Supporting Information for:

Deoxyfluoro-D-trehalose (FDTre) analogues as potential PET probes for imaging mycobacterial infection: rapid synthesis and purification, conformational analysis, and uptake by mycobacteria

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Supplementary figures

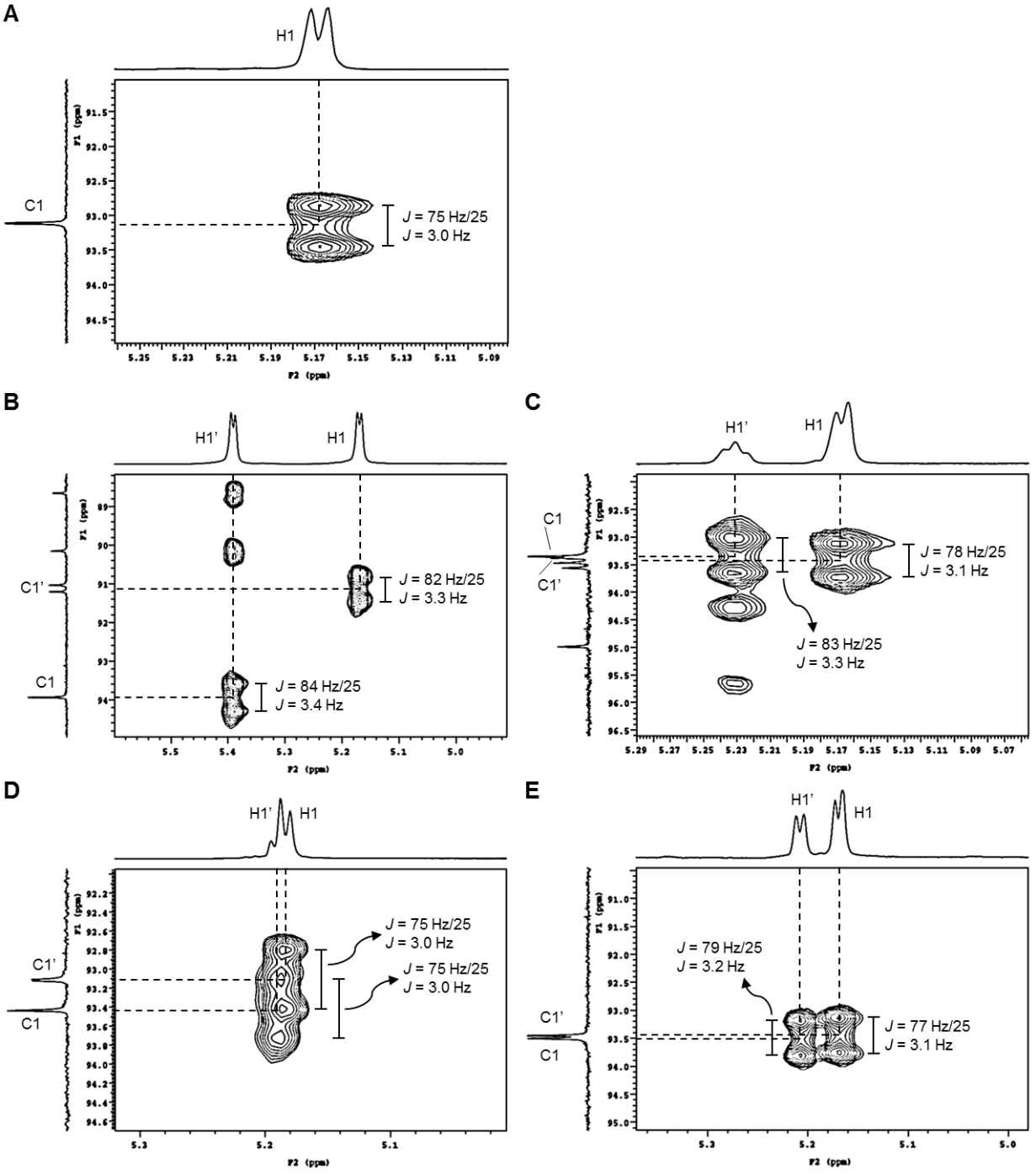

Figure S1. EXSIDE spectra showing long-range heteronuclear couplings across the glycosidic bond for (A) trehalose, (B) ^{19}F-2-FDTre, (C) ^{19}F-3-FDTre, (D) ^{19}F-4-FDTre, and (E) ^{19}F-6-FDTre. Dashed lines indicate cross-peaks between H1–C1' and H1'–C1 for each compound. $^{3}J_{COCH}$ constants were measured from EXSIDE spectra doublets using a scaling factor of 25, as shown.

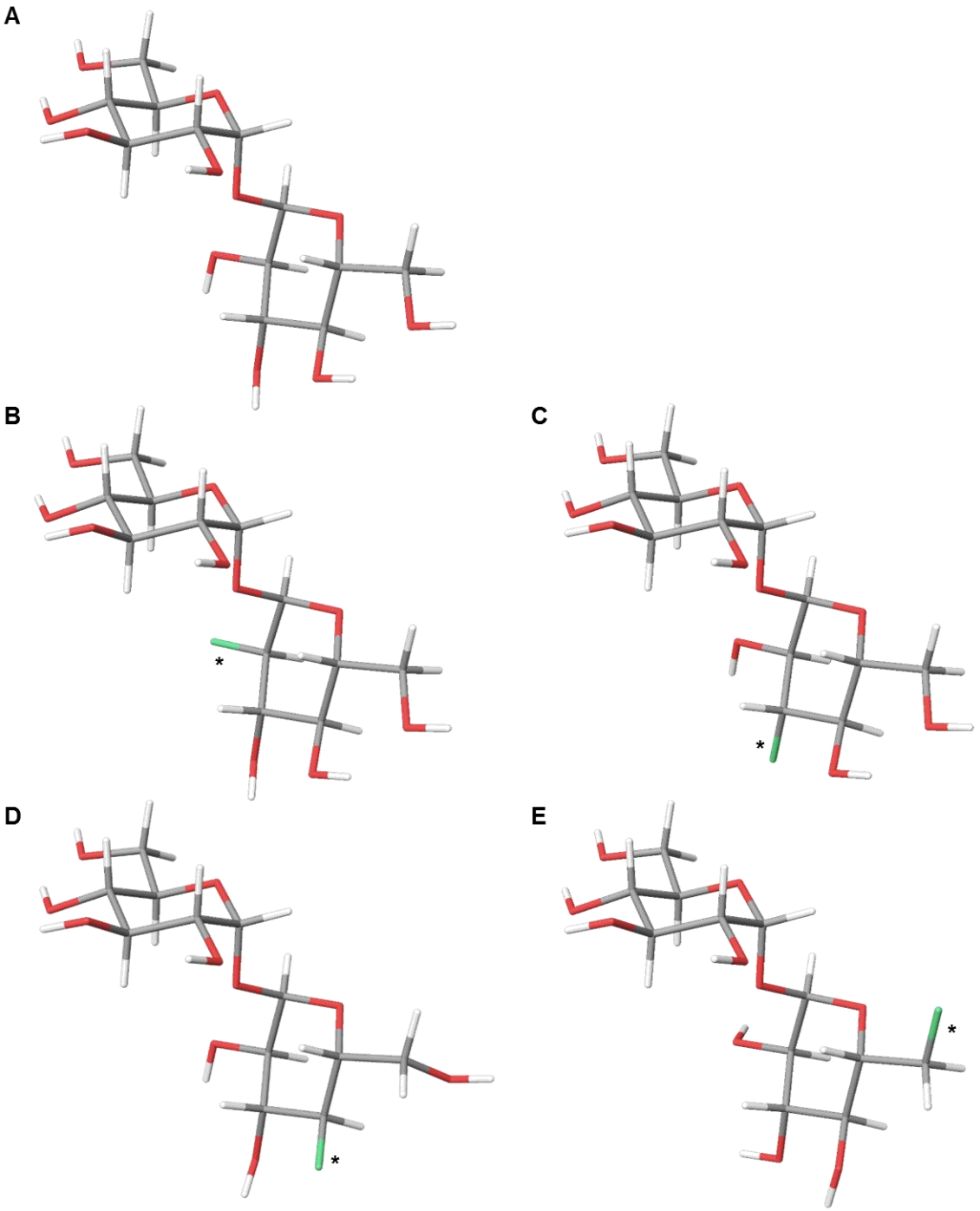

Figure S2. MM3*-derived lowest energy conformers of (A) trehalose, (B) ^{19}F-2-FDTre, (C) ^{19}F-3-FDTre, (D) ^{19}F-4-FDTre, and (E) ^{19}F-6-FDTre using NMR-determined glycosidic dihedral angles (see Table 1 in manuscript) as constraints. The fluorine atom is colored green and marked with an asterisk (*) in (B–E). Images generated in MacroModel version 11.2.

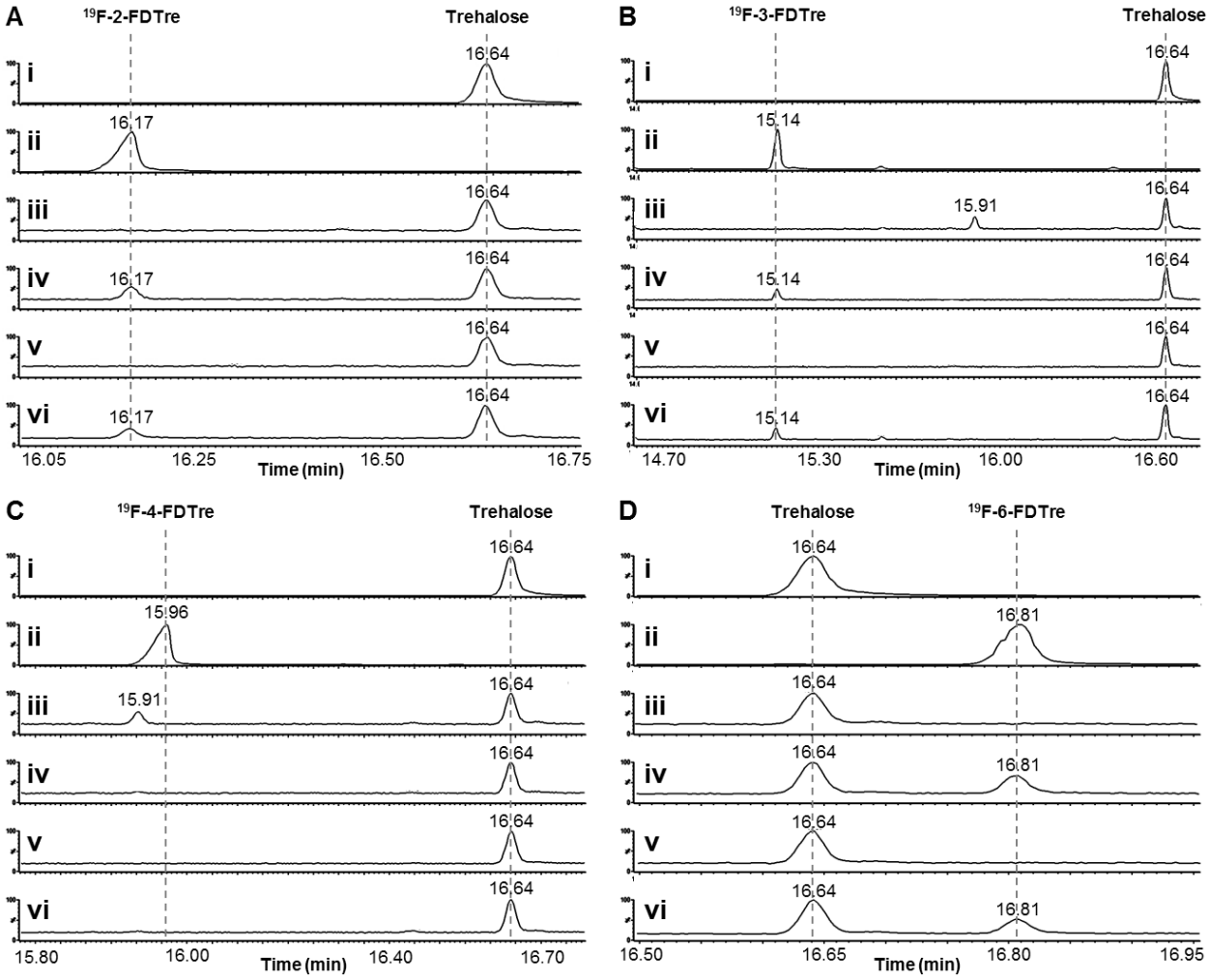

Figure S3. GC-MS data evaluating SugABC-LpqY-dependent uptake of (A) ^{19}F-2-FDTre, (B) ^{19}F-3-FDTre, (C) ^{19}F-4-FDTre, and (D) ^{19}F-6-FDTre by *M. smegmatis*. Standards or cytosolic extracts from untreated or ^{19}F-FDTre-treated (25 μM) cells were dried, TMS-derivatized, and analyzed by GC-MS according to the procedure on page S10. (i) Trehalose standard; (ii) ^{19}F-FDTre standard; (iii) untreated *M. smegmatis* wild type; (iv) ^{19}F-FDTre-treated *M. smegmatis* wild type; (v) ^{19}F-FDTre-treated *M. smegmatis* Δ*sugC* mutant; ^{19}F-FDTre-treated *M. smegmatis* Δ*sugC::sugC* complement.

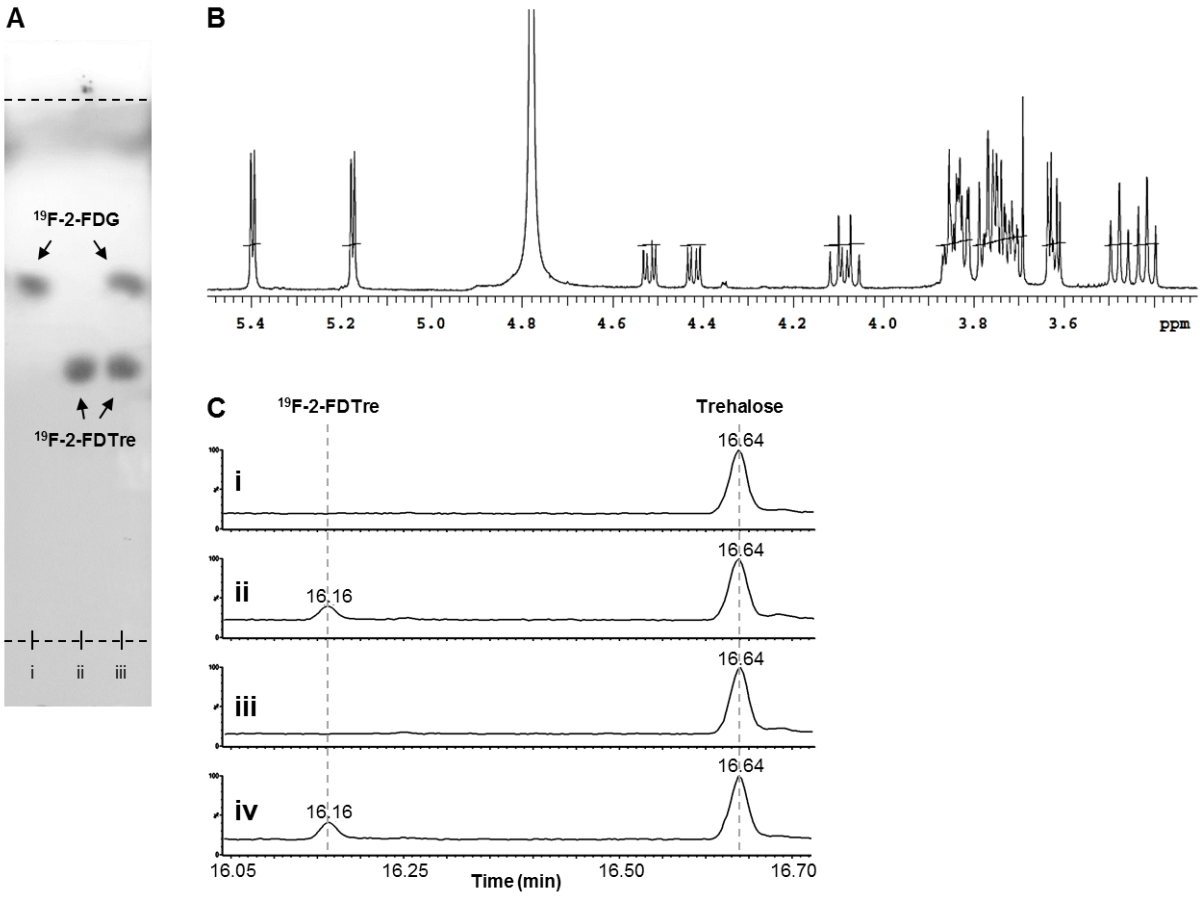

Figure S4. Data for rapid synthesis, purification, and administration of ^{19}F-2-FDTre to mycobacteria. According to the procedure on page S11, TreT catalysis was used to convert ^{19}F-2-FDG to ^{19}F-2-FDTre (15 min), which was then purified by spin dialysis/ion exchange (45 min) and immediately administered to *M. smegmatis* cultures. (A) TLC analysis of the reaction product confirmed quantitative conversion of ^{19}F-2-FDG to ^{19}F-2-FDTre. Lanes: (i) ^{19}F-2-FDG standard; (ii) TreT reaction product; (iii) co-spot. The TLC plate was developed in *n*-butanol/ethanol/water 5:3:2 and stained with 5% H_2SO_4 in ethanol. Dashed lines represent the origin (bottom) and solvent front (top). (B) ^{1}H NMR spectrum (500 MHz, D_2O) of ^{19}F-2-FDTre generated through the accelerated synthesis process. (C) GC-MS data of SugABC-LpqY-dependent uptake of rapidly prepared ^{19}F-2-FDTre by *M. smegmatis*. Samples were prepared according to the procedures on pages S10 and S11. (i) Untreated *M. smegmatis* wild type; (ii) ^{19}F-2-FDTre-treated *M. smegmatis* wild type; (iii) ^{19}F-2-FDTre-treated *M. smegmatis* Δ*sugC* mutant; (iv) ^{19}F-2-FDTre-treated *M. smegmatis* Δ*sugC::sugC* complement. Retention times for trehalose and ^{19}F-2-FDTre, indicated by dashed lines, matched those of authentic standards (see Figure S3A).

General experimental for synthesis

Materials and reagents were obtained from commercial sources without further purification unless otherwise noted. ^{19}F-FDG analogues were obtained from CarboSynth. UDP-Glucose was obtained from Abcam. Anhydrous solvents were obtained either commercially or from an alumina column solvent purification system. Chemical synthesis reactions were carried out in oven-dried glassware under inert gas. Analytical TLC was performed on glass-backed silica 60 Å plates (thickness 250 µm) from Dynamic Adsorbents and detected by charring with 5% H_2SO_4 in ethanol. Column chromatography was performed using flash-grade silica gel 32-63 µm (230-400 mesh) from Dynamic Adsorbents. NMR spectra were obtained at room temperature on Varian INOVA 500 (^{1}H, ^{13}C) or Varian Mercury 300 (^{19}F) instruments, with NMR spectra recorded at 500, 125, and 282 MHz, respectively. Coupling constants (*J*) are reported in hertz (Hz). See Page S9 for more details on NMR experiments. High-resolution electrospray ionization (HR ESI) mass spectra were obtained in negative ion mode using a Waters LCT Premier XE with raffinose as the lock mass for accurate mass determinations.

Expression and purification of TreT

TreT was expressed and purified as described[1] with some modifications. Top10 *E. coli* expressing His-tagged TreT[1] was plated on LB agar containing 100 µg/mL ampicillin. A single colony was picked and used to inoculate a 3 mL LB/ampicillin culture, which was grown overnight in a shaking incubator at 37 °C. This 3 mL culture was then used to inoculate a 750 mL culture of Terrific Broth containing 100 µg/mL ampicillin. Once the culture reached mid-log phase, TreT expression was induced by addition of arabinose to a final concentration of 1 mM. The culture was grown in a shaking incubator overnight at 37 °C. Cells were centrifuged at 4,000 x g at 4 °C and washed with PBS. After centrifugation at 4,000 x g at 4 °C, the pellet was resuspended by vortexing in 20 mL of equilibration/lysis/wash buffer (500 mM NaCl, 50 mM NaH_2PO_4, 20 mM imidazole, pH 8.0) containing a dissolved EDTA-free protease inhibitor mini tablet (Pierce). Resuspended pellets were transferred to a beaker and sonicated (3 x 45 s, 75% amplitude). To clarify the lysate, sonicated cells were centrifuged at 15,000 x g for 30 min at 4 °C and then passed through a syringe filter (0.45 µm). Next, TreT was purified from lysates using an Akta fast protein liquid chromatography (FPLC) system equipped with a 5 mL nickel affinity column (GE Healthcare HisTrap HP). After equilibration of the column with wash buffer (50 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole, pH 8.0), TreT was eluted using a linear gradient of elution buffer (50 mM NaH_2PO_4, 500 mM NaCl, 250 mM imidazole, pH 8.0) from 1–100% over 60 min at a flow rate of 1 mL/min. Fractions containing TreT were collected and dialyzed into Tris-HCl buffer (300 mM NaCl, 50 mM Tris, pH 8.0), analyzed by SDS-

PAGE to verify purity, and assessed for concentration using UV-Vis spectroscopy (NanoDrop 2000). TreT was stored in Tris-HCl buffer at 4 °C.

Luminescence glycosyltransferase assay

TreT activity was measured using the UDP-Glo glycosyltransferase assay (Promega). For each experimental condition, three replicates of TreT reaction mixture containing 1 µg TreT, 200 mM NaCl (or other concentration), 20 mM $MgCl_2$, 10 mM glucose (or analogue), and 0.4 mM UDP-glucose in 25 µL 50 mM Tris-HCl buffer at pH 7.0 (or other buffer/pH) were set up in a white 384-well microplate. The reactions were incubated for 2 min at room temperature (or other temperature). After equilibrating to room temperature (if necessary), 25 µL UDP detection reagent were added, which coupled UDP production to a luciferase reaction. After incubation at room temperature for 60 min, the luminescence signal was recorded using a microplate reader (Tecan Infinite F200 Pro). The luminescence signal was fitted to a standard curve made from a dilution series of known UDP concentrations measured in the same 384-well microplate. Relative light units (RLUs) given by the luminescence reader were converted to percent enzyme activity. In all experiments, reactions without acceptor substrate were used as negative controls.

Chemoenzymatic synthesis of FDTre analogues

General method for chemoenzymatic synthesis. To a 15 mL conical tube was added ^{19}F-FDG analogue (0.080 mmol, 14.5 mg), UDP-glucose (0.160 mmol, 97.6 mg), and $MgCl_2$ (0.080 mmol, 16.3 mg). TreT in Tris-HCl buffer (50 mM Tris, 300 mM NaCl, pH 8.0), plus additional Tris-HCl buffer if needed, were added to achieve a final volume of 4 mL and a final protein concentration of 10 µM. The reaction was incubated at 70 °C with shaking at 300 rpm for 1 h, then the tube was cooled by placing it on ice. An Amicon Ultra-15 centrifugal filter unit (nominal molecular weight limit (NMWL) 10 kDa) was pre-rinsed with 3 mL deionized water three times by centrifugation at 3214 x g for 20 min to remove trace glycerol in the membrane. After transferring the cooled enzymatic reaction mixture to the pre-rinsed centrifugal filter unit, it was spun at 3214 x g for 20 min. The upper chamber of the centrifugal filter unit was rinsed two times with 3 mL of deionized water and centrifuged again using the same speed and time. After discarding the upper chamber of the centrifugal filter unit, mixed-bed ion-exchange resin (3 g of Bio-Rad Bio-Rex RG 501-X8) was added to the tube and stirred for 1 h at room temperature. Next, the supernatant was decanted and filtered. The remaining resin was rinsed two times with 5 mL of deionized water and the supernatant was decanted, filtered, and combined with the rest of the product. TLC was performed using *n*-butanol/ethanol/deionized water 5:3:2. The purified product was concentrated by rotary evaporation or lyophilization.

2-Deoxy-2-fluoro-α,α-D-trehalose (*^{19}*F-2-FDTre, compound 9). From 14.2 mg of ^{19}F-2-FDG, obtained 21.1 mg ^{19}F-2-FDTre (79%). ^{1}H NMR (500 MHz, D_2O): δ 5.39 (d, J = 3.5 Hz, 1 H, H1'), 5.17 (d, J = 3.5 Hz, 1 H, H1), 4.46 (ddd, J = 3.5, 9.5 Hz, $J_{H,F}$ = 49.0 Hz, 1 H, H2'), 4.08 (dt, J = 9.0 Hz, $J_{H,F}$ = 13.0 Hz, 1 H, H3'), 3.88–3.80 (m, 3 H, H5', H6$_{a/b}$ or H6$_{a/b}$'), 3.80–3.69 (m, 4 H, H3, H5, H6$_{a/b}$ or H6$_{a/b}$'), 3.62 (dd, J = 3.5, 10.0 Hz, 1 H, H2), 3.47 (t, J = 10.0 Hz, 1 H, H4'), 3.41 (t, J = 10.0 Hz, 1 H, H4). ^{13}C NMR (125 Hz, D_2O): δ 93.93 (C1), 91.11 (d, $J_{C,F}$ = 21 Hz, C1'), 89.40 (d, $J_{C,F}$ = 187 Hz, C2'), 72.50 (C5), 72.12 (C5'), 71.01 (d, $J_{C,F}$ = 17 Hz, C3'), 70.86 (C2), 69.47 (C4), 68.98 (C3), 68.92 (C4'), 60.38 (C6 or C6'), 60.18 (C6 or C6'). ^{19}F NMR (282 MHz, D_2O): δ –201.8 (dd, $J_{H,F}$ = 13.5, 49.0 Hz). HR ESI MS negative mode: calcd. for $C_{12}H_{21}ClFO_{10}$ $[M+Cl]^-$ *m/z,* 379.0807; found, 379.0800.

3-Deoxy-3-fluoro-α,α-D-trehalose (*^{19}*F-3-FDTre, compound 10). From 15.5 mg of ^{19}F-3-FDG, obtained 21.8 mg ^{19}F-3-FDTre (74%). ^{1}H NMR (500 MHz, D_2O): δ 5.23 (t, J = 4.0 Hz, 1 H, H1'), 5.17 (d, J = 3.5 Hz, 1 H, H1), 4.74 (dt, J = 9.5 Hz, $J_{H,F}$ = 54.5 Hz 1 H, H3'), 3.92 (ddd, J = 4.0, 9.5 Hz, $J_{H,F}$ = 13.5 Hz, 1 H, H2'), 3.87–3.73 (m, 8 H, H3, H5, H6$_{ab}$, H4', H5', H6$_{ab}$'), 3.63 (dd, J = 4.0, 9.5 Hz, $J_{H,F}$ = 13.5 Hz, 1 H, H2), 3.44 (t, J = 9.5 Hz, 1 H, H4). ^{13}C NMR (125 Hz, D_2O): δ 94.28 (d, $J_{C,F}$ = 178 Hz, C3'), 93.43 (d, $J_{C,F}$ = 11.4 Hz, C1'), 93.35 (C1), 72.41 (C3), 72.14 (C5), 71.59 (d, $J_{C,F}$ = 6.6 Hz, C5'), 70.88 (C2), 69.54 (C4), 69.45 (d, $J_{H,F}$ = 18.0 Hz, C2'), 67.80 (d, $J_{C,F}$ = 17.1 Hz, C4'), 60.40 (C6 or C6'), 60.00 (C6 or C6'). ^{19}F NMR (282 MHz, D_2O): δ –200.4 (ddt, $J_{H,F}$ = 2.8, 13.0, 54.2 Hz). HR ESI MS negative mode: calcd. for $C_{12}H_{21}ClFO_{10}$ $[M+Cl]^-$ *m/z,* 379.0807; found, 379.0794.

6-Deoxy-6-fluoro-α,α-D-trehalose (*^{19}*F-6-FDTre, compound 12). From 15.6 mg of ^{19}F-6-FDG, obtained 21.9 mg ^{19}F-6-FDG (74%). ^{1}H NMR (500 MHz, D_2O): δ 5.21 (d, J = 3.5 Hz, 1 H, H1'), 5.17 (d, J = 4.0 Hz, 1 H, H1), 4.74 (ddd, 1 H, J = 2.5, 10.0 Hz, $J_{H,F}$ = 47.0 Hz, H6$_a$' or H6$_b$'), 4.67 (dd, 1 H, J = 10.0 Hz, $J_{H,F}$ = 48.0 Hz, H6$_a$' or H6$_b$'), 3.96 (dd, J = 9.0 Hz, $J_{H,F}$ = 28.0 Hz, 1 H, H5'), 3.87–3.80 (m, 2 H, H5, H6$_a$ or H6$_b$), 3.86 (t, J = 10.0 Hz, 1 H, H3'), 3.84 (t, J = 9.5 Hz, 1 H, H3), 3.75 (dd, J = 5.0, 12.5 Hz, 1 H, H6$_a$ or H6$_b$), 3.66 (dd, J = 3.5, 9.5 Hz, 1 H, H2'), 3.63 (dd, J = 3.5,10.5 Hz, 1 H, H2), 3.55 (t, J = 9.5 Hz, 1 H, H4'), 3.44 (t, J = 10.0 Hz, 1 H, H4). ^{13}C NMR (125 Hz, D_2O): δ 93.50 (C1), 93.45 (C1'), 82.03 (d, $J_{C,F}$ = 168 Hz, C6'), 72.43 (C3'), 72.29 (C3), 72.15 (C5), 70.94 (d, $J_{C,F}$ = 10.5 Hz, C5'), 70.84 (C2 and C2' overlapping), 69.58 (C4), 68.45 (d, J = 6.6 Hz, C4'), 60.42 (C6). ^{19}F NMR (282 MHz, D_2O): δ –236.2 (dt, $J_{H,F}$ = 28.5, 47.1 Hz). HR ESI MS negative mode: calcd. for $C_{12}H_{21}ClFO_{10}$ $[M+Cl]^-$ *m/z,* 379.0807; found, 379.0786.

Chemical Synthesis of ^{19}F-4-FDTre

4-Deoxy-4-fluoro-α,α-D-trehalose (*^{19}*F-4-FDTre, compound 11). To a stirring solution of compound **18**[2] (500 mg, 0.461 mmol) in anhydrous tetrahydrofuran (0.95 mL) under an argon atmosphere at room temperature was added bis(2-methoxyethyl)aminosulfur trifluoride (BAST, 0.11 mL, 0.500 mmol)

dropwise. The solution was heated to 50 °C and allowed to stir overnight. TLC showed that the reaction was incomplete. An excess of BAST (0.54 mL, 2.50 mmol) was added dropwise and stirred for an additional 3 h at 50 °C, after which TLC showed completion. The product was diluted with ethyl acetate, then poured into a separatory funnel and washed sequentially with saturated aqueous $NaHCO_3$ and water. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated by rotary evaporation. The crude material was chromatographed on silica gel (toluene/ethyl acetate 20:1) to give the fluorinated intermediate (0.371 g, 74%). A portion of the intermediate (0.160 g, 0.149 mmol) was dissolved in anhydrous CH_2Cl_2 (1 mL), and a freshly prepared solution of $NaOCH_3$ in CH_3OH (2 mL) was added dropwise to achieve a final $NaOCH_3$ concentration of 0.05 M. After stirring overnight, TLC showed complete conversion of the starting material to a single polar product. Dowex H^+ resin was used to neutralize the reaction, after which the resin was filtered off and the solution was concentrated by rotary evaporation. The crude material was chromatographed on silica gel (CH_2Cl_2/CH_3OH 2.5:1) to give the desired product, which was re-suspended in deionized water, passed through a 0.2 µm syringe filter, and dried to give ^{19}F-4-FDTre (0.051 g, 98%; 73% from **18** over 2 steps). ^{1}H NMR (500 MHz, D_2O): δ 5.19 (d, J = 4.0 Hz, 1 H, H1'), 5.18 (d, J = 4.0 Hz, 1 H, H1), 4.36 (dt, J = 9.5 Hz, $J_{H,F}$ = 51.5 Hz, 1 H, H4'), 4.14 (dt, J = 10.5 Hz, $J_{H,F}$ = 15.5 Hz, 1 H, H3'), 4.04–3.98 (m, 1 H, H5'), 3.86–3.72 (m, 6 H, H3, H5, $H6_{ab}$, $H6_{ab}$'), 3.68 (dd, J = 4.0, 10.5 Hz, 1 H, H2'), 3.64 (dd, J = 4.0, 9.5 Hz, 1 H, H2), 3.44 (t, J = 9.5 Hz, 1 H, H4). ^{13}C NMR (125 Hz, D_2O): δ 93.43 (C1), 93.10 (C1'), 89.05 (d, $J_{C,F}$ = 179.3 Hz, C4'), 72.41 (C3 or C5), 72.14 (C3 or C5), 70.90 (C2), 70.67 (d, $J_{C,F}$ = 8.6 Hz, C3'), 70.42 (d, J = 18.1 Hz, C2'), 69.55 (C4), 69.54 (d, $J_{H,F}$ = 23.9 Hz, C5') 60.41 (C6 or C6'), 59.74 (C6 or C6'). ^{19}F NMR (282 MHz, D_2O): δ –199.0 (dd, $J_{H,F}$ = 15.8, 51.4 Hz). HR ESI MS negative mode: calcd. for $C_{12}H_{20}FO_{10}$ $[M-H]^-$ *m/z*, 343.1041; found, 343.1048.

Conformational analysis of FDTre analogues

NMR experiments. Deuterium-exchanged trehalose and ^{19}F-FDTre analogues were dissolved in D_2O (99.8%) to a final concentration of 100 mM. 300 µL of the solution were transferred to a Shigemi NMR tube matched to D_2O. Argon was bubbled through the solution for 1 min and then the tube was sealed with parafilm. To obtain ^{1}H, ^{13}C, COSY, and HSQC spectra, samples were analyzed on a Varian INOVA 500 instrument at room temperature. For HSQC spectra, in-phase cross-peaks correspond to CH/CH_3 carbons, out-of-phase cross-peaks correspond to CH_2 carbons. ^{1}H NMR spectra were referenced to residual HDO peak at δ 4.78 ppm. To obtain ^{1}H-coupled ^{19}F NMR spectra, samples were analyzed on a Varian Mercury 300 instrument at room temperature. ^{19}F NMR spectra were referenced to trifluoroacetic acid at δ –76.55 ppm. Standard Varian pulse sequences were used for 1D and 2D experiments. NMR data were processed, analyzed, and plotted using Varian VnmrJ version 4.2 revision A.

For experimental determination of $^{3}J_{COCH}$ values, a Varian selexcit experiment with multifrequency excitation of the anomeric H1 and H1' protons was set up using a previously acquired 1D proton spectrum of the appropriate disaccharide. The selexcit experiment was used to configure a 2D excitation-sculptured indirect-detection NMR experiment (EXSIDE),[3] which enabled the measurement of long-range hetereonuclear coupling constants. Both the selexcit and EXSIDE experiments were part of the standard Varian VnmrJ software package. EXSIDE spectra were obtained using 18 scans in the F2 (^{1}H) dimension, 256 increments in the F1 dimension (^{13}C), a jnxh setting of 4, and a *J* scaling factor (N) of 25. Long-range couplings were observed as in-phase pairs in the F1 dimension of the 2D spectrum. $^{3}J_{COCH}$ values were determined from the EXSIDE spectra using the following equation:

$$J = \frac{([\delta_2 - \delta_1] * 125.7\ MHz)}{\mathrm{N}}$$

where δ_2 and δ_2 are the chemical shifts of each cross-peak in a pair, 125.7 MHz is the ^{13}C NMR frequency, and N is the *J* scaling factor 25. Next, the $^{3}J_{COCH}$ values were converted into approximate dihedral angles using the Karplus equation developed by Tvaroška and co-workers[4]:

$$J = 5.7cos^2(\theta) - 0.6cos^2(\theta) + 0.5$$

Molecular modeling. Molecular mechanics calculations employing the MM3* force field[5] were used to predict the solution conformations of trehalose and the ^{19}F-FDTre analogues. All MM3* calculations were performed in MacroModel version 11.2 in the Maestro environment (version 10.5) running on the Windows 10 operating system. First, structures for trehalose and the ^{19}F-FDTre analogues were built with the appropriate stereochemical configurations. The structures were energy-minimized with MM3* using the Polak-Ribiere Conjugate Gradient (PRCG) method and a maximum of 2500 iterations. Next, these structures were used to initiate conformational searches using the Monte Carlo Multiple Minimum (MCMM) torsional sampling algorithm on all rotatable bonds, including ring closure. 1000 structures were sampled using MCMM. All MM3* calculations were performed with water selected as the solvent. MCMM conformational searches were performed either without constraints or with glycosidic dihedral angle constraints determined experimentally by NMR as described above. The lowest-energy conformers were selected for visual comparison in the manuscript and Supporting Information.

^{19}F-FDTre uptake analysis in *M. smegmatis*

Cultures of *M. smegmatis* wild type, Δ*sugC* mutant, or Δ*sugC*::*sugC* complement[1] were generated by inoculating a single colony from a freshly streaked agar plate (with appropriate antibiotic, if needed) into 3 mL Middlebrook 7H9 liquid medium supplemented with ADC (albumin, dextrose, and catalase), 0.5%

glycerol, and 0.05% Tween-80 in a culture tube. Starter cultures were incubated at 37 °C with shaking until reaching log phase. Cultures were diluted to an OD_{600} of 0.5, then ^{19}F-FDTre was added from a 1 mM aqueous stock solution to a final concentration of 25 µM. Control cells received vehicle solution. Cultures were incubated for 3 h at 37 °C with shaking to achieve a final OD_{600} of 0.8–1.0. Cells were centrifuged (6500 rpm, 5 min) and washed three times, twice with PBS and once with deionized water. Cell pellets were resuspended in deionized water and lysed by boiling at 100 °C for 4 h with shaking (300 rpm). Insoluble debris from lysed cells was pelleted by centrifugation (3600 rpm, 5 min), after which the supernatant was transferred to a microcentrifuge tube and dried on a speedvac. The resulting residue, which contained water-soluble cytosolic metabolites, was trimethylsilyl (TMS)-derivatized by treatment with 50 µL anhydrous pyridine and 50 µL *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA). After incubation overnight at room temperature, samples were transferred to low-volume vials with embedded 250 µL inserts and analyzed by GC-MS. GC-MS was performed on a Waters GCT Premier with an Agilent 7890A equipped with a CTC Analytics PAL autosampler and an SGE Forte BPX5 capillary column (10 m length, 0.1 mm inner diameter, 0.1 µm film thickness). A temperature gradient of 100–300 °C (hold at 100 °C for 1 min, linear increase of 10 °C/min for 20 min, hold at 300 °C for 4 min) and a split ratio of 1:50 were used. Chromatograms and spectra collected were analyzed using MassLynx 4.1 software. Standards of ^{19}F-FDTre and trehalose were derivatized and analyzed identically. Data shown are representative of at least two independent experiments.

Protocol for the rapid synthesis, purification, and administration of FDTre probes

Cultures of *M. smegmatis* wild type, Δ*sugC* mutant, or Δ*sugC*::*sugC* complement in 7H9 liquid medium were incubated at 37 °C with shaking until reaching log phase. Meanwhile, a 1 mL TreT reaction employing ^{19}F-2-FDG as the acceptor substrate was carried out to synthesize ^{19}F-2-FDTre. The general method for chemoenzymatic synthesis (page S7) was used with some modifications to accelerate the synthesis and purification processes. After TreT enzyme was added to initiate the reaction, it was incubated with shaking for 15 min, then transferred to a pre-rinsed Amicon Ultra-15 centrifugal filter unit (NMWL 10 kDa). The reaction tube was washed two times with 1.5 mL deionized water. The washings were transferred to the centrifugal filter unit, which was then spun at 3214 x g for 10 min. The upper chamber of the filter unit was discarded, and mixed-bed ion exchange resin (0.75 g of Bio-Rad Bio-Rex RG 501-X8) was added to the tube and stirred for 25 min at room temperature. The supernatant was decanted and filtered through a 0.2 µm syringe filter, giving a 4 mL aqueous stock solution of ^{19}F-2-FDTre with an approximate concentration of 5 mM (the initial substrate concentration of 20 mM was diluted 4x from washing the reaction tube). 15 µL of the ^{19}F-2-FDTre solution were added to each strain of *M. smegmatis* (adjusted to an OD_{600} of 0.5) to achieve a final concentration of 25 µM (the remainder of the ^{19}F-2-FDTre was analyzed by TLC and ^{1}H

NMR as shown in Figure S4A and S4B, respectively). The cultures were incubated for 1 h at 37 °C with shaking, after which the cells were processed and analyzed by GC-MS as described above. See Figure S4C for uptake data.

References for Supporting Information

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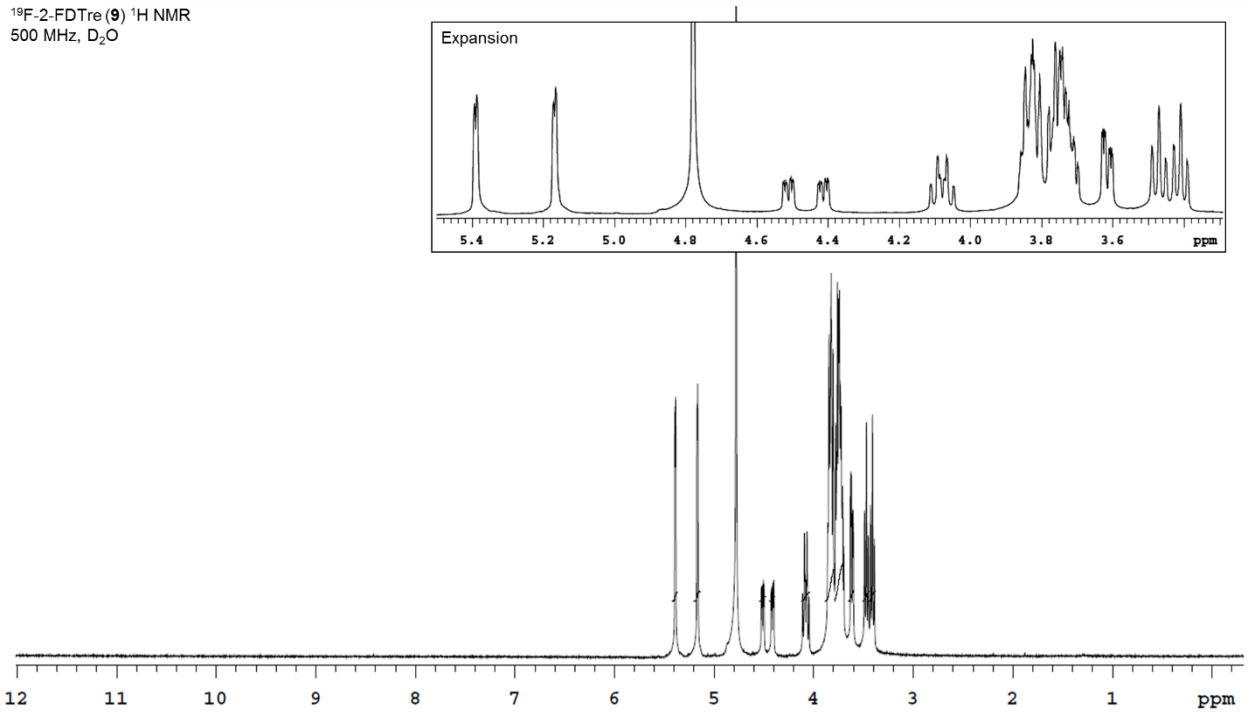
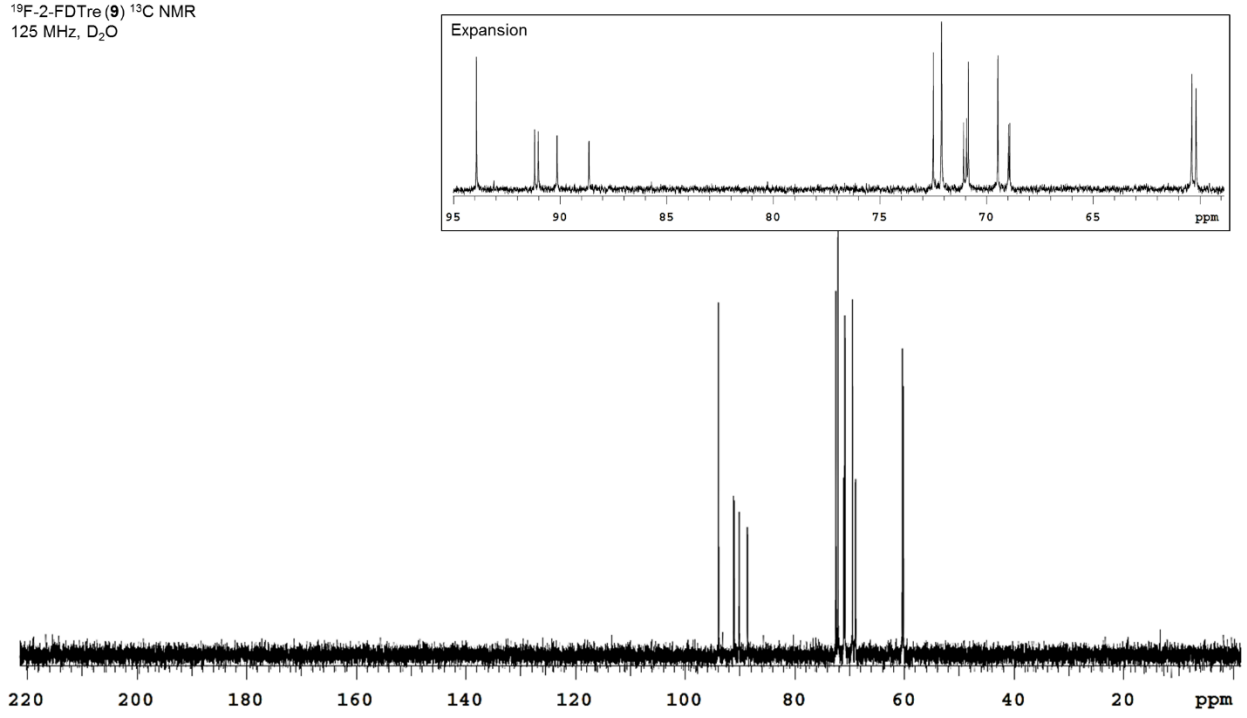
NMR spectra for ^{19}F-FDTre analogues

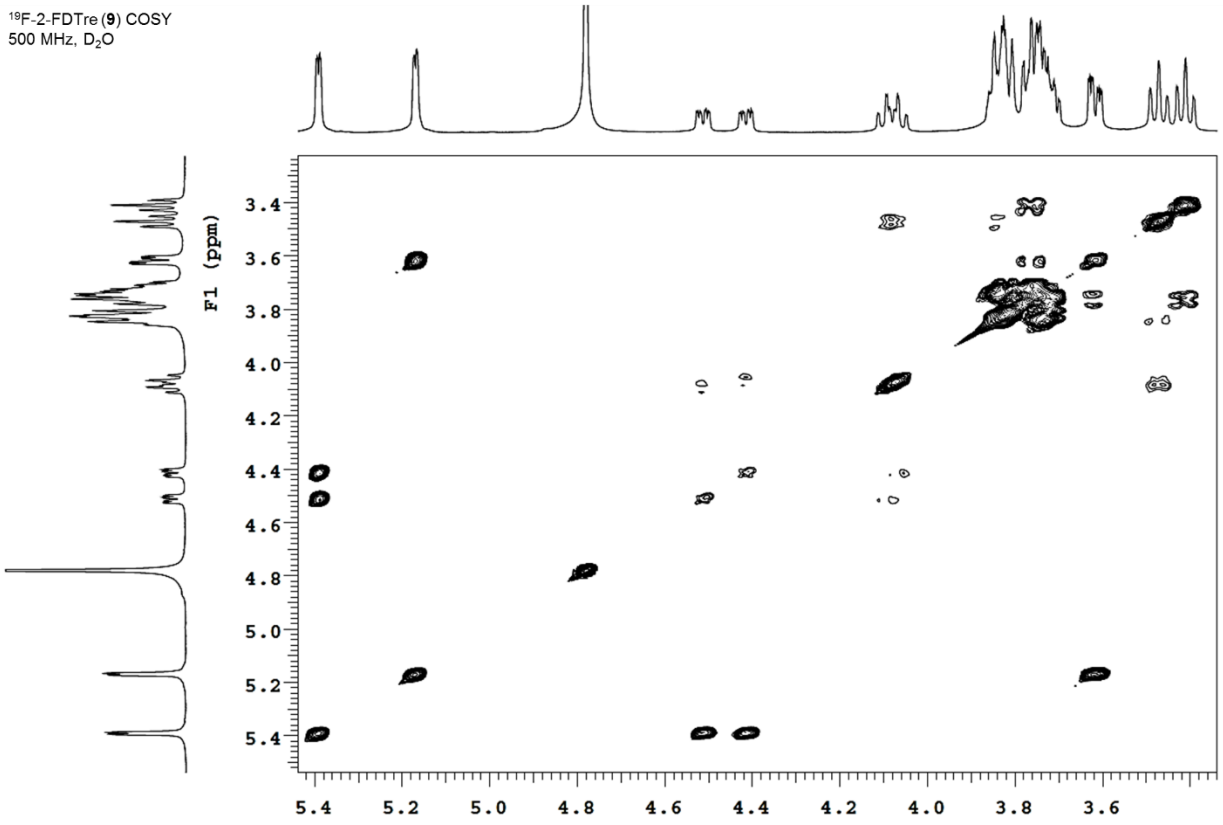
^{19}F-2-FDTre (**9**) ^{1}H NMR
500 MHz, D_2O



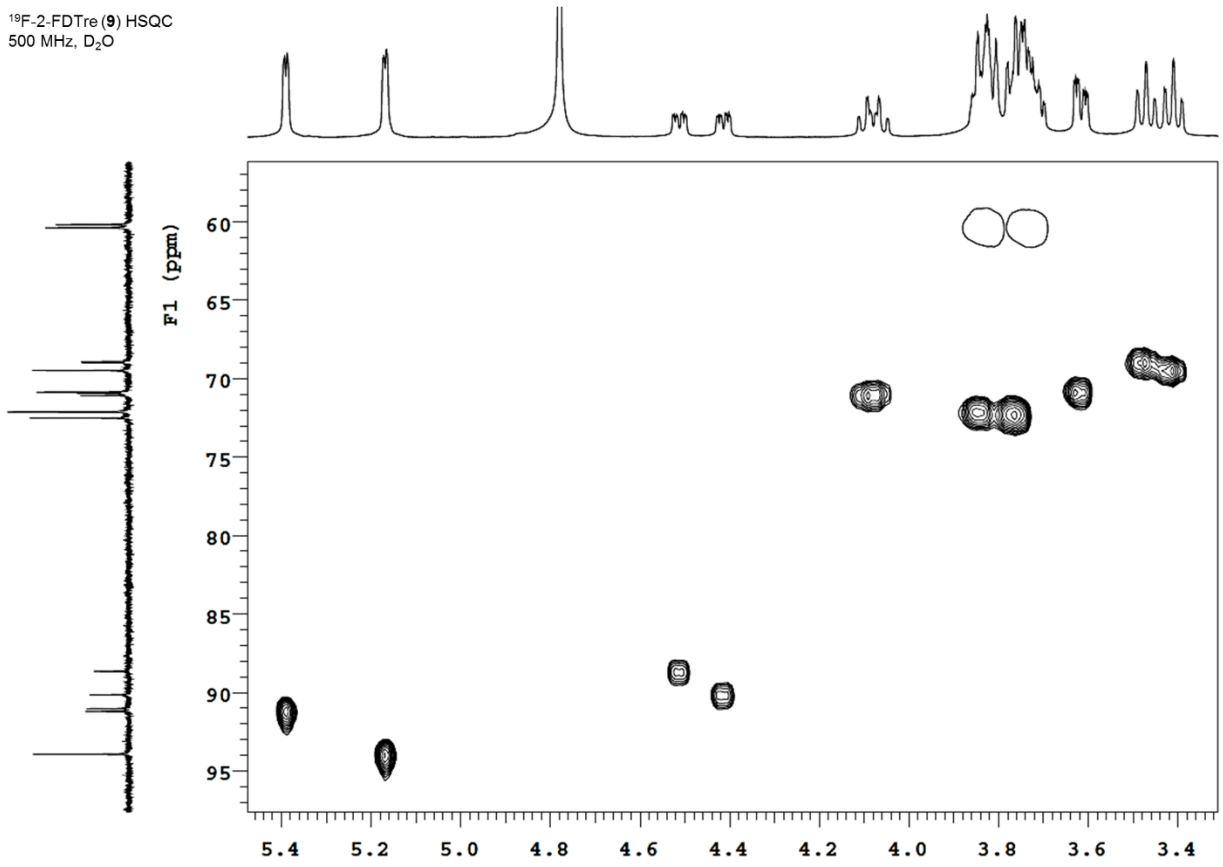
^{19}F-2-FDTre (**9**) ^{13}C NMR
125 MHz, D_2O

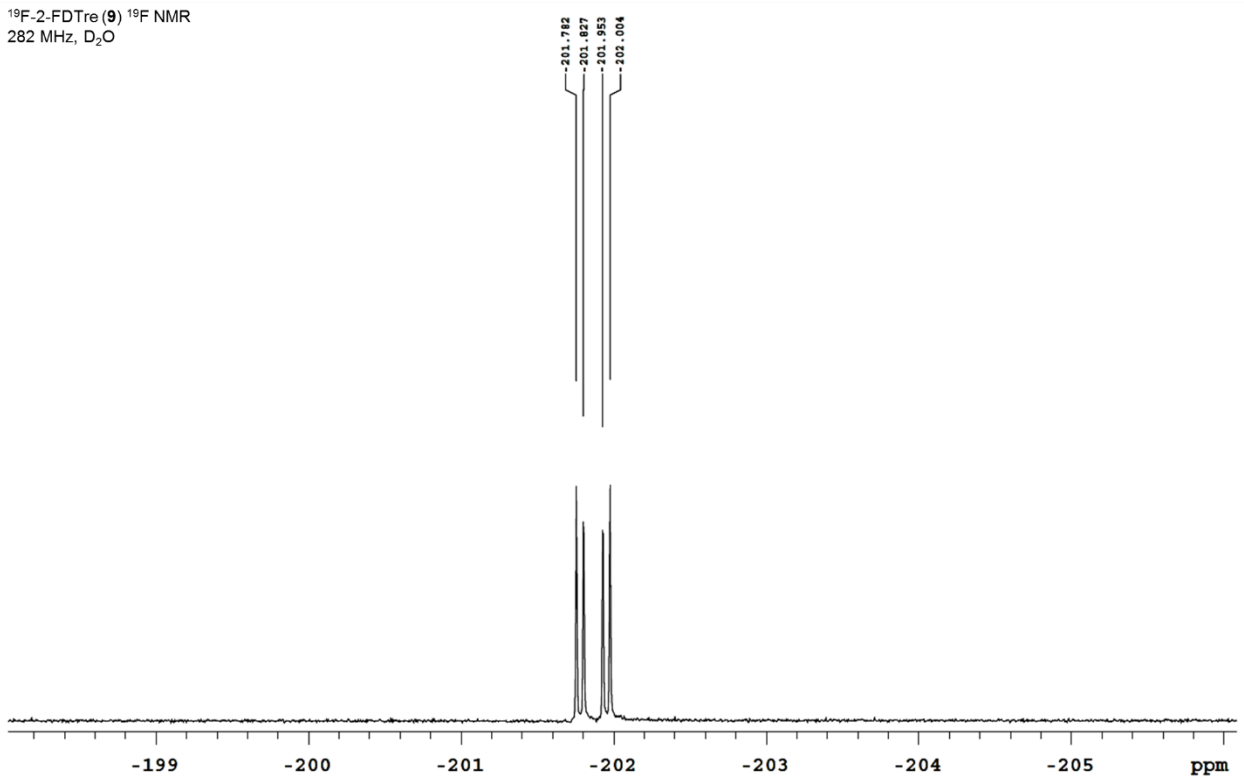


^{19}F-2-FDTre (**9**) COSY
500 MHz, D_2O

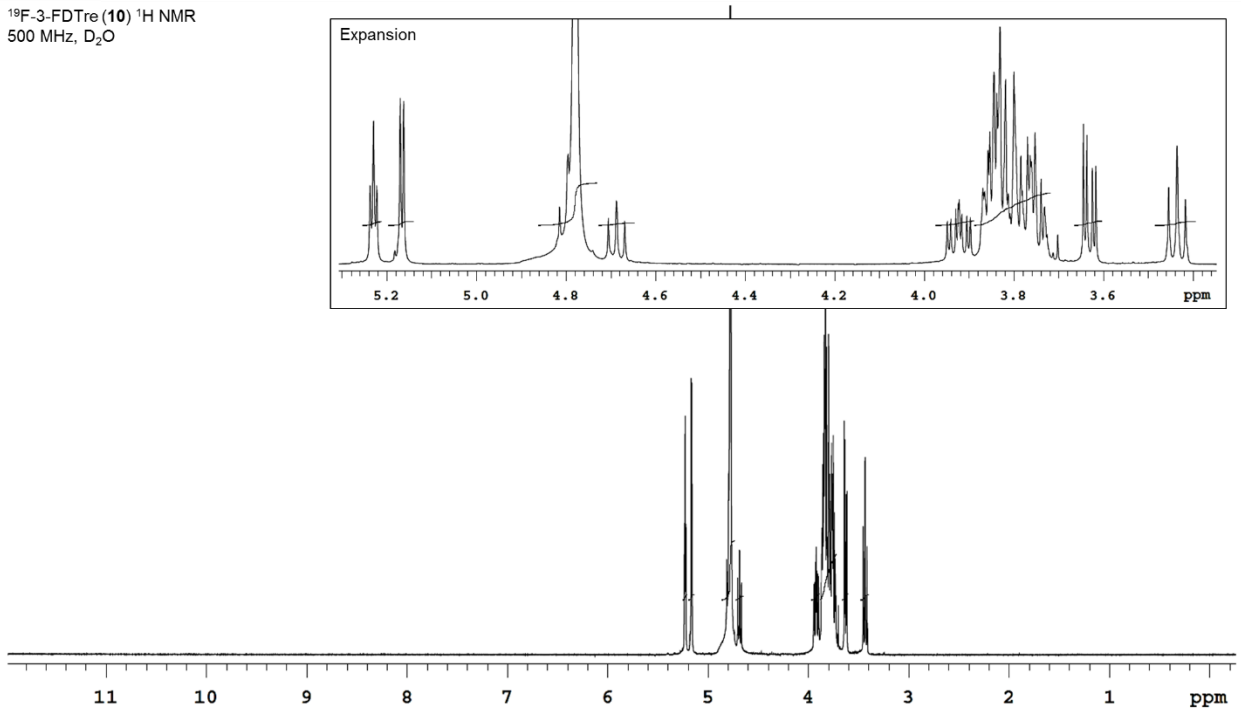


^{19}F-2-FDTre (**9**) HSQC
500 MHz, D_2O

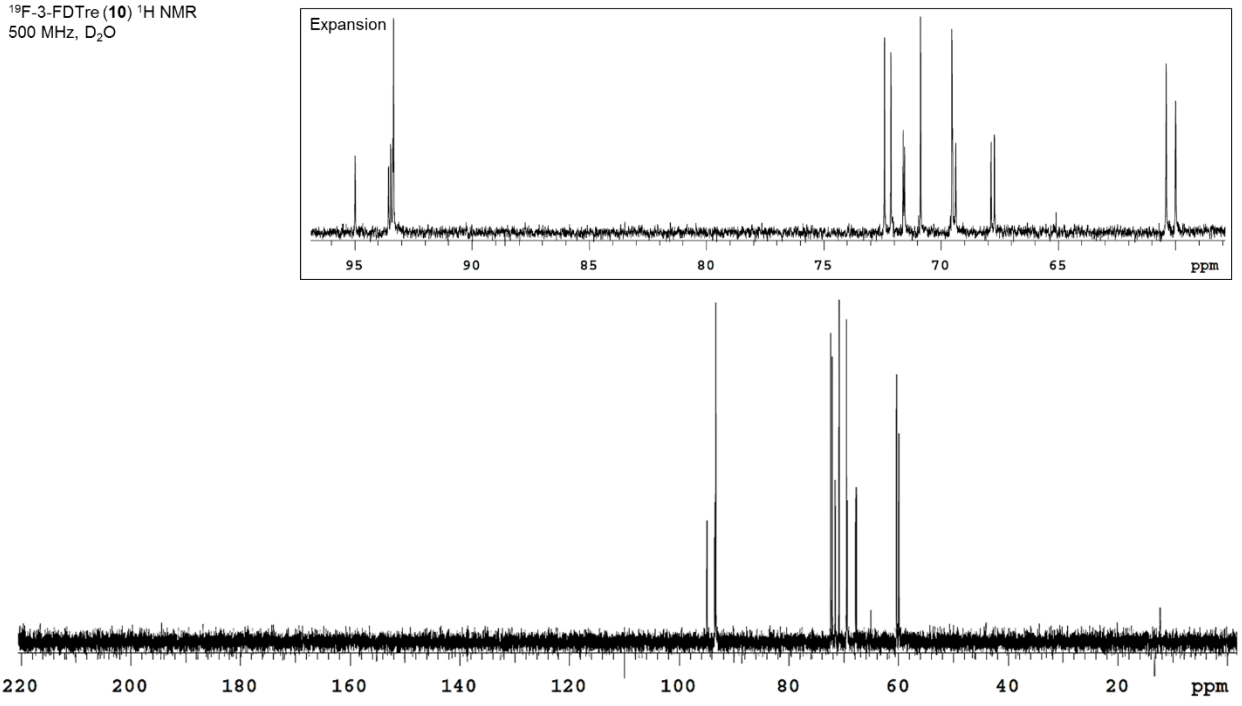



^{19}F-2-FDTre (**9**) ^{19}F NMR
282 MHz, D_2O
-201.782
-201.827
-201.953
-202.004
-199
-200
-201
-202
-203
-204
-205
ppm

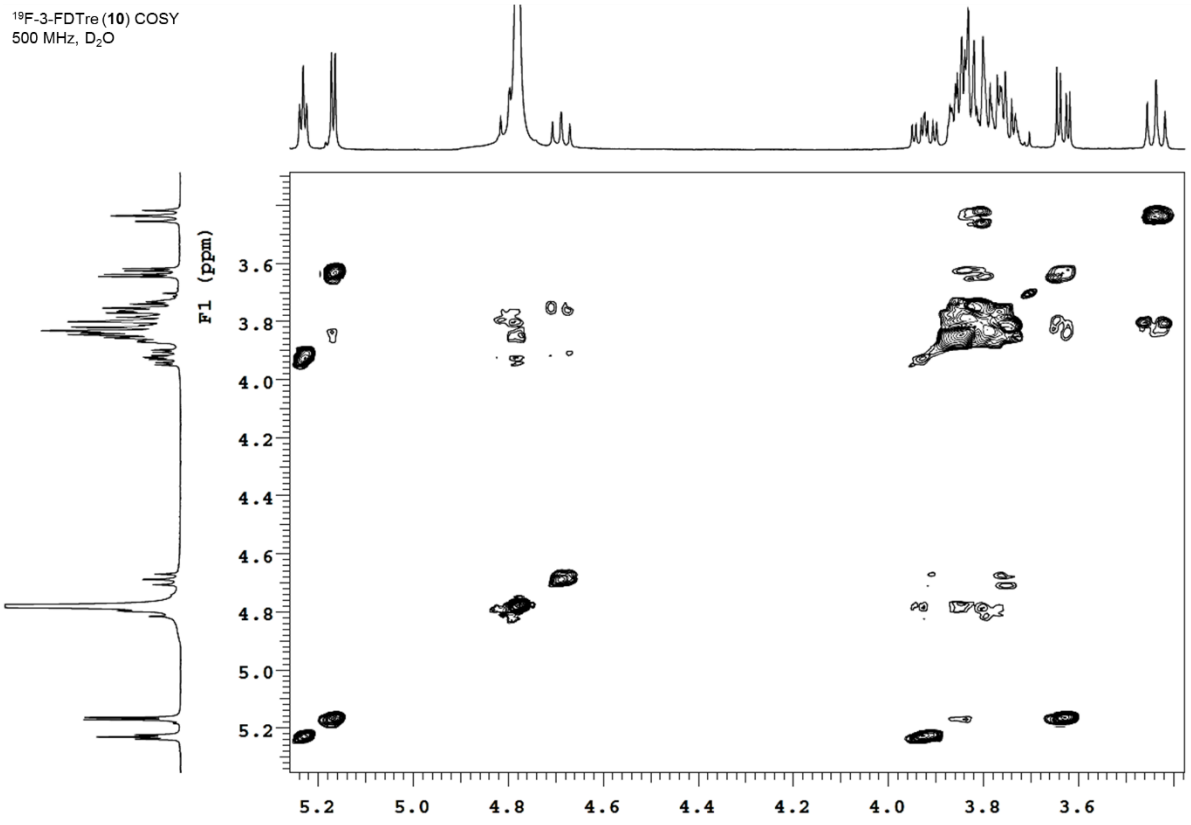
^{19}F-3-FDTre (**10**) ^{1}H NMR
500 MHz, D_2O



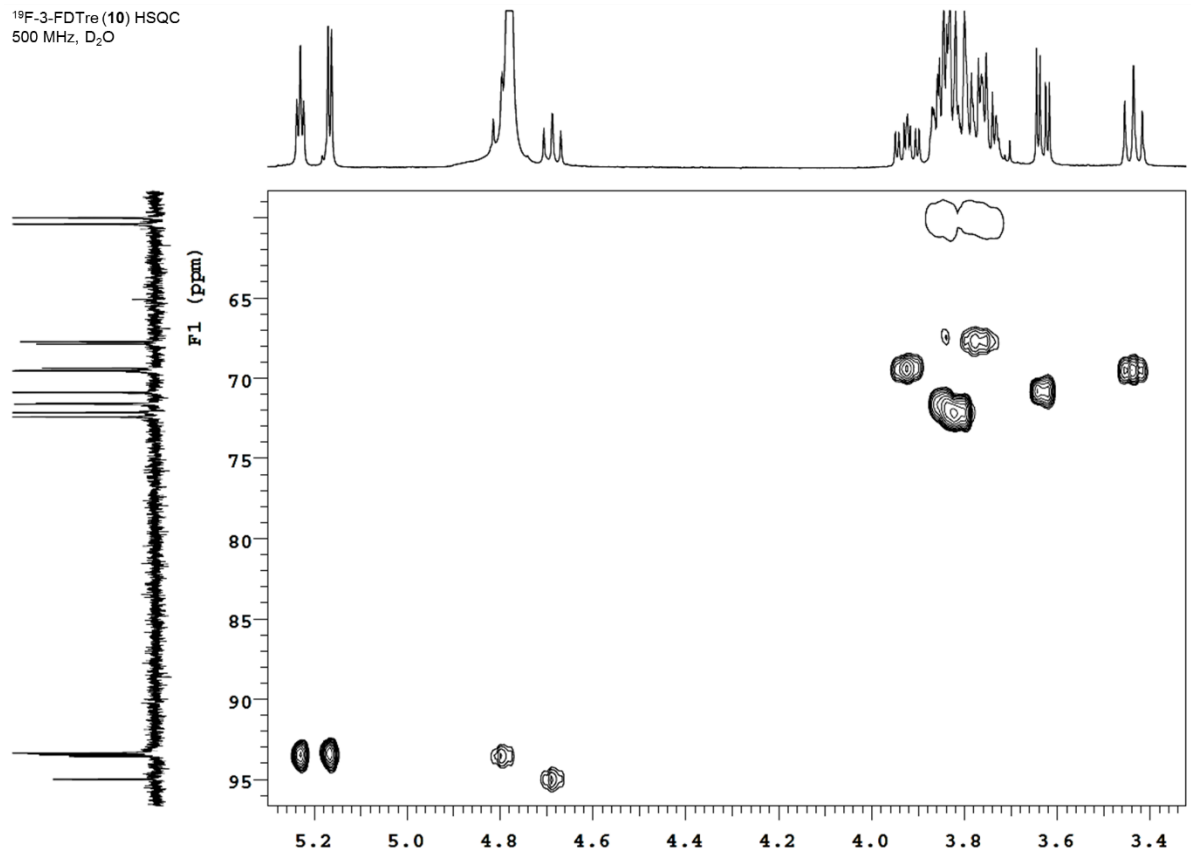
^{19}F-3-FDTre (**10**) ^{1}H NMR
500 MHz, D_2O



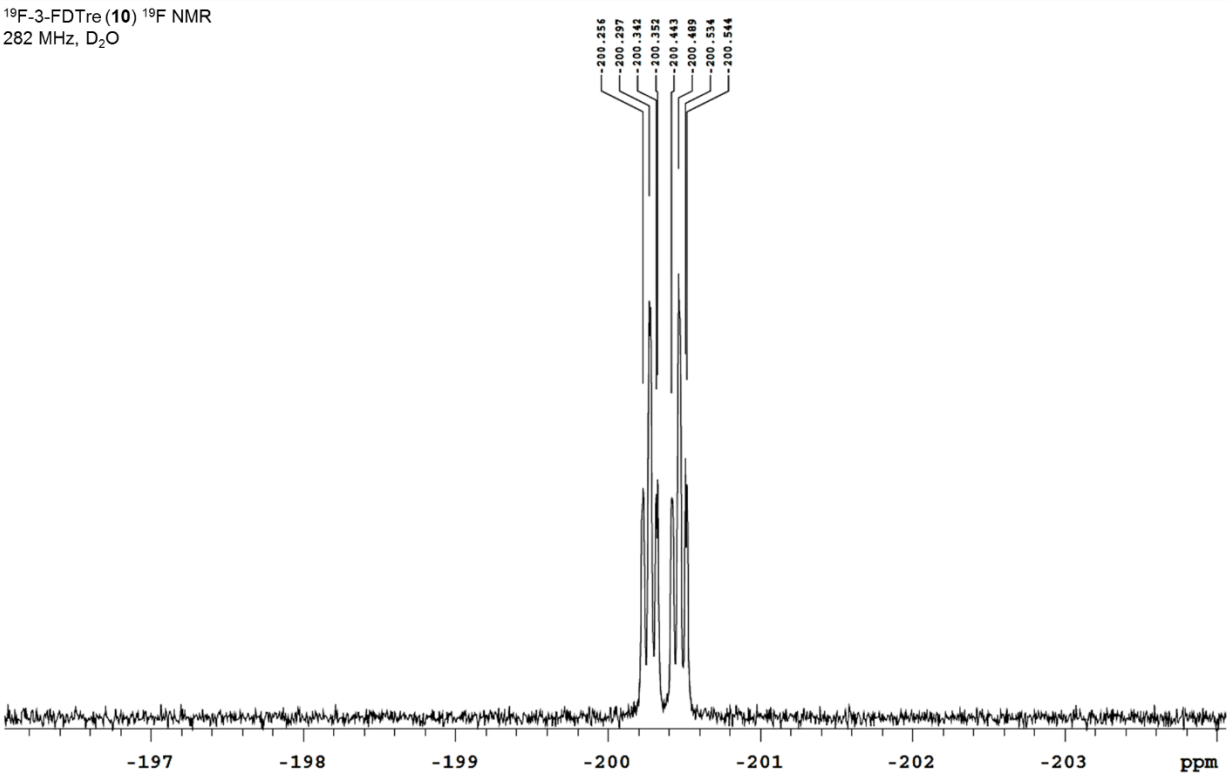
^{19}F-3-FDTre (**10**) COSY
500 MHz, D_2O



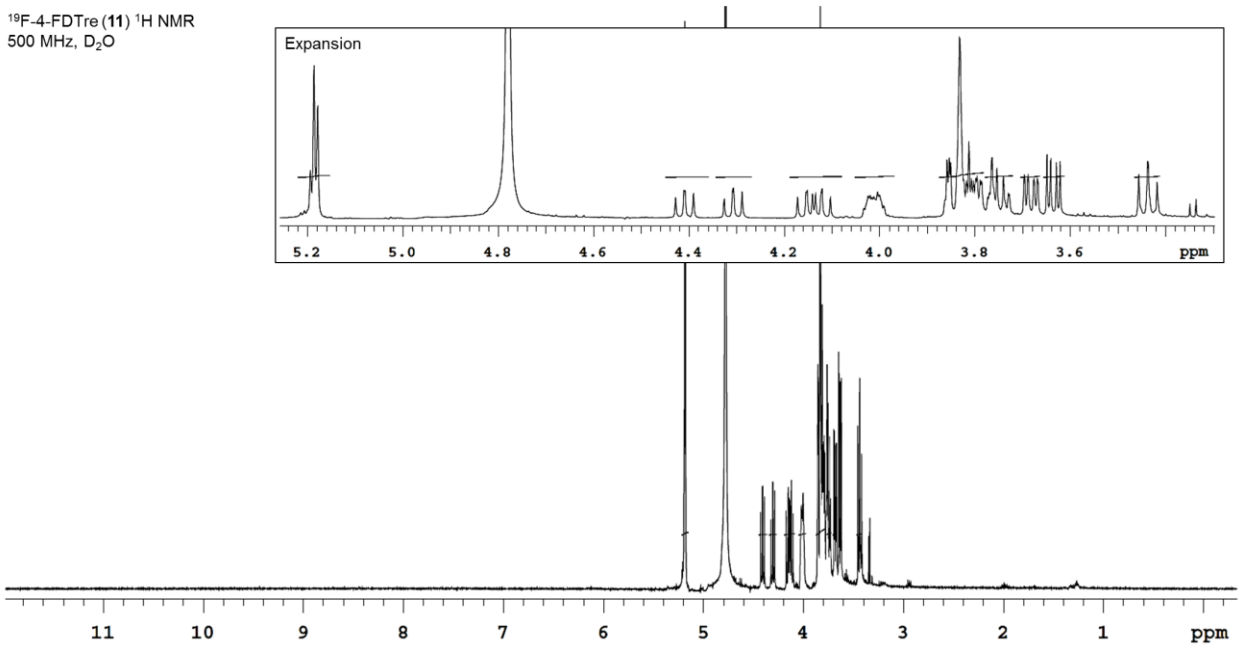
^{19}F-3-FDTre (**10**) HSQC
500 MHz, D_2O



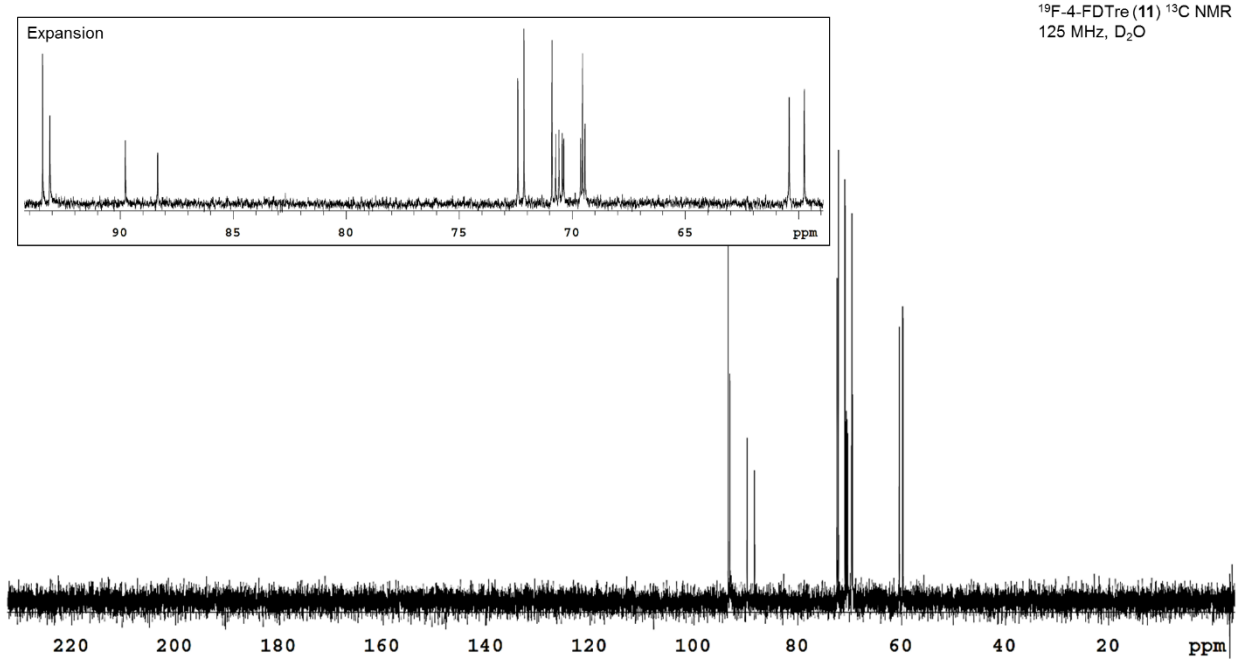
^{19}F-3-FDTre (**10**) ^{19}F NMR
282 MHz, D_2O

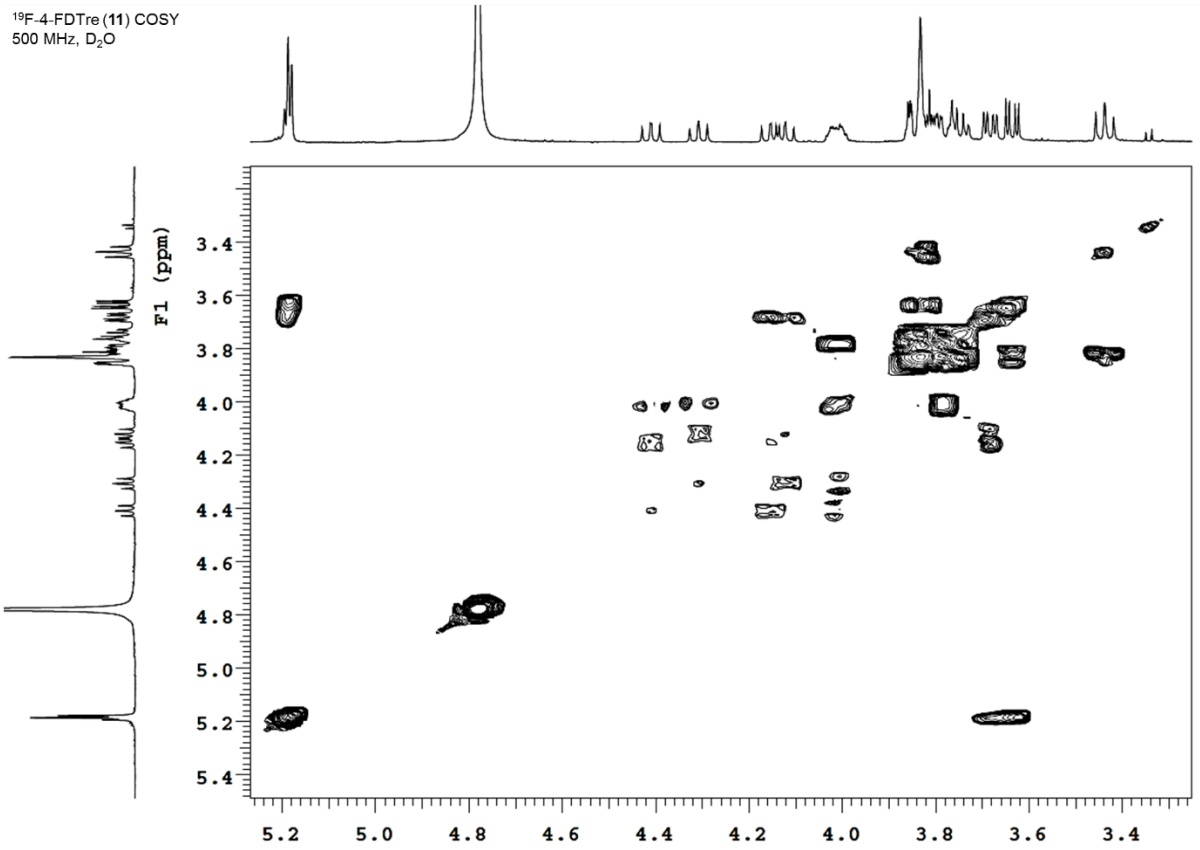


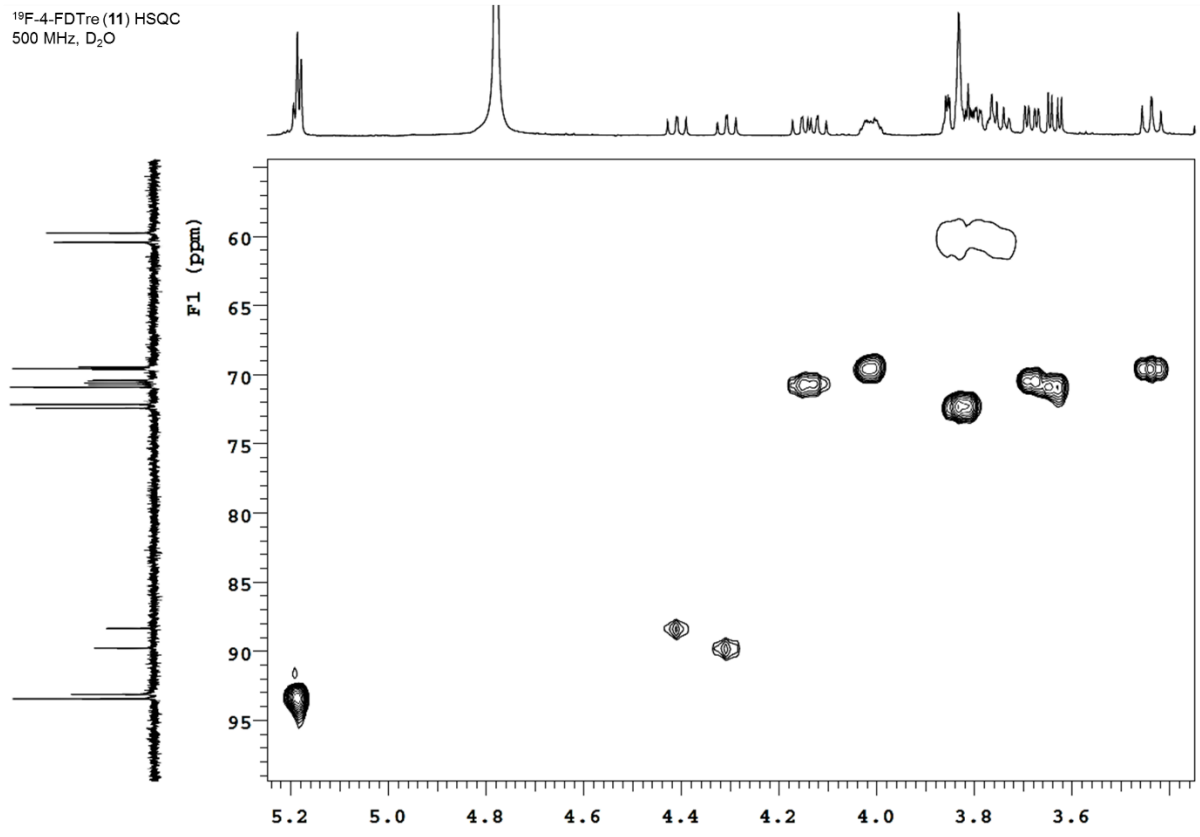
^{19}F-4-FDTre (**11**) ^{1}H NMR
500 MHz, D_2O



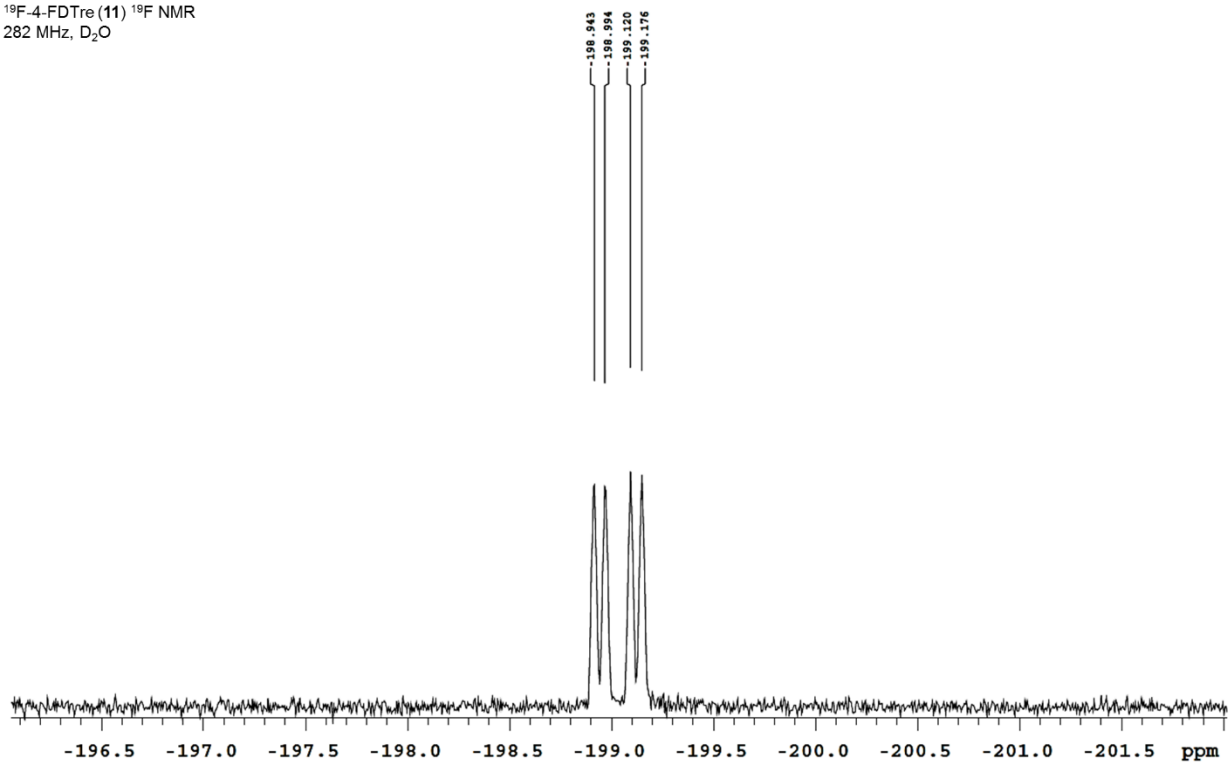
^{19}F-4-FDTre (**11**) ^{13}C NMR
125 MHz, D_2O



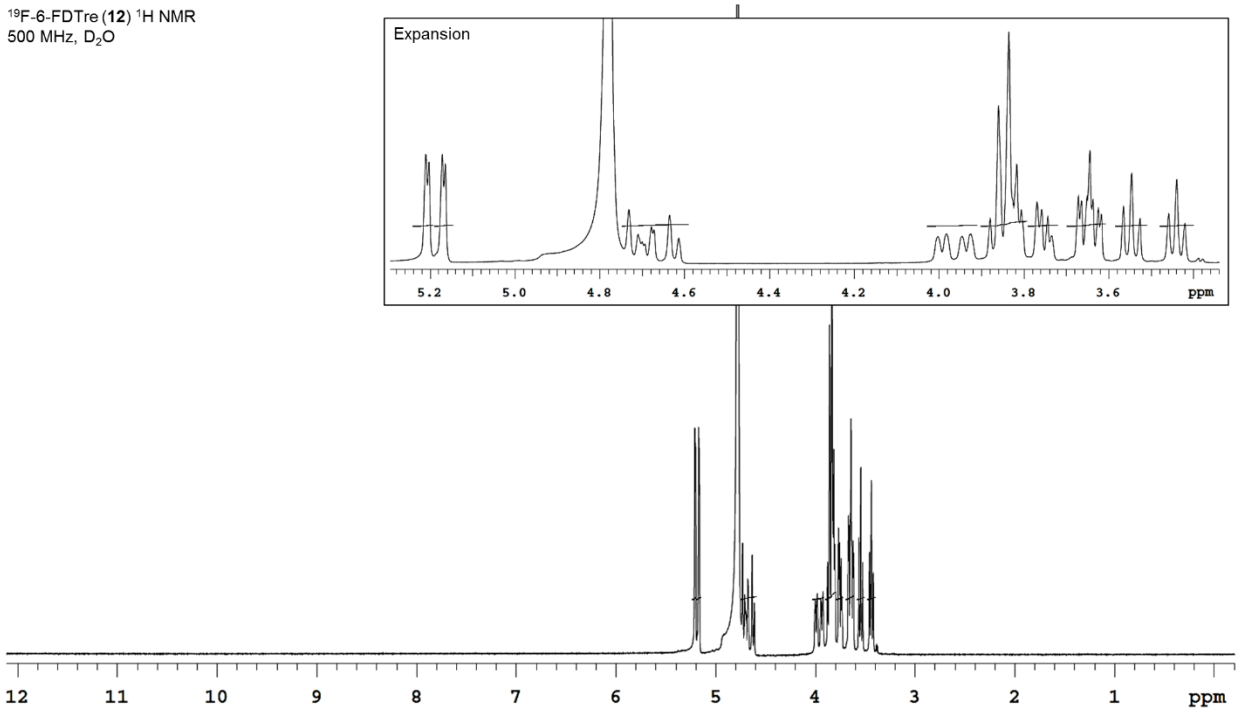

19F-4-FDTre (11) COSY
500 MHz, D2O
F1 (ppm)
3.4
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
5.2
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4


19F-4-FDTre (11) HSQC
500 MHz, D2O
F1 (ppm)
60
65
70
75
80
85
90
95
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6

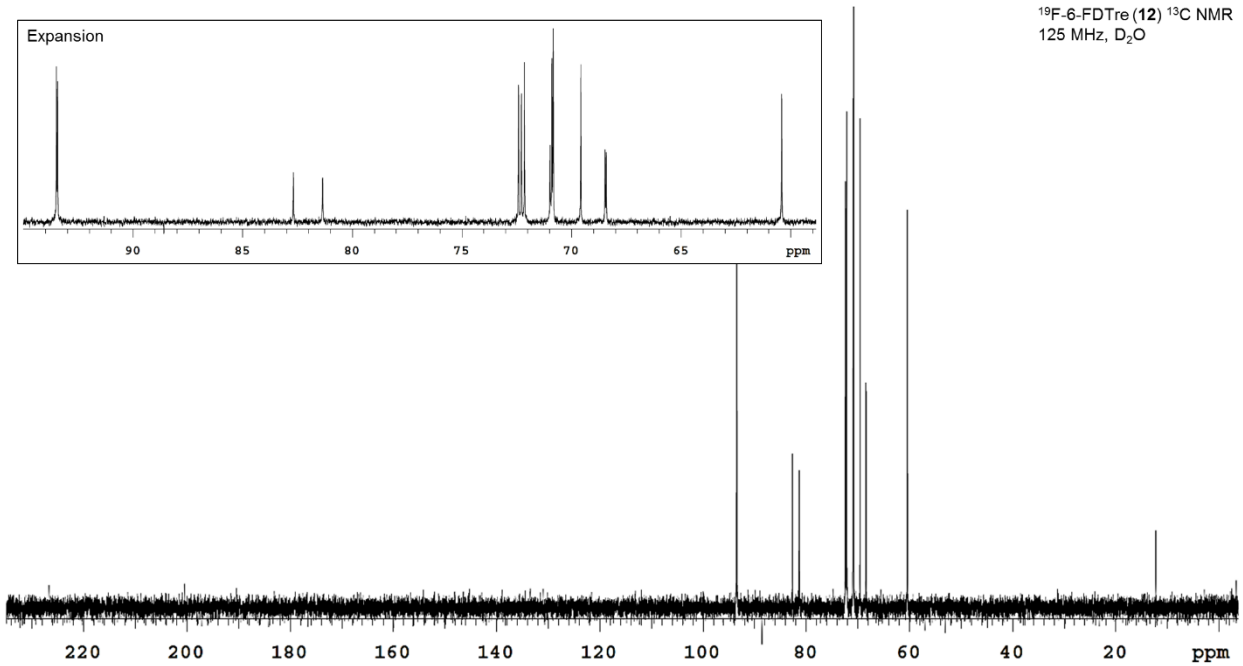
^{19}F-4-FDTre (**11**) ^{19}F NMR
282 MHz, D_2O



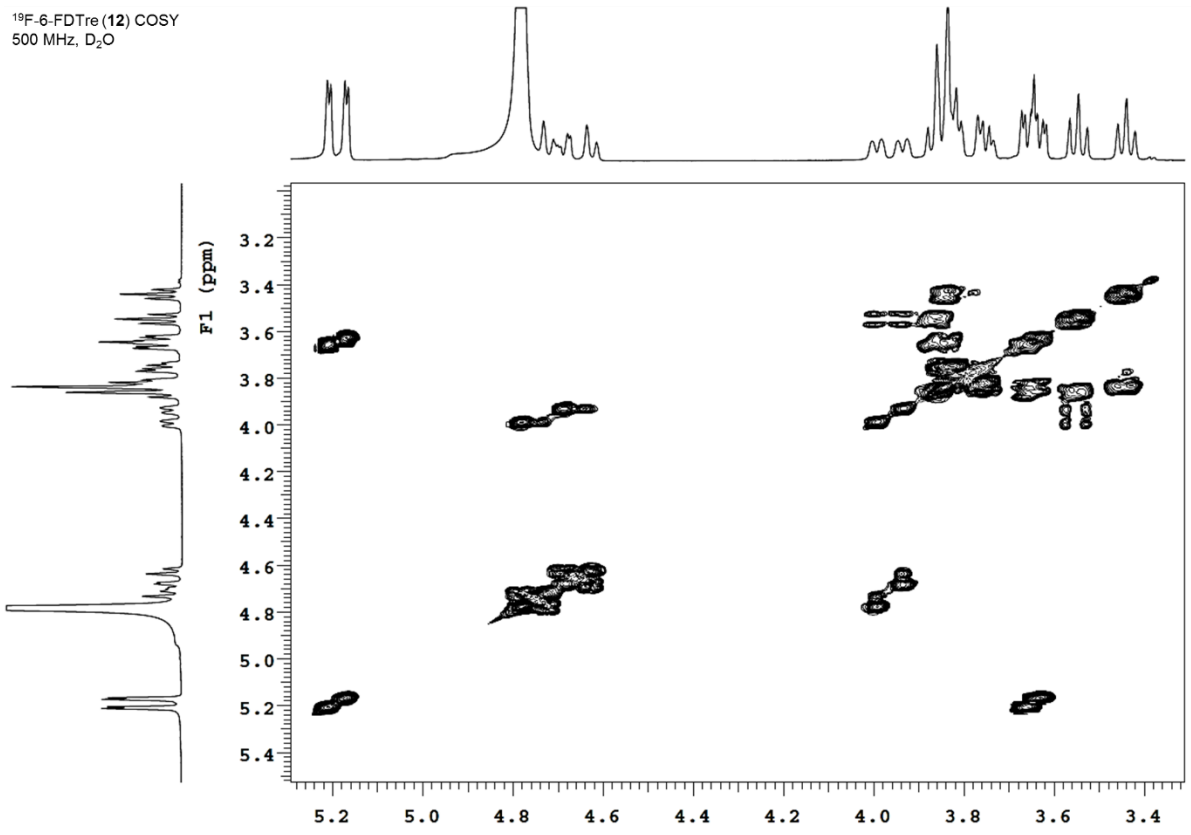
^{19}F-6-FDTre (**12**) ^{1}H NMR
500 MHz, D_2O



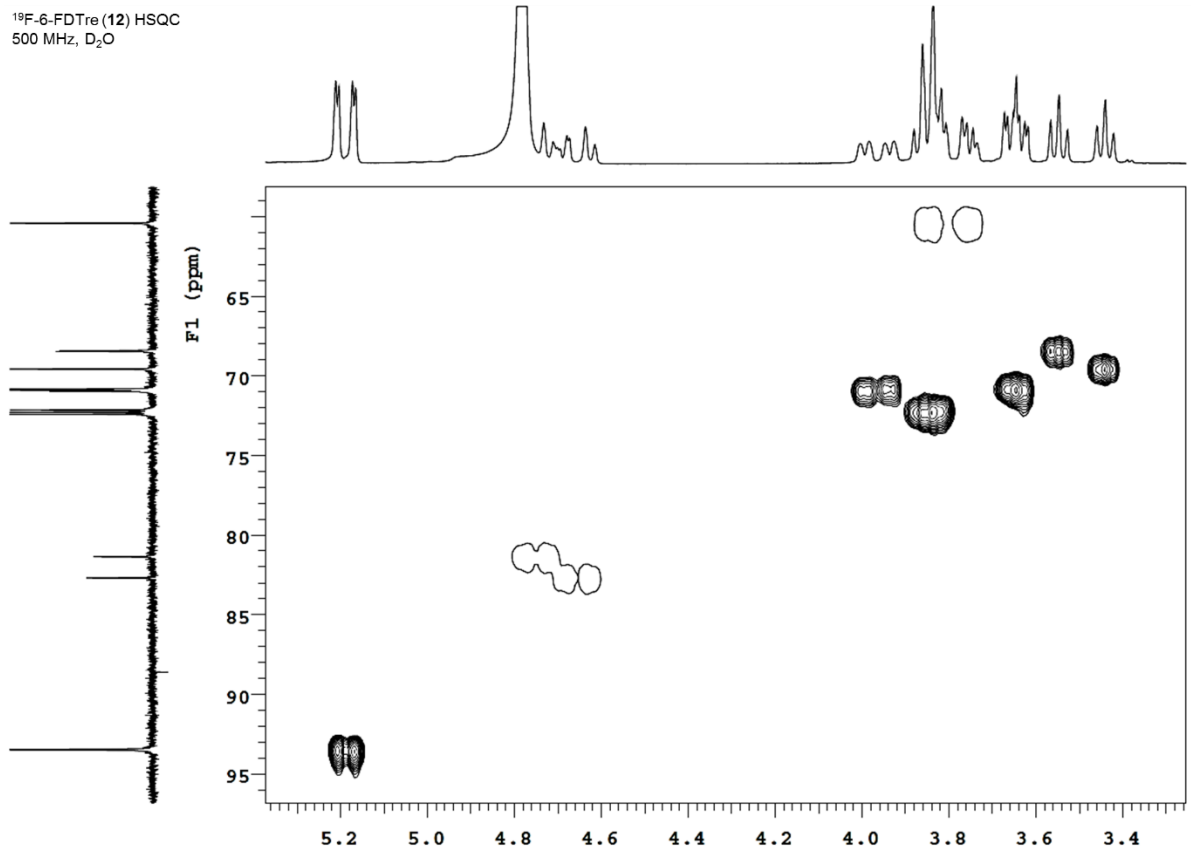
^{19}F-6-FDTre (**12**) ^{13}C NMR
125 MHz, D_2O



^{19}F-6-FDTre (**12**) COSY
500 MHz, D_2O



^{19}F-6-FDTre (**12**) HSQC
500 MHz, D_2O



^{19}F-6-FDTre (**12**) ^{19}F NMR
282 MHz, D_2O