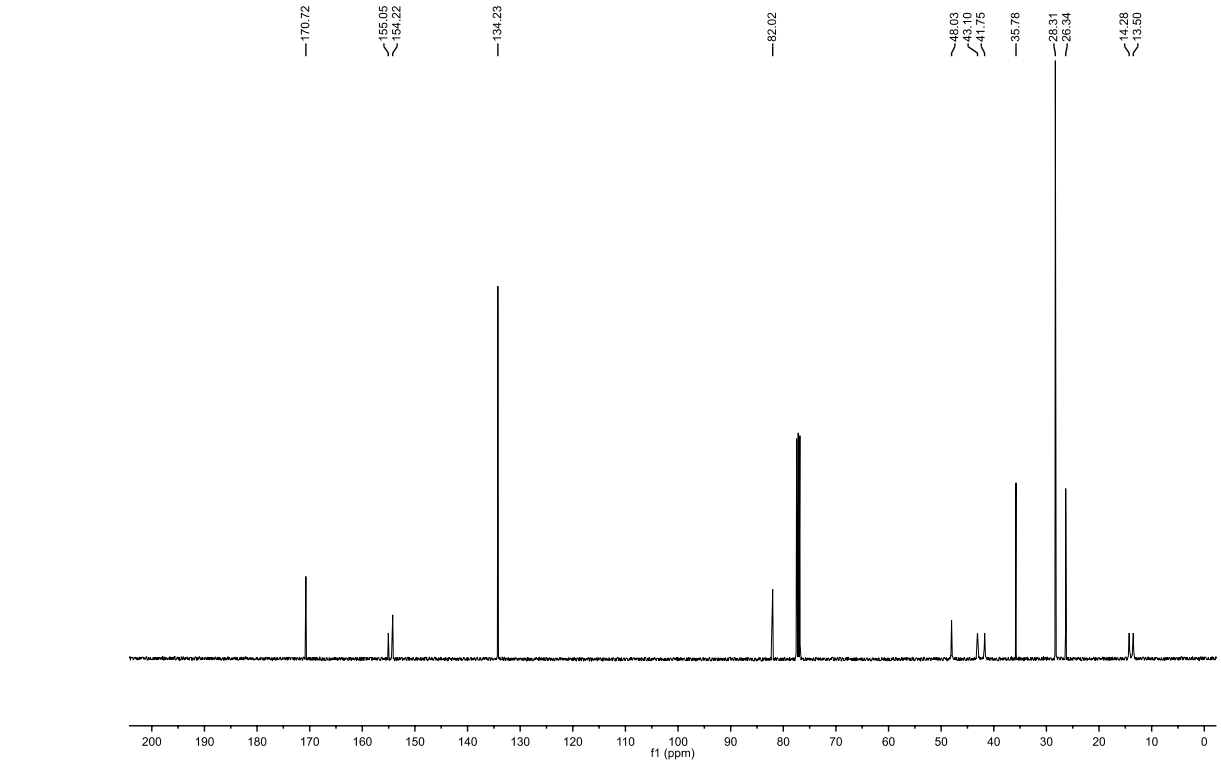
Compound 009 ^{13}C NMR (101 MHz, $CDCl_3$)



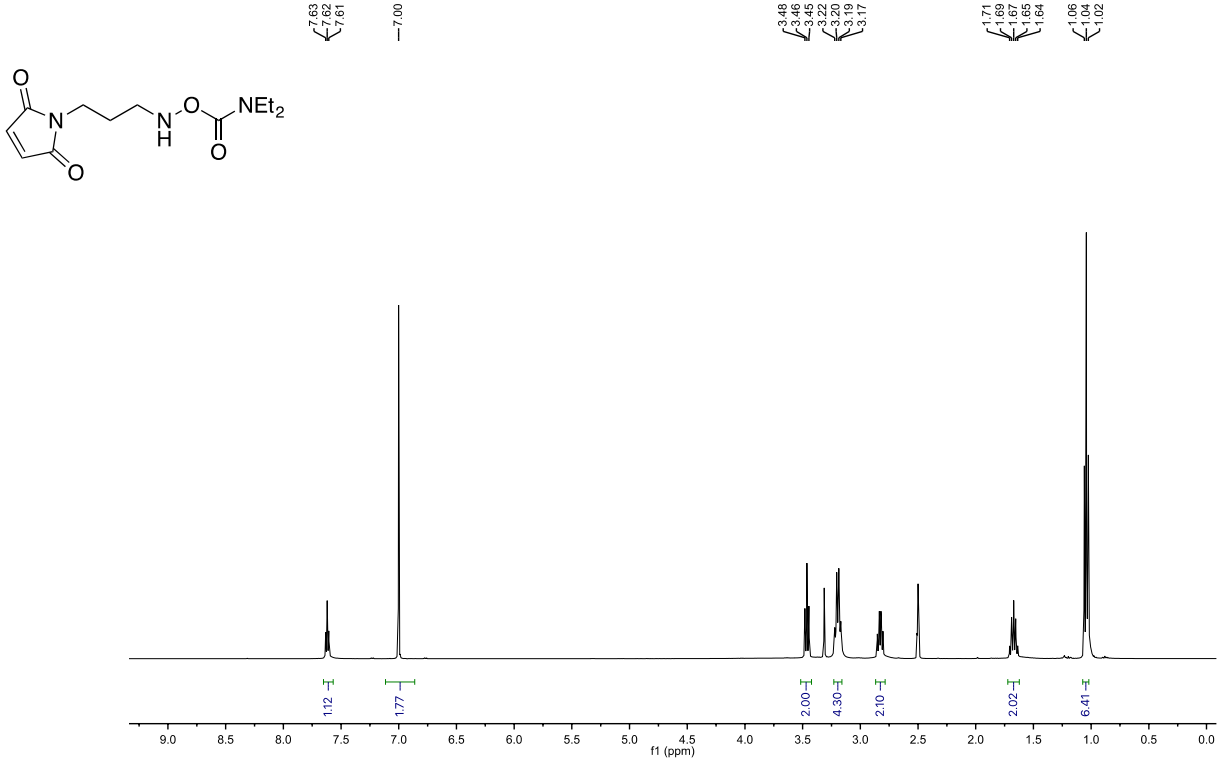
Compound 010 ^{1}H NMR (400 MHz, $CDCl_3$)



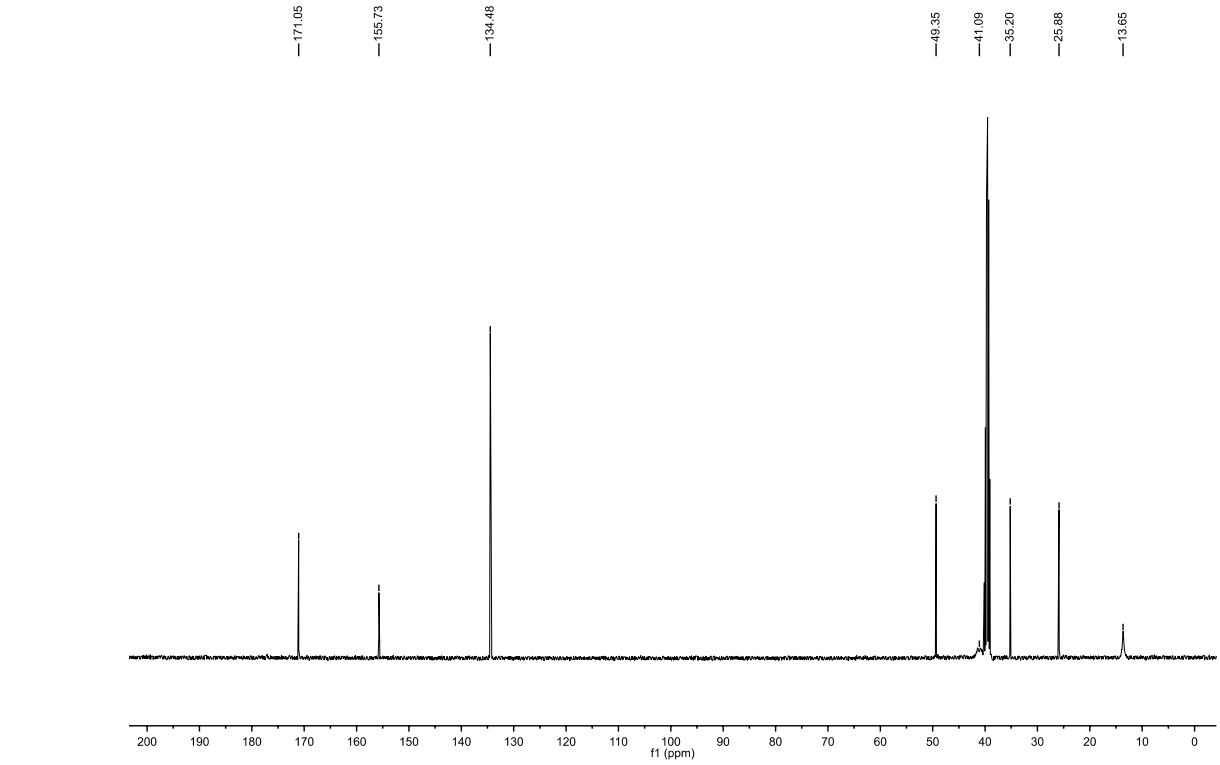
Compound 010 ^{13}C NMR (101 MHz, $CDCl_3$)



Compound 011 ^{1}H NMR (400 MHz, d_6-DMSO)

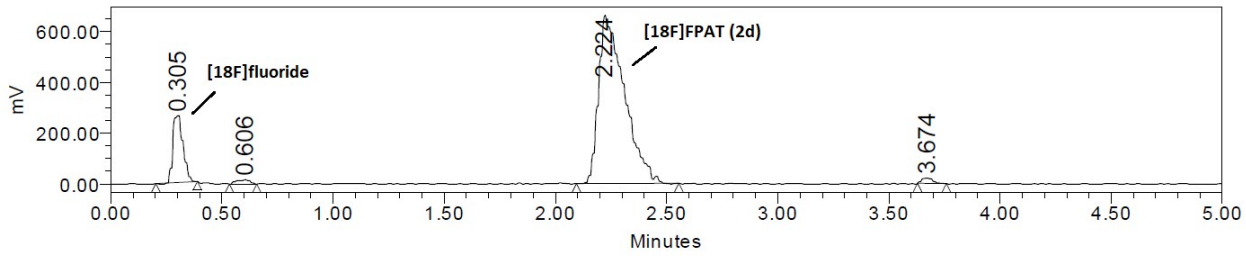


Compound 011 ^{13}C NMR (101 MHz, d_6-DMSO)



7. γ- and UV-HPLC chromatograms

UPLC analysis during optimization of 2d radiosynthesis



Channel Description: Channel 1

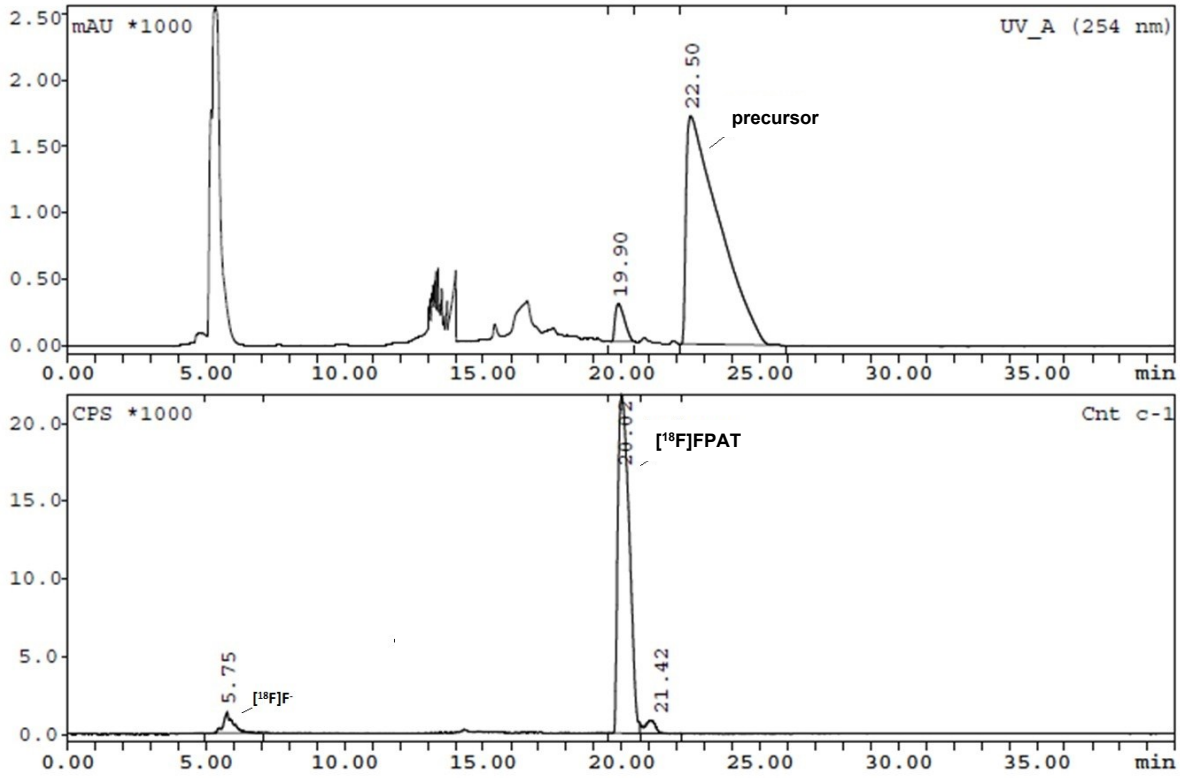
Channel Description:

	RT	Area	% Area	Height
1	0.305	843855	13.29	262654
2	0.606	67614	1.06	16019
3	2.224	5363075	84.46	661069
4	3.674	75358	1.19	21994

column: Acquity UPLC BEH C18, 2.1 × 50 mm, 1.7 µm (Waters)

mobile phase (system A): aq NH_4HCO_3 10mM, pH (solvent A), CH_3CN (solvent B); flow 0.6 mL/min; 0–0.5 min: 5% B, 0.5–2.5 min: 5–60% B, 2.5 – 2.8 min: 60% B, 2.8 – 2.9 min: 60 – 5% B, 2.9 – 3.0 min: 5% B; UV = 280/254 nm

Semipreparative HPLC purification of [^{18}F]FPAT



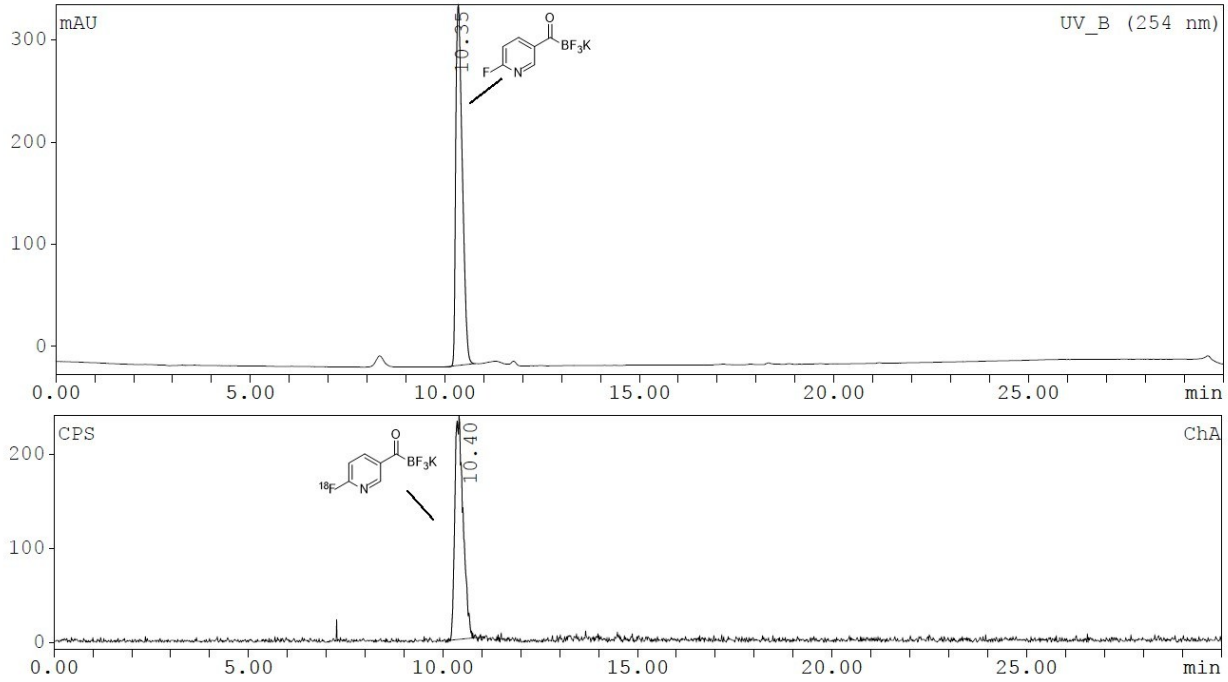
Integration Cnt c-1

Substance	R/T min	Type	Area Counts	%Area %
Reg #1	5.75	BB	32492.4	4.82
Reg #2	20.02	BD	617573.5	91.69
Reg #3	21.42	DB	23449.3	3.48
Sum in ROI			673515.2	
Area			775209.0	
Ext. BKG			0.00 CPS	

column: Gemini, C18, 10 × 250 mm, 5 µm

mobile phase (**system B**): aq. KOAc 10 mM, adjusted to pH 4 by addition of AcOH (solvent A), CH_3CN (solvent B); flow 4 mL/min; 0 – 5 min: 5% B, 5 – 15 min: 5 – 27% B, 15 – 30 min: 27% B, 30 – 35 min: 27 – 40% B, 35 – 40 min 40% B; UV = 254 nm

-γ-HPLC of compound 2d ([^{18}F]FPAT) and UV-HPLC of compound [^{19}F]2d (co-injection)



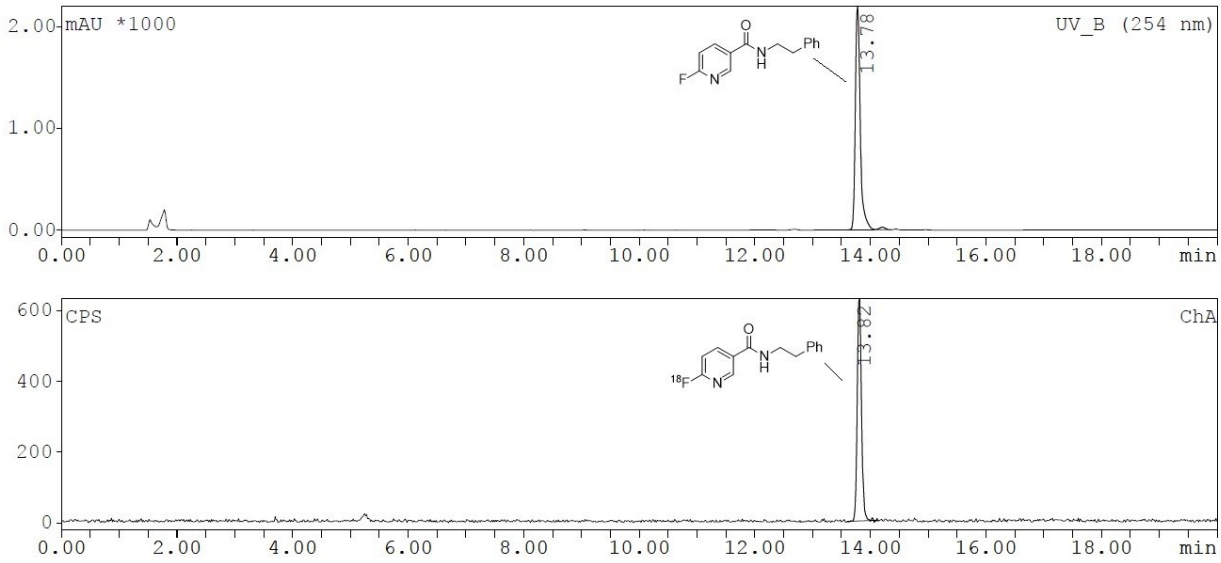
Integration ChA

Substance	R/T min	Type	Area Counts	%Area %
Reg #1	10.40	BB	3513.531	100.00
Sum in ROI			3513.531	100.00
Area (total)			7810.564	
Ext. BKG.			0.00 CPS	

column: LiChrospher 100RP-18, 4 mm × 250 mm, 5 µm

mobile phase (system D): H_2O (0.1% TFA) (solvent A), CH_3CN (0.1% TFA) (solvent B); flow 1 mL/min; 0 – 4 min: 5% B, 4 – 22 min: 5 – 90% B, 22 – 26 min: 90% B, 26 – 28 min: 90 – 5% B; 28 – 30 min: 5% B; UV = 254/280 nm

-γ-HPLC of compound 4a and UV-HPLC of compound [^{19}F]4a (co-injection)

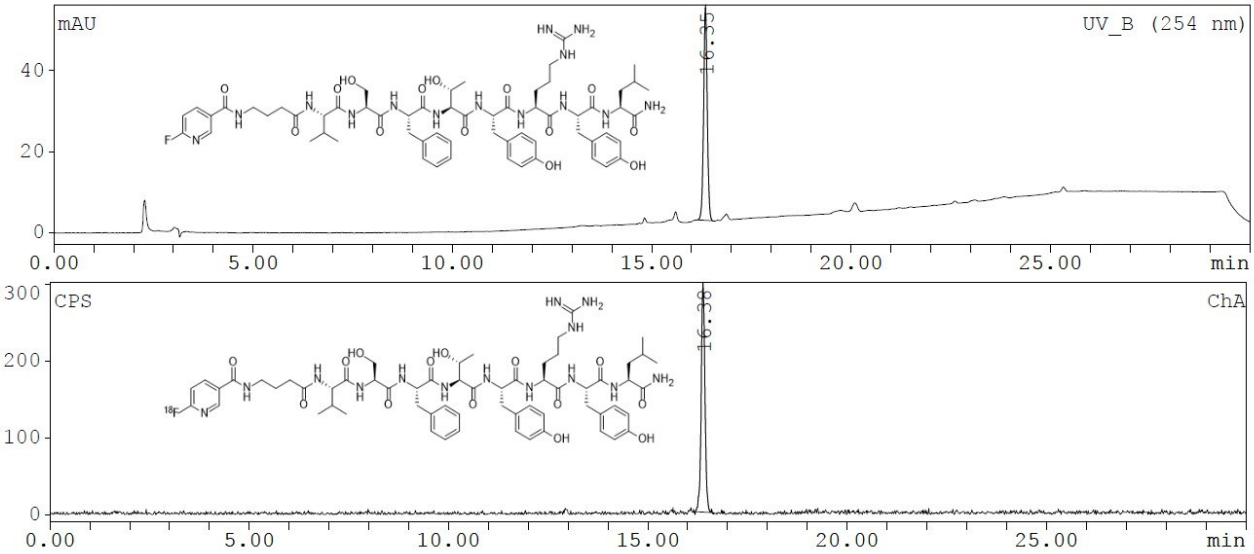


Integration ChA

Substance	R/T min	Type	Area Counts	%Area %
Reg #1	13.82	BB	3198.168	100.00
Sum in ROI			3198.168	100.00
Area (total)			9776.303	
Ext. BKG.			0.00 CPS	

column: Atlantis T3, 4.6×150 mm, 3 µm
mobile phase (system C): aq. NH_4OAc 3.75 mM + 0.05% AcOH (solvent A), CH_3CN (solvent B); flow 1 mL/min; 0 – 2 min: 5% B, 2 – 12 min: 5 – 60% B, 12 – 13 min: 60-5% B, 13 – 20 min: 5% B; UV = 254/280 nm

-γ-HPLC of compound 4b and UV-HPLC of compound [^{19}F]4b (co-injection)



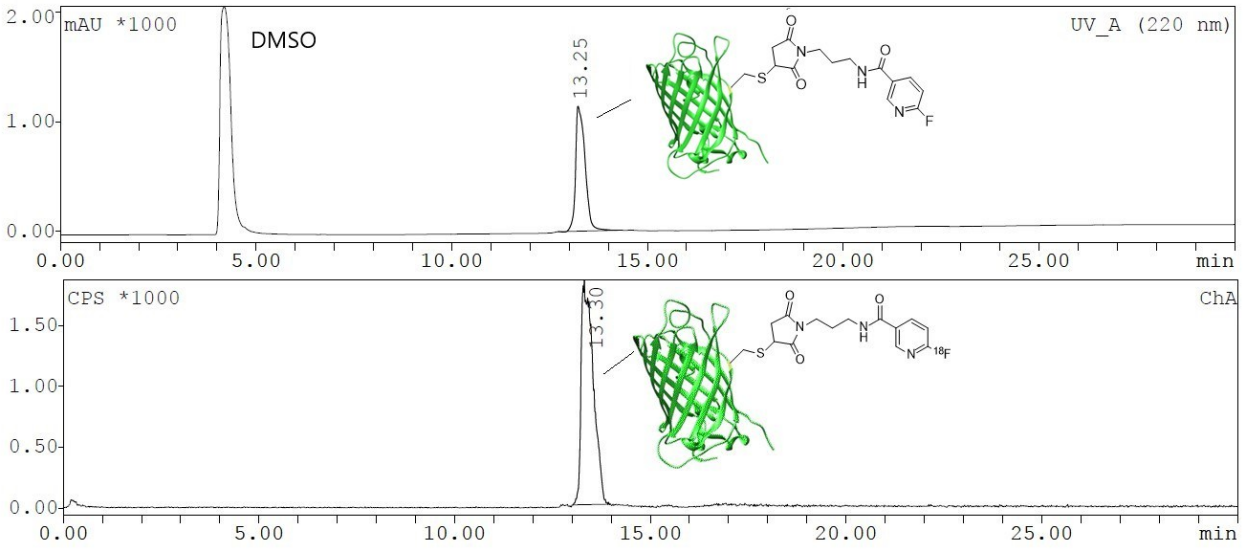
Integration UV_B (254 nm)

Substance	R/T min	Type	Area mAU*min	%Area %
Reg #1	16.35	BB	336.4429	100.00
Sum in ROI			336.4429	100.00

column: LiChrospher 100RP-18, 4 mm × 250 mm, 5 µm

mobile phase (system D): H_2O (0.1% TFA) (solvent A), CH_3CN (0.1% TFA) (solvent B); flow 1 mL/min; 0 – 4 min: 5% B, 4 – 22 min: 5 – 90% B, 22 – 26 min: 90% B, 26 – 28 min: 90 – 5% B; 28 – 30 min: 5% B; UV = 254/280 nm

-γ-HPLC of compound 4c and UV-HPLC of compound [^{19}F]4c (co-injection)



Integration ChA

Substance	R/T min	Type	Area Counts	%Area %
Reg #1	13.30	BB	39581.39	100.00
Sum in ROI			39581.39	100.00
Area (total)			61726.30	
Ext. BKG.			0.00 CPS	

column: Zorbax 300SB-C18, 4.6 × 150 mm, 5 µm

mobile phase (system E): H_2O (0.1% TFA) (solvent A), CH_3CN (0.1% TFA) (solvent B); flow 1 mL/min; 0 – 3 min: 10% B, 3 – 20 min: 10 – 95% B, 20 – 25 min: 95% B, 25 – 27 min: 95 – 10% B; 27 – 30 min: 10% B; UV = 254/280 nm

8. References

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