Origin of the Stability Conferred upon Collagen by Fluorination

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General Experimental. Commercial chemicals were of reagent grade or better, and were used without further purification. Anhydrous DMF and CH_2Cl_2 were obtained from a CYCLE-TAINER® solvent delivery system (J. T. Baker, Phillipsburg, NJ). Semi-preparative HPLC was performed with a Macherey-Nagel C18 reversed-phase column. Analytical HPLC was performed with an Agilent C8 reversed-phase column. Linear gradients of solvent A (H_2O with 0.1% v/v TFA) and solvent B (CH_3CN with 0.1% v/v TFA) were used for HPLC analysis and purification.

NMR spectra were recorded on a Bruker DMX-400 Avance spectrometer (^{1}H, 400 MHz; ^{13}C, 100.6 MHz) at the National Magnetic Resonance Facility at Madison (NMRFAM), unless indicated otherwise. NMR spectra were obtained at ambient temperatures. Values of coupling constants (*J*) are in Hertz. Compounds with a carbamate protecting group (*e.g.*, Boc or Fmoc) and *N*-acylated proline derivatives exist as mixtures of *Z* and *E* isomers that do not interconvert on the NMR time scale at ambient temperature. Accordingly, these compounds exhibit two sets of NMR signals. Mass spectrometry was performed with either a Micromass LCT (electrospray ionization, ESI) or a Waters Autospec (electron impact, EI) in the Mass Spectrometry Facility in the University of Wisconsin Department of Chemistry, or an Applied Biosystems Voyager DE-Pro (matrix-assisted laser desorption/ionization, MALDI) mass spectrometer in the University of Wisconsin Biophysics Instrumentation Facility.

The term "concentrated under reduced pressure" refers to the removal of solvents and other volatile materials using a rotary evaporator at water aspirator pressure (<20 torr) while maintaining the water-bath temperature below 50 °C. Residual solvent was removed from samples at high vacuum (<0.1 torr). The term "high vacuum" refers to vacuum achieved by a mechanical belt-drive oil pump.

***N*-*tert*-Butoxycarbonyl-(2*S*)-4,4-difluoroprolylglycine Benzyl Ester (Boc-DfpGly-OBn).** Gly-OBn·TsOH (4.63 g, 13.71 mmol) and DIEA (6.37 mL, 36.56 mmol) were added to a solution of Boc-Dfp-OH (2.30 g, 9.14 mmol) in DCM (170 mL) at 0 °C under Ar(g) and stirred for 5 min. PyBroP (4.26 g, 9.14 mmol) was added and the solution was allowed to warm to rt overnight. The resulting solution was washed with citric acid(aq) (10% w/v, 200 mL), saturated sodium bicarbonate (200 mL), and brine (200 mL), then dried over $MgSO_4$(s) and concentrated under reduced pressure. The product was isolated by flash chromatography (1:9→6:4 v/v EtOAc/hexanes) affording Boc-Dfp-Gly-OBn (2.54 g, 70%) as a sticky white paste. ^{1}H NMR (DMSO-d_6): δ 8.58–8.44 (m, 0.8 H), 7.42–7.29 (m, 5H), 5.19–5.08 (m, 2H), 4.45–4.31 (m, 1H), 4.02–3.62 (m, 4H), 2.86–2.70 (m, 1H), 2.36–2.19 (m, 1H), 1.40 and 1.34 (2 s, 9H); ^{13}C NMR (DMSO-d_6): δ 171.1, 170.8, 169.5, 153.2, 152.9, 135.8, 128.4, 128.1, 128.0, 79.9, 65.9, 57.6, 57.1, 53.4, 53.2, 52.9, 52.6, 40.7, (peaks under DMSO), 38.6, 38.3, 38.2, 27.9, 27.7; ESI–EMM: *m/z* ([M+Na]$^+$) calcd for $C_{19}H_{24}F_2N_2O_5Na$ 421.1546, found 421.1528.

***N*-9-Fluorenylmethoxycarbonyl-(2*S*)-prolyl-(2*S*)-4,4-difluoroprolylglycine Benzyl Ester (Fmoc-ProDfpGly-OBn).** Boc-DfpGly-OBn (2.54 g, 6.37 mmol) was dissolved in 100 mL of 4N HCl in dioxane (400 mmol) and the solution was stirred under Ar(g) for 2 h. The resulting solution was concentrated under reduced pressure. Solvent was removed from the remaining solid (HCl·DfpGly-OBn) as an azeotrope with toluene, and the residue was dried under high vacuum. The solid was dissolved in DMF (100 mL), Fmoc-Pro-OPfp (5.00 g, 9.93 mmol) and DIEA (3.65 mL, 20.93 mmol) were added, and the mixture was stirred under Ar(g) for 18 h. The resulting solution was concentrated by rotary evaporation under high vacuum, and the product was isolated by flash chromatography (4:6→9:1 v/v EtOAc/hexanes) to afford Fmoc-ProDfpGly-OBn containing a slight impurity that was removed in the succeeding step (1.20 g, 34%). ESI–EMM: *m/z* ([M+Na]$^+$) calcd for $C_{34}H_{33}F_2N_3O_6Na$ 640.2230, found 640.2229.

***N*-9-Fluorenylmethoxycarbonyl-(2*S*)-prolyl-(2*S*)-4,4-difluoroprolylglycine (Fmoc-ProDfpGly-OH)**. MeOH (130 mL) was added carefully to a mixture of Fmoc-ProDfpGly-OBn (1.17 g, 1.89 mmol) and Pd/C (0.29 g) under Ar(g). The resulting suspension was stirred under H_2(g) for 1.5 h. The mixture was filtered through a Celite® pad and concentrated under reduced pressure. The product was isolated by flash chromatography (1:9 EtOAc/hexanes→1:19 MeOH/DCM in 0.1% formic acid). Formic acid was removed by azeotroping repeatedly with toluene, affording Fmoc-ProDfpGly-OH (0.39 g, 39%). ^{1}H NMR (MeOH-d_4): δ 7.85–7.75 (m, 2H), 7.68–7.51 (m, 2H), 7.46–7.25 (m, 4H), 4.71 (dd, J = 6.2 and 9.2, 0.5H), 4.64–3.67 (m, 8.5H), 3.67–3.34 (m, 2H), 2.96–1.74 (m, 6H); ^{13}C NMR (MeOH-d_4): δ 173.7, 173.3, 172.7, 172.5, 156.7, 156.2, 145.6, 145.4, 145.1, 145.1, 142.6, 128.9, 128.3, 128.2, 126.2, 126.0, 125.9, 121.0, 120.9, 68.8, 68.0, 59.6, 59.2, 59.1, 59.0, 54.8, 54.4, 50.3, 48.5, 41.8, 38.3, 38.0, 37.8, 30.8, 30.1, 25.3, 24.1; ESI–EMM: *m/z* ([M+Na]$^+$) calcd for $C_{27}H_{27}F_2N_3O_6Na$ 550.1761, found 550.1774.

***N*-Acetyl-(2*S*)-4,4-difluoroproline Methyl Ester (Ac-Dfp-OMe).** Following the method of Nudelman et al.,[S1] a solution of *N*-Boc-(2*S*)-4,4-difluoroproline (612 mg, 2.44 mmol) in anhydrous MeOH (25 mL) was cooled to 0 °C. Acetyl chloride (25 mL) was added dropwise while stirring, the mixture was allowed to warm to room temperature and stirred under Ar(g) for 3 h. The resulting solution was concentrated under reduced pressure, and the residue (HCl·Dfp-OMe) was dried under high vacuum. The residue was dissolved in CH_2Cl_2 (50 mL), *N,N*-4-dimethylaminopyridine (2.46 g, 20.13 mmol) and acetyl chloride (1.34 mL, 18.91 mmol) were added, and the mixture was stirred under Ar(g) overnight. DCM (30 mL) was added, and the mixture was washed twice with citric acid(aq) (10% w/v), then dried over $MgSO_4$(s) and concentrated under reduced pressure. The product was isolated by flash chromatography (7:3 EtOAc/hexanes→100% EtOAc) affording Ac-Dfp-OMe (170 mg, 34%). ^{1}H NMR (500 MHz-$CDCl_3$): δ 4.72 and 4.60 (dd, J =5.3 and 9.4 and J =3.1 and 9.4, 1H), 4.10–3.72 (m, 5H), 2.92–2.42 (m, 2H), 2.10 and 2.10 (2 s, 3H);); ^{19}F{^{1}H} NMR (470.6 MHz-$CDCl_3$, referenced to the ^{1}H NMR spectrum): δ –96.6 (d, J = 235), –98.0 (d, J = 233), –98.9 (d, J = 233), –101.3 (d, J = 235); ^{13}C NMR ($CDCl_3$): δ 171.0, 170.7, 169.9, 169.5, 129.0, 128.4, 126.5, 126.0, 124.0, 123.5, 77.6, 77.3, 77.0, 58.3, 56.5, 54.9, 54.6, 54.2, 53.5, 53.4, 53.2, 53.0. 52.8, 39.9, 39.7, 39.4, 38.0, 37.7, 37.5, 22.3, 21.2; EI–EMM: *m/z* calcd for $C_8H_{11}F_2NO_3^{+\cdot}$ 207.0702, found 207.0697.

Measurement of $K_{trans/cis}$ for Ac-Dfp-OMe. Ac-Dfp-OMe (~2 mg) was dissolved in D_2O (~0.8 mL). ^{1}H NMR spectra were acquired and worked up using the software NUTS.[S2] The value of $K_{trans/cis}$ was determined from the relative areas of the trans and cis peaks. An NOE difference experiment was performed to confirm the assignments of the trans and cis peaks.

Conformational Analysis of Ac-Dfp-OMe by NMR. Ac-Dfp-OMe (~6 mg) was dissolved in $CDCl_3$ (~0.8 mL) or Ac-Dfp-OMe (~2 mg) was dissolved in D_2O (~0.8 mL). In an effort to extract all ^{1}H,^{1}H coupling constants, ^{1}H, ^{1}H{^{19}F}, ^{19}F, and ^{19}F{^{1}H} spectra were recorded on a Varian INOVA 500 using a Varian 5 mm ^{1}H/^{19}F high-field probe in the Magnetic Resonance Facility, Department of Chemistry, University of Wisconsin–Madison. Coupling constants are reported in Table S1. Assuming that the dominant conformations of the pyrrolidine rings in Dfp are C$^\gamma$-endo and C$^\gamma$-exo, the preferred conformation of Ac-Dfp-OMe in solution can be determined unambiguously by analysis of ^{1}H,^{1}H coupling constants, if one conformation is strongly favored over the other.[S3,S4] Nonetheless, the equilibrium between C$^\gamma$-endo and C$^\gamma$-exo ring puckers is fast on the NMR timescale. Therefore, if the C$^\gamma$-endo and C$^\gamma$-exo ring puckers are similar in energy, an averaged NMR spectrum will be observed and this type of conformational analysis will be ambiguous. In agreement with our computational analyses, we find that the

conformational manifold for Ac-Dfp-OMe is slightly different in the trans and cis conformations, in both $CDCl_3$ and D_2O. For the trans conformer in $CDCl_3$, the long-range ^4J coupling between Hβ and Hδ indicates their pseudoequatorial positions (see Figure S1 for proton assignments), which is possible only in the C$^\gamma$-exo conformation. This result suggests that the C$^\gamma$-exo conformation is favored by trans Ac-Dfp-OMe in $CDCl_3$. For the cis conformer in $CDCl_3$, the small ^3J coupling between Hα and Hβ' is indicative of the C$^\gamma$-endo conformation, but in the absence of a long-range ^4J coupling between Hβ' and Hδ' any further analysis is ambiguous. It appears, then, that both the C$^\gamma$-endo and C$^\gamma$-exo ring puckers are similar in energy for cis Ac-Dfp-OMe in $CDCl_3$. For the trans conformer in D_2O, Hδ and Hδ' overlap and coupling constants could not be determined. Therefore, the only measure of conformation available is the moderate size of the ^3J coupling between Hα and Hβ'. This ambiguous analysis suggests that the C$^\gamma$-exo and C$^\gamma$-endo ring puckers of trans Ac-Dfp-OMe are similar in energy in D_2O. For the cis conformer in D_2O, the apparent long-range ^4J coupling between Hβ' and Hδ' indicates their pseudoequatorial position, which is possible only in the C$^\gamma$-endo ring pucker. In addition, the small ^3J coupling between Hα and Hβ' convincingly indicates that the C$^\gamma$-endo conformation is favored. In summary, these results confirm that Ac-Dfp-OMe has a high population of at least two ring puckers in solution, as does Ac-Pro-OMe.

Peptide Synthesis. Ac-(ProProGly)$_4$-(ProDfpGly)-(ProProGly)$_5$-NH_2 (Dfp-CRP), Ac-(ProProGly)$_4$-(ProFlpGly)-(ProProGly)$_5$-NH_2 (Flp-CRP), and Ac-(ProProGly)$_{10}$-NH_2 (Pro-CRP) were synthesized by segment condensation of Fmoc-ProProGly-OH (described previously[S5]), Fmoc-ProDfpGly-OH, and Fmoc-ProFlpGly-OH (described previously[S6]) as appropriate on solid phase using an Applied Biosystems Synergy 432A Peptide Synthesizer at the University of Wisconsin–Madison Biotechnology Center. The first trimer was loaded onto Fmoc-amide resin under standard coupling conditions. Unreacted sites on the resin were subsequently capped by acetylation, if necessary. Fmoc-deprotections were achieved by treatment with 20% v/v piperidine in DMF. The Fmoc-tripeptides (3 equiv) were converted to active esters by treatment with HBTU, DIEA, and HOBt. Couplings were allowed to proceed for 45–60 min at room temperature. N-Terminal acetylations were performed using 3 equiv of acetic acid and standard coupling reagents. Peptides were cleaved from the resin in 38:1:1 TFA:H_2O:triisopropylsilane (3 mL), precipitated from *tert*-butylmethylether at 0 °C, and isolated by centrifugation. Semi-preparative HPLC was used to purify the peptides Pro-CRP (gradient: 10% B to 75% B over 45 min), Dfp-CRP (gradient: 10% B to 60% B over 45 min), and Flp-CRP (gradient: 20% B to 70% B over 50 min). All three peptides were >90% pure by analytical HPLC and MALDI–TOF mass spectrometry (m/z) $[M + Na]^+$ calcd for $C_{122}H_{175}N_{31}O_{31}$ 2594.3, found 2595.4 for Pro-CRP; calcd for $C_{122}H_{173}F_2N_{31}O_{31}$ 2630.3, found 2630.5 for Dfp-CRP; calcd for $C_{122}H_{174}FN_{31}O_{31}$ 2612.3, found 2612.9 for Flp-CRP.

Circular Dichroism (CD) Spectroscopy. CD spectra were recorded with an Aviv 202SF circular dichroism spectrometer in the University of Wisconsin Biophysics Instrumentation Facility. For the analyses, peptide solutions (90 μM in 50 mM HOAc, pH 2.9) that had been incubated at ≤4°C for ≥48 h were used. Peptide concentrations were standardized by dilution of stock solutions of known concentrations, determined by amino acid analysis at the Biomolecular Resource Facility, University of Texas–Medical Branch, Galveston, TX. CD spectra were recorded in 1-nm increments with a 5-s averaging time, 1-nm bandpass, and 0.1-cm pathlength. The solutions were then heated from 4 °C to 82 °C (for samples in 50 mM HOAc(aq), pH 2.9) at 3-°C increments with a 5-min equilibration at each step. The ellipticity at 225 nm was monitored

with a 5-s averaging time, 1-nm bandpass, and 0.1-cm pathlength. Values of T_m were determined by fitting the data to a two-state model.[S7]

Computational Methodology. The conformational preferences of Ac-Dfp-OMe were examined by hybrid density functional theory as implemented in Gaussian 03.[S8] Geometry optimizations and frequency calculations at the B3LYP/6-311+G(2d,p) level of theory were performed on endo and exo conformers in both the trans and cis geometries. Frequency calculations on the optimized structures yielded no imaginary frequencies, indicating true stationary points on the potential energy surface. The resulting self-consistent field (SCF) energies were corrected by the zero-point vibrational energy (ZPVE) determined in the frequency calculations, and are listed in Table S2.

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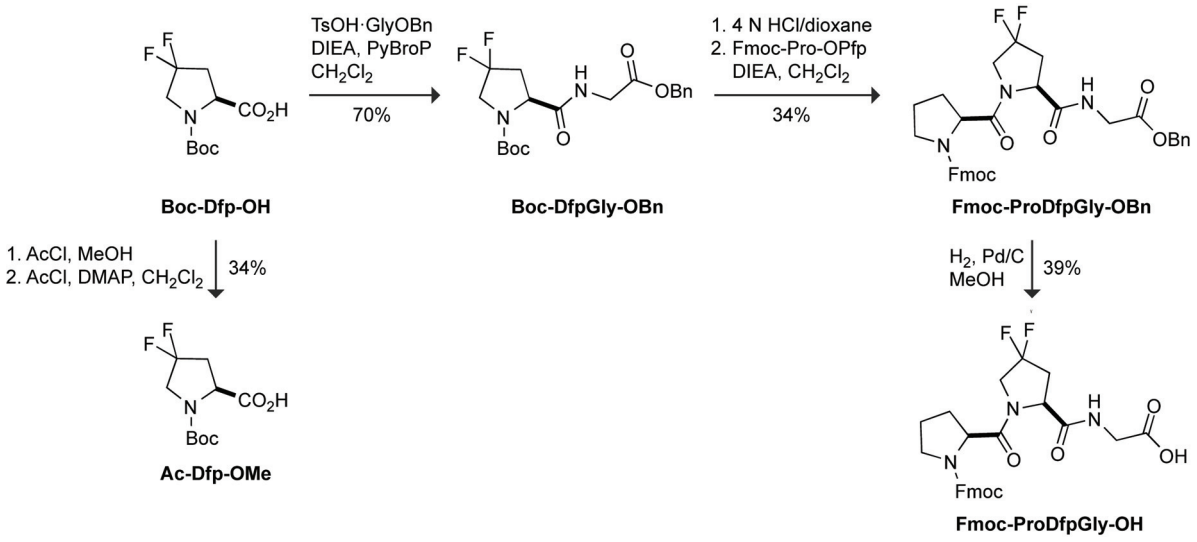


Scheme S1. Synthesis of Ac-Dfp-OMe and Fmoc-ProDfpGly-OH.

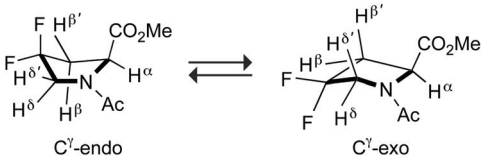


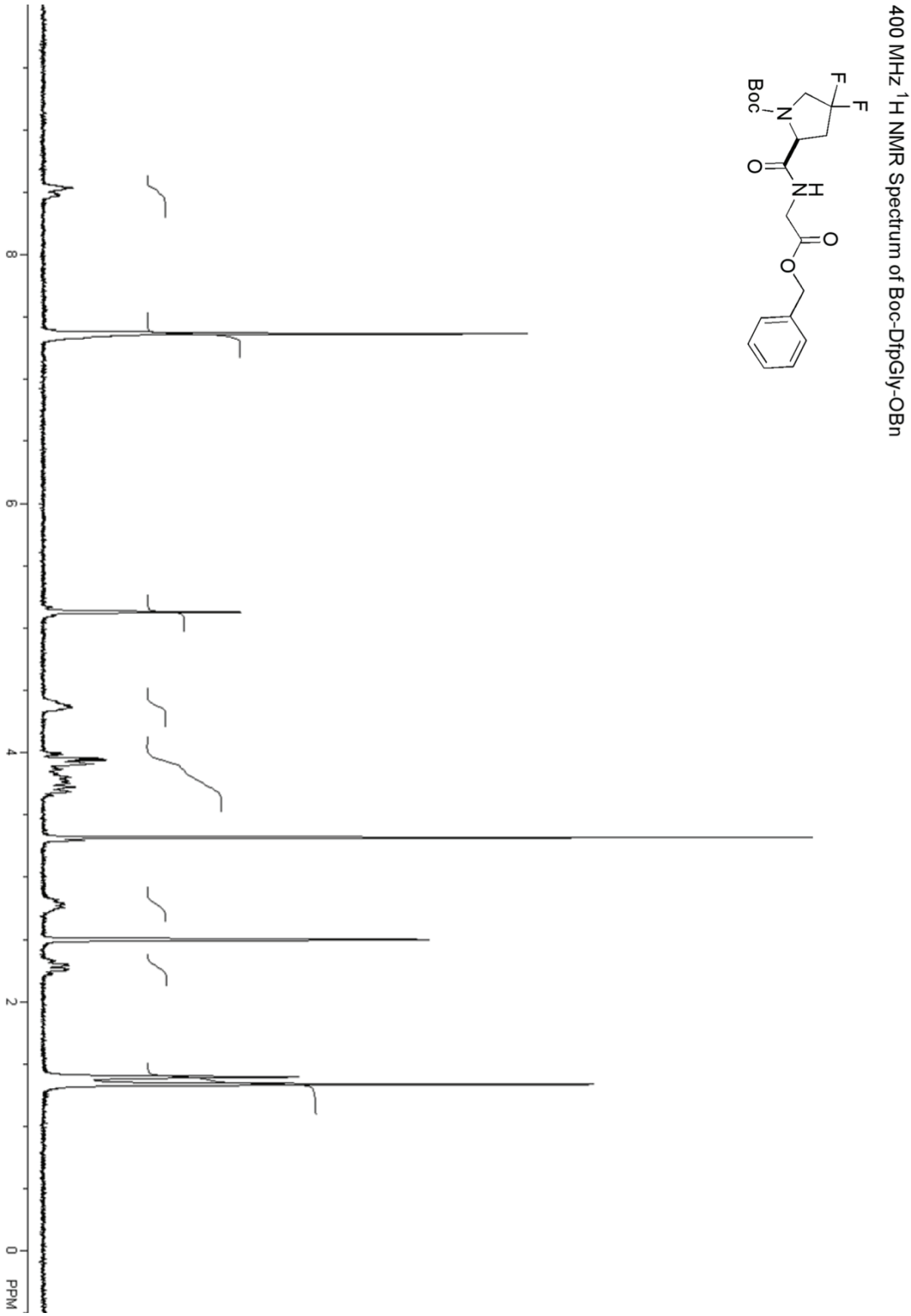
Figure S1. C^{γ}-endo and C^{γ}-exo conformations of Ac-Dfp-OMe.

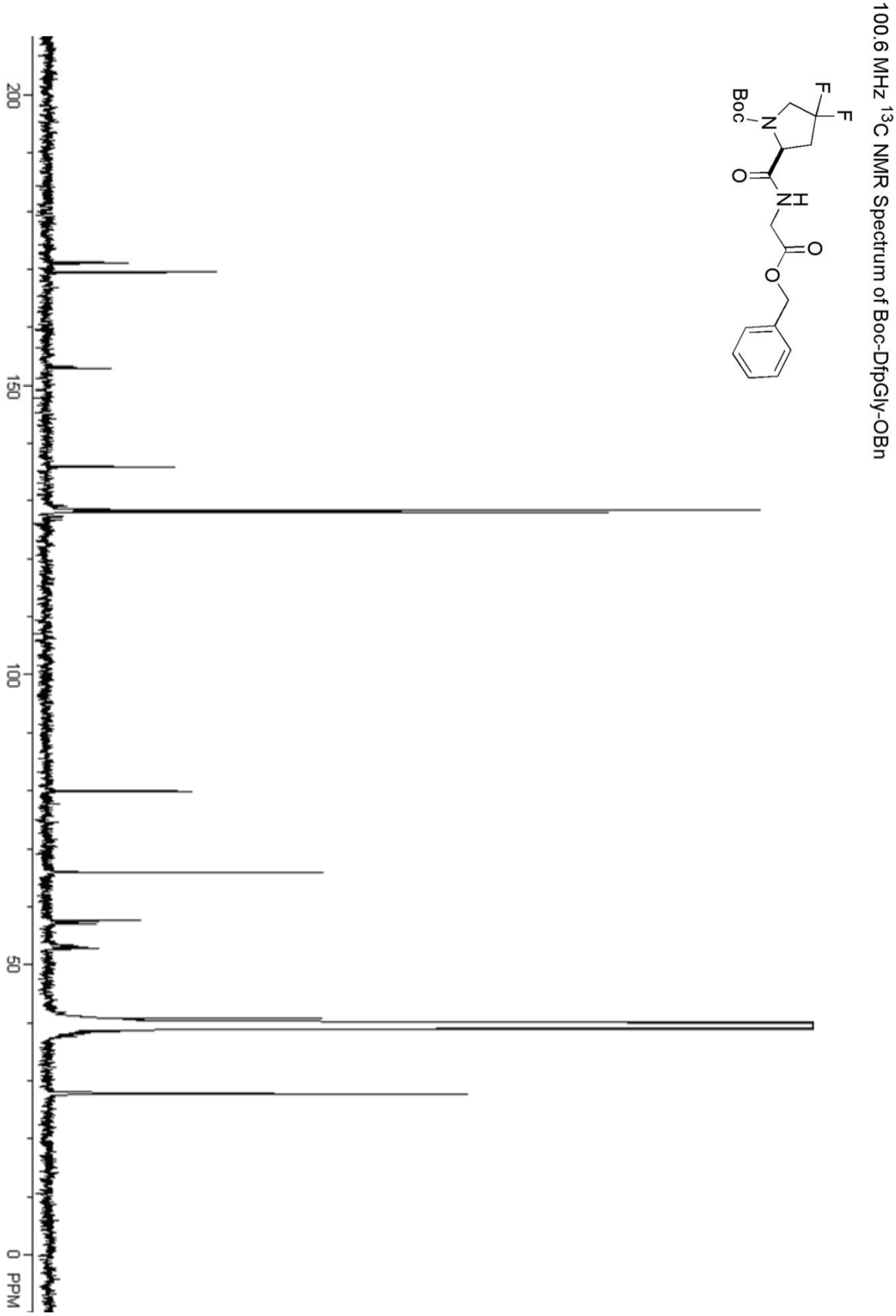
Table S1. Coupling constants of the trans and cis conformers of Ac-Dfp-OMe in D_2O and $CDCl_3$

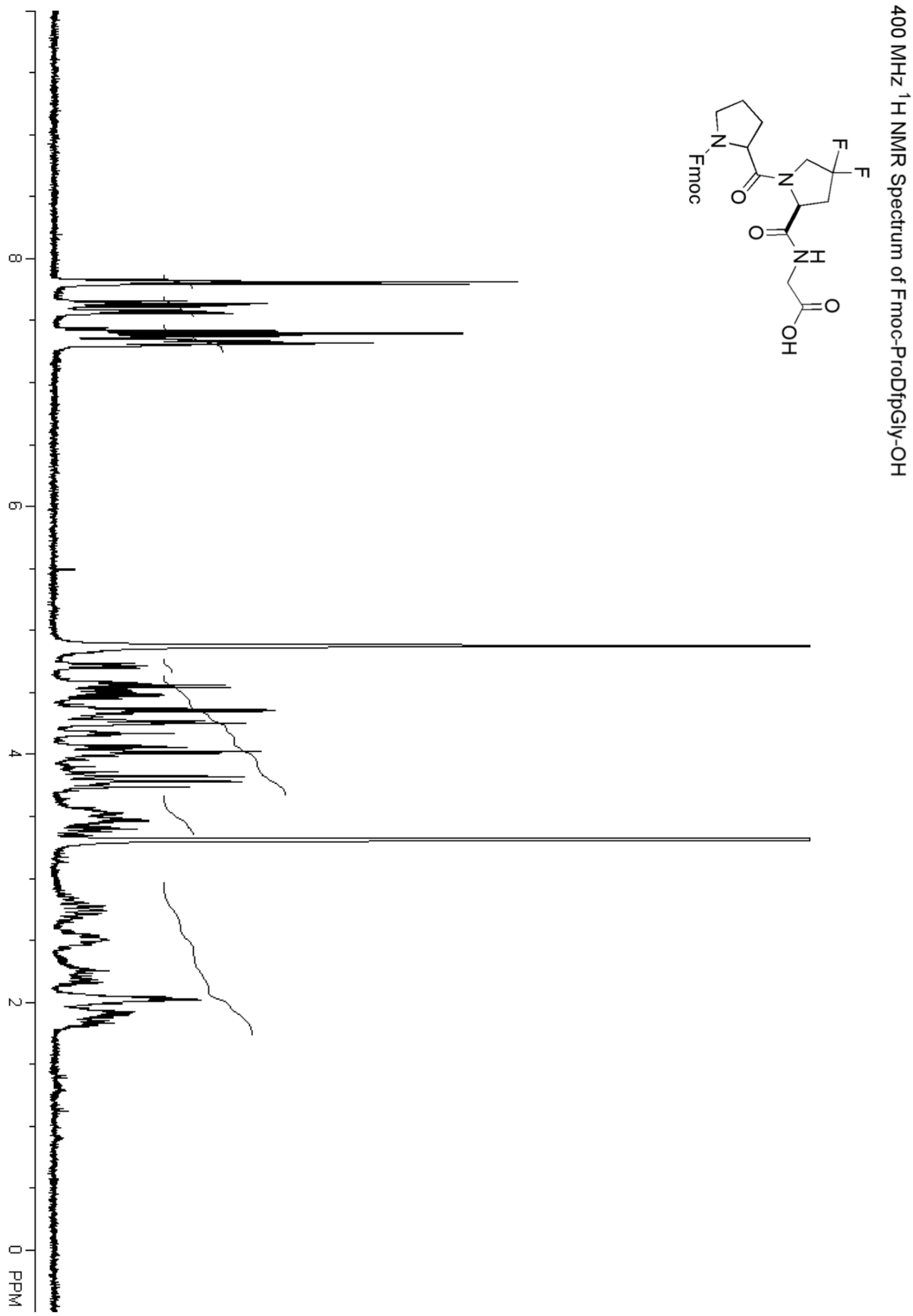
	D_2O		$CDCl_3$	
$J_{(H–H)}$	trans	trans	cis	cis
Hα–Hβ	9.9	9.3	9.5	9.8
Hα–Hβ'	4.5	5.4	3.2	1.7
Hβ–Hβ'	14.5	14.3	14.2	14.4
Hβ–Hδ	—	1.2	—	—
Hβ'–Hδ'	—	—	—	1.7
Hδ–Hδ'	—	11.5	13.5	13.7

Table S2. SCF energies (atomic units; au) calculated at the B3LYP/6-311+G(2d,p) level of theory

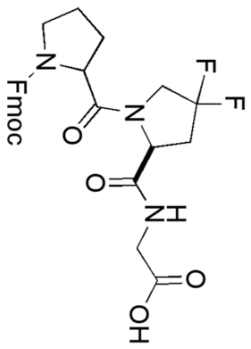
Ac-Dfp-OMe conformer	Energy	ZPE correction	Energy (corrected)
trans, exo	–791.8660836	0.192957	–791.6731266
trans endo	–791.8648927	0.192588	–791.6723047
cis exo	–791.8634706	0.192598	–791.6708726
cis endo	–791.8638557	0.192569	–791.6712867

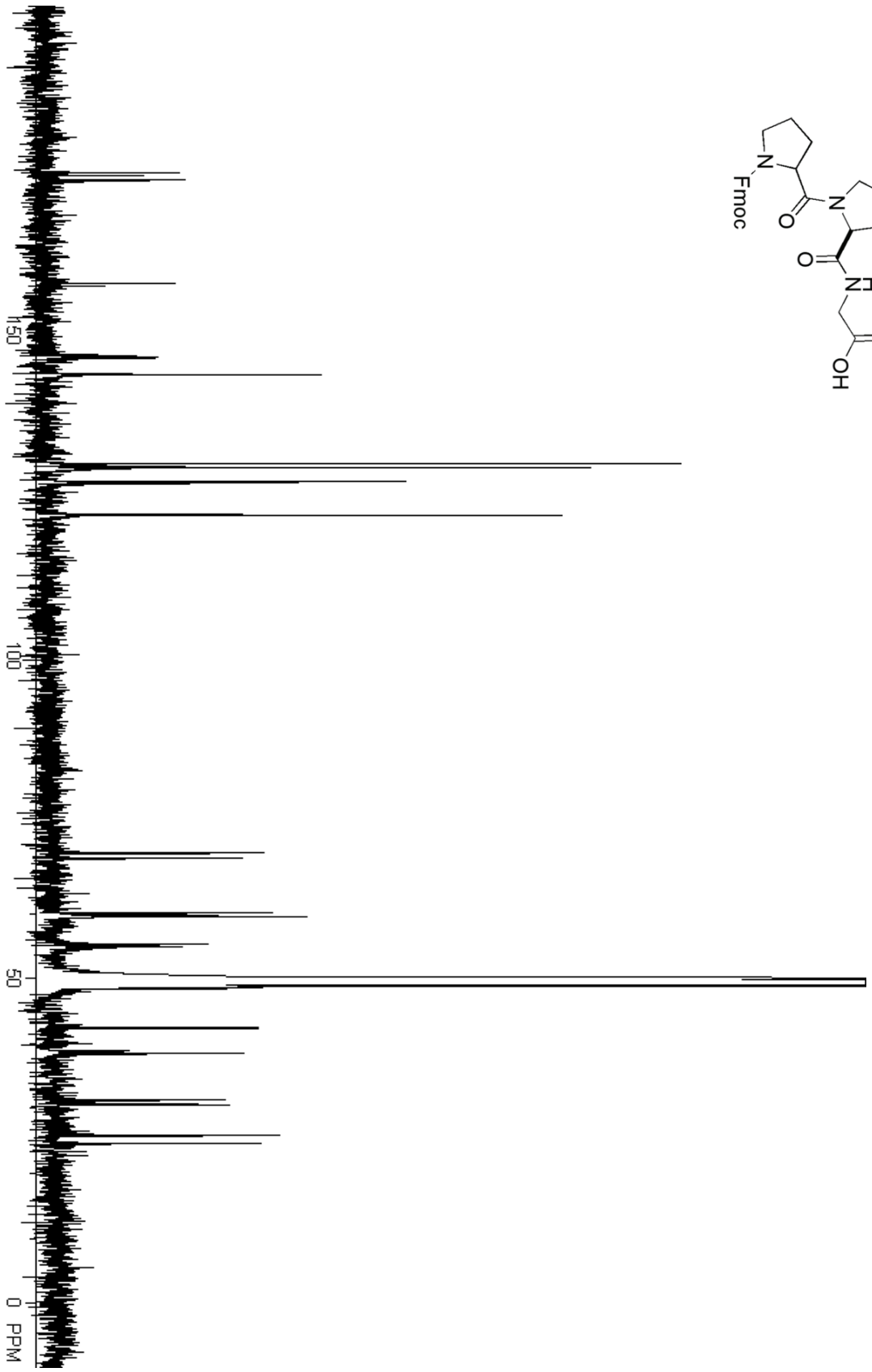

400 MHz ^{1}H NMR Spectrum of Boc-DfpGly-OBn
F
F
Boc
N
O
H
N
O
O
8
6
4
2
0
PPM


100.6 MHz 13C NMR Spectrum of Boc-DfpGly-OBn
F
F
Boc
N
O
N
H
O
O
200
150
100
50
0 PPM

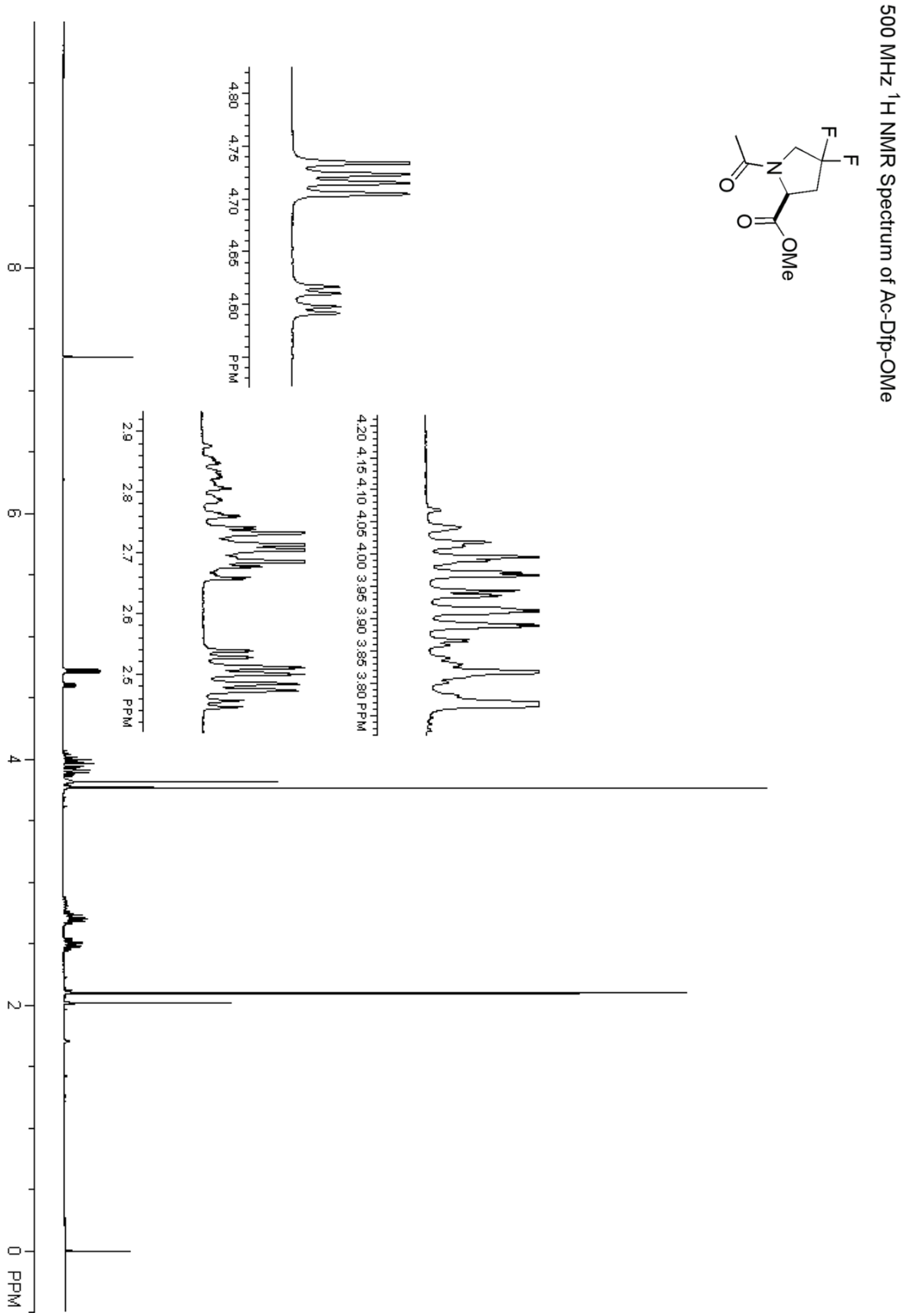

400 MHz 1H NMR Spectrum of Fmoc-ProDfpGly-OH
F
F
N
O
O
H
N
O
OH
N
Fmoc
8
6
4
2
0 PPM

100.6 MHz ^{13}C NMR Spectrum of Fmoc-ProDfpGly-OH

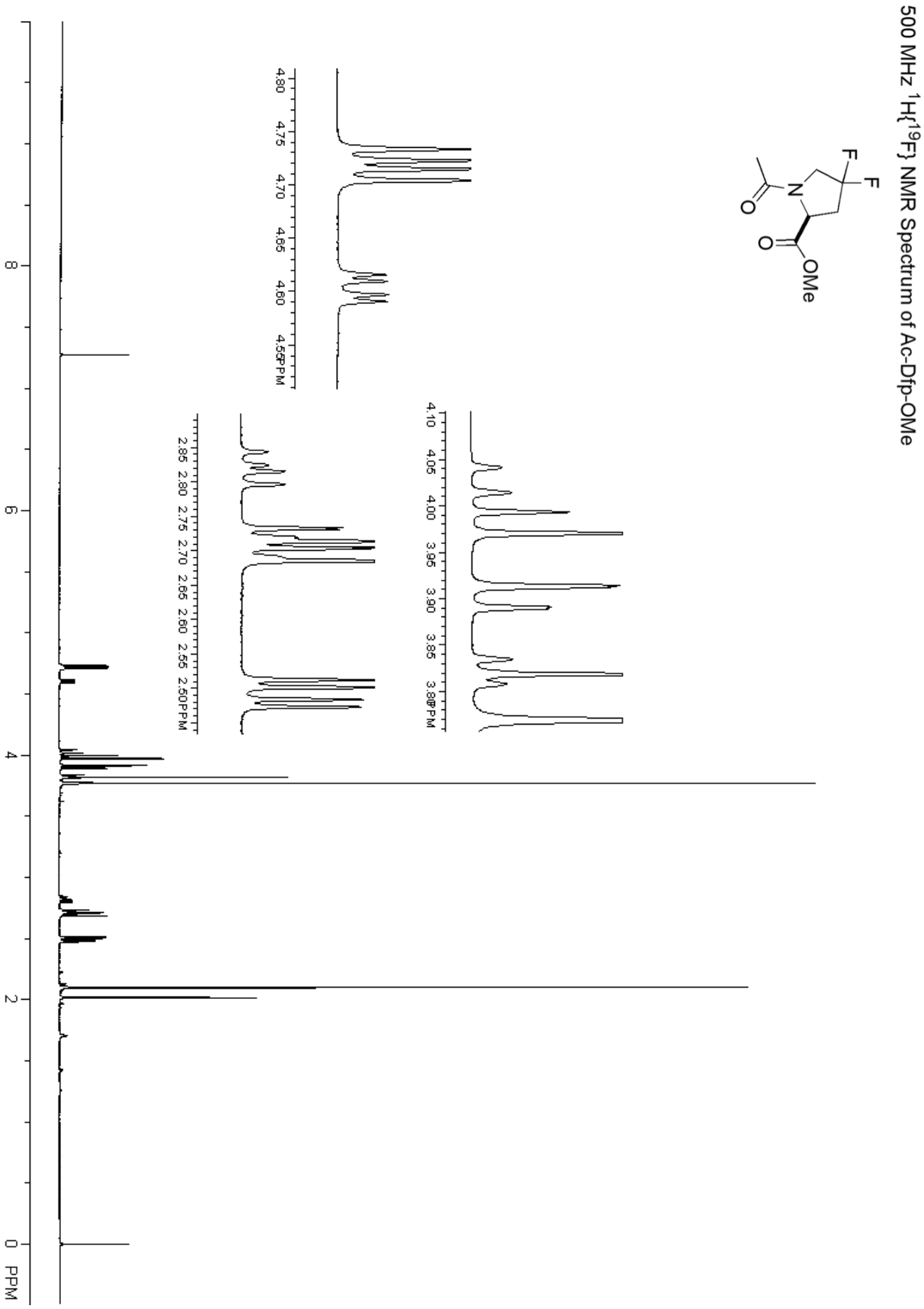




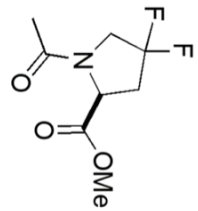
500 MHz ^{1}H NMR Spectrum of Ac-Dfp-OMe

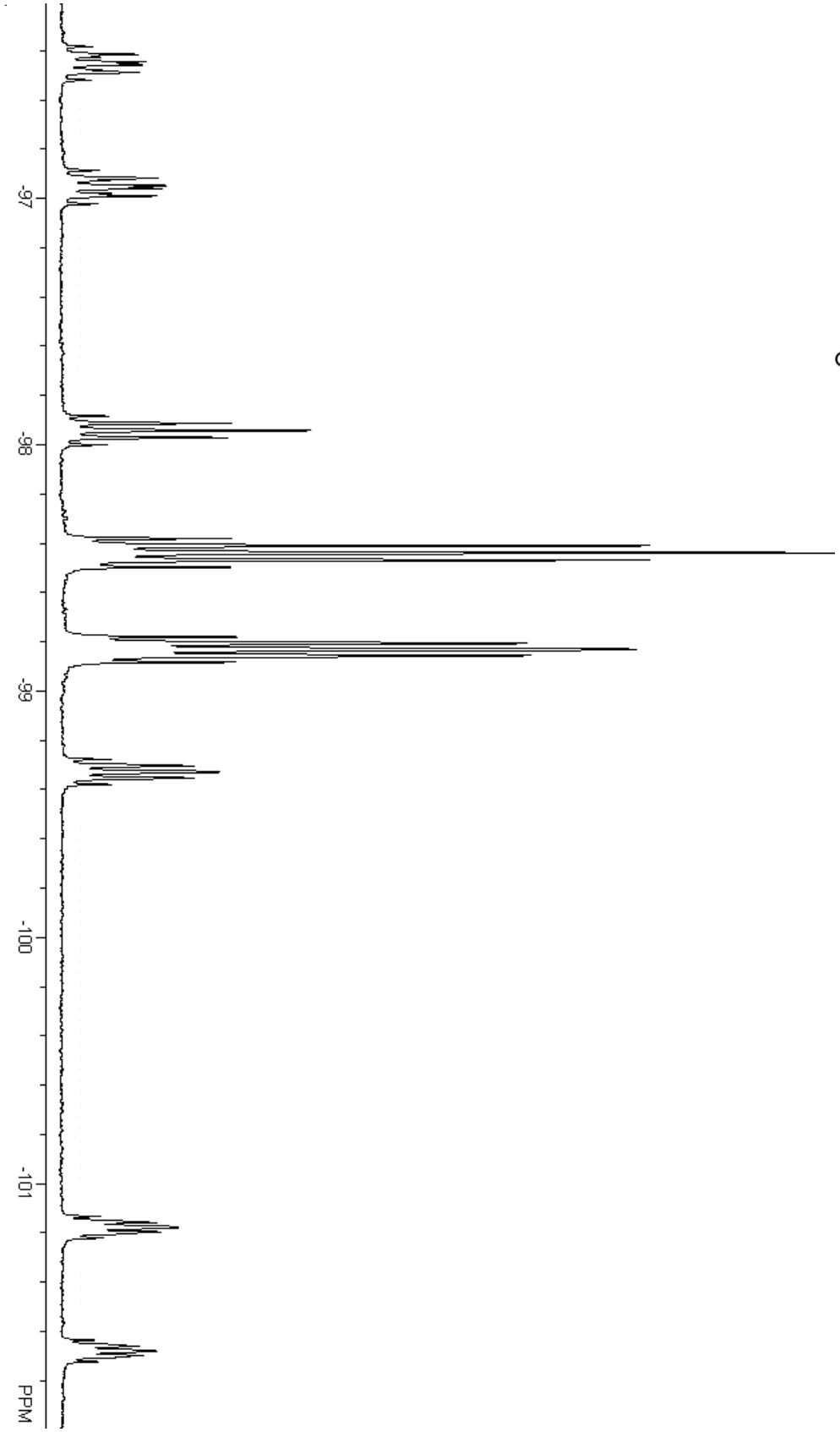


500 MHz $^{1}H\{^{19}F\}$ NMR Spectrum of Ac-Dfp-OMe

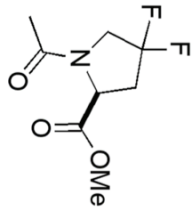


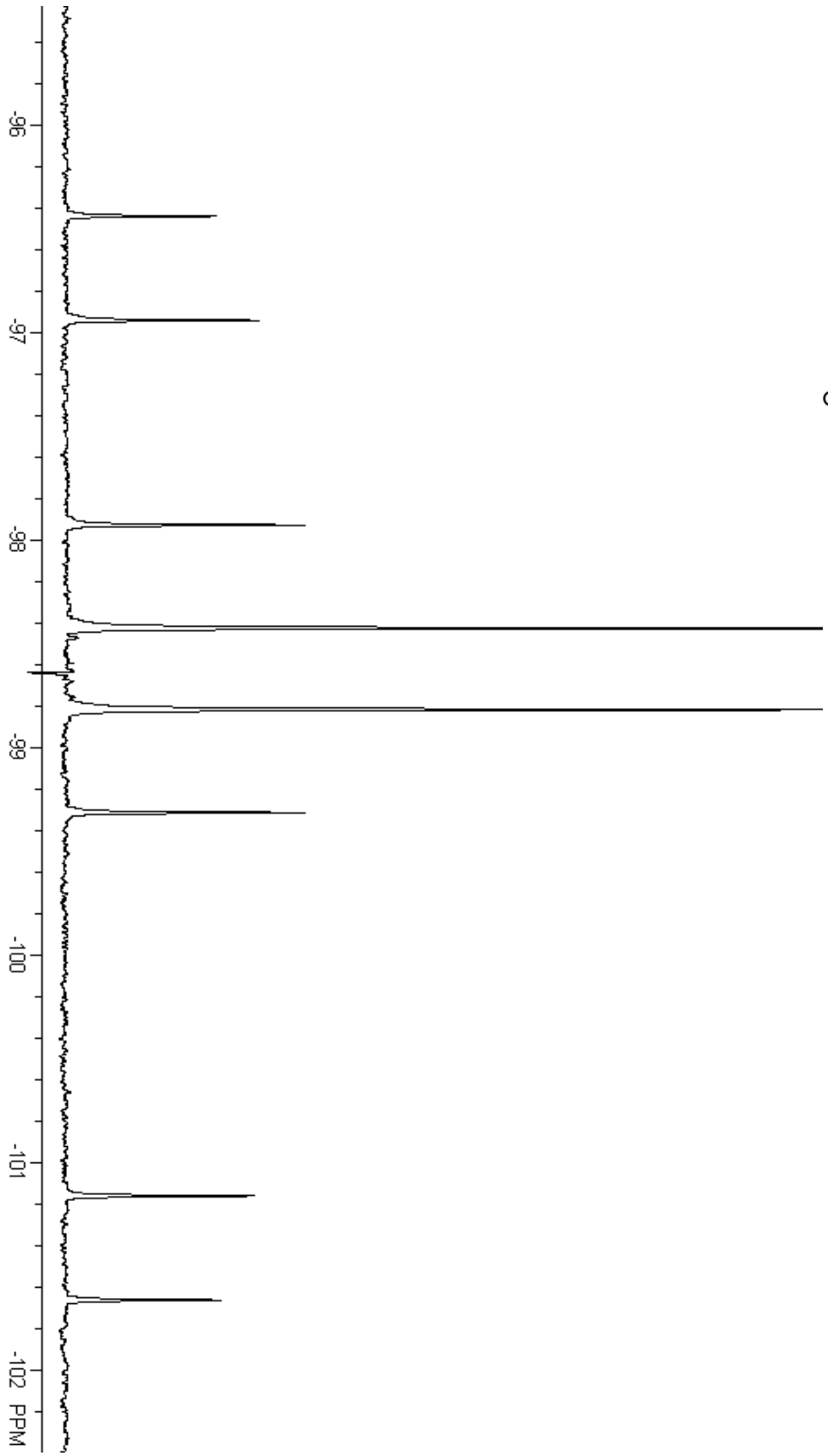
470.6 MHz ^{19}F NMR Spectrum of Ac-Dfp-OMe Referenced to ^{1}H NMR Spectrum



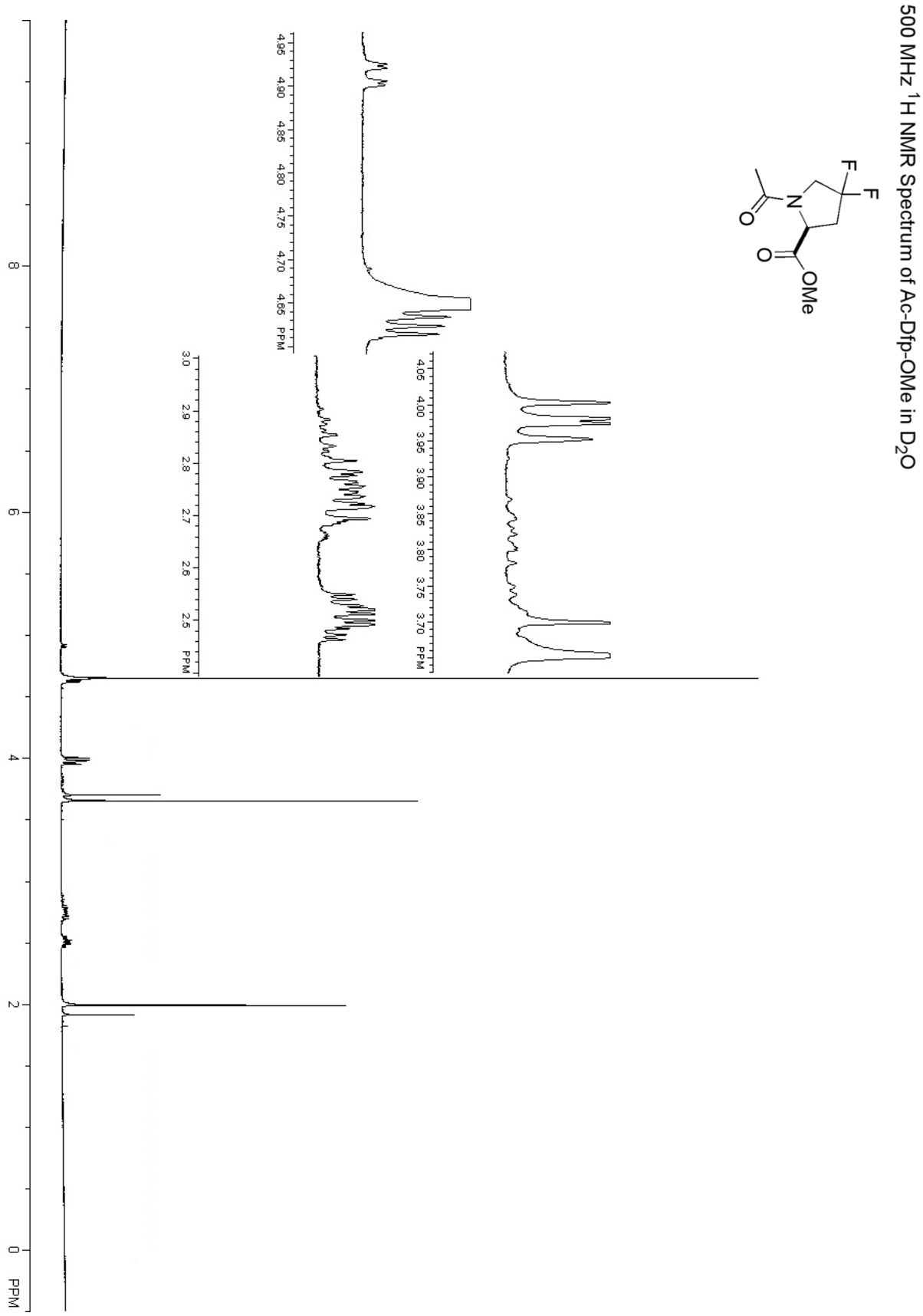


470.6 MHz $^{19}F\{^{1}H\}$ NMR Spectrum of Ac-Dfp-OMe Referenced to ^{1}H NMR Spectrum

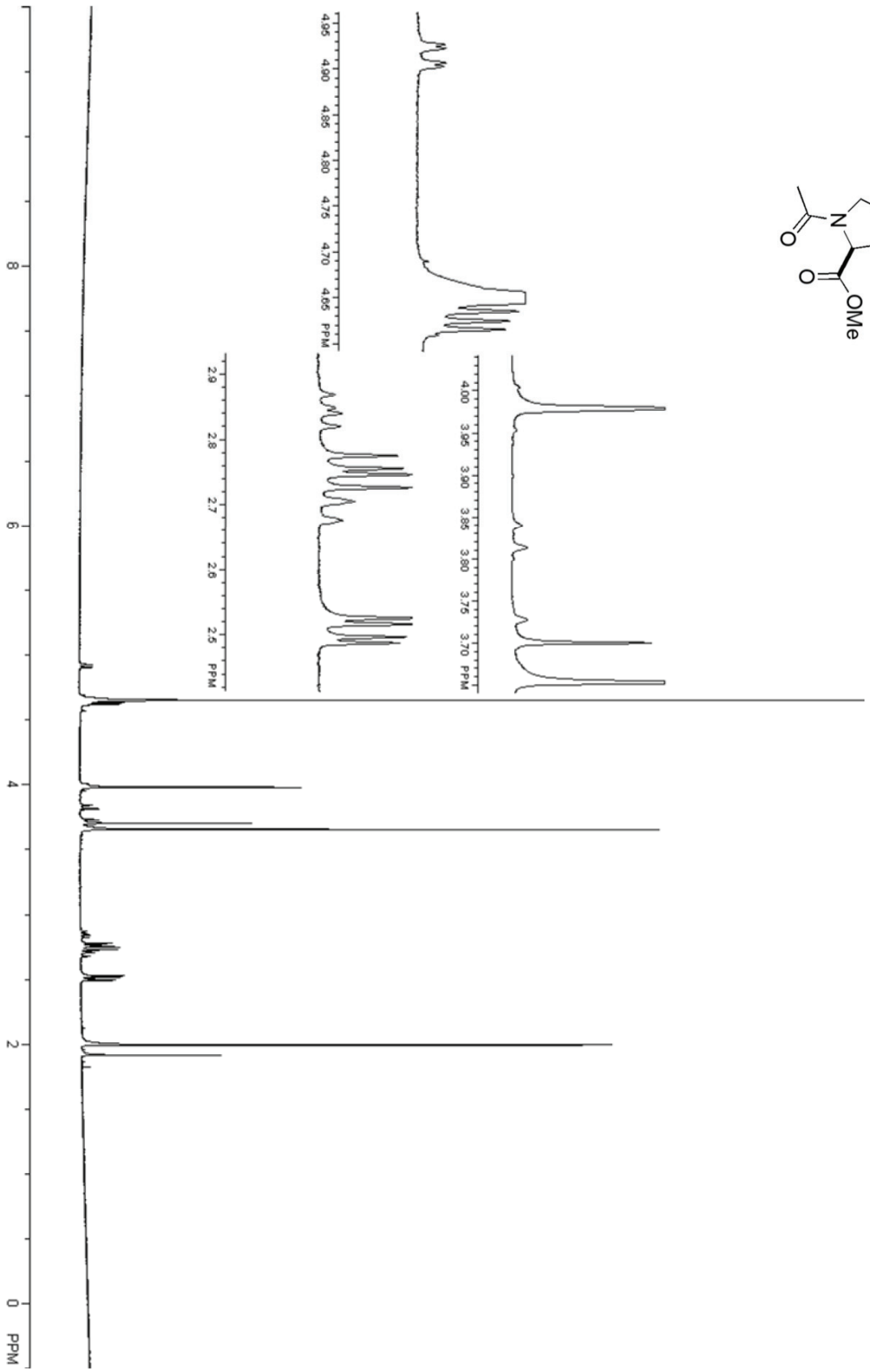




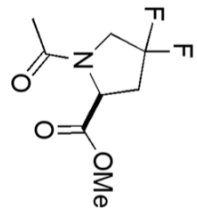
500 MHz ^{1}H NMR Spectrum of Ac-Dfp-OMe in D_2O

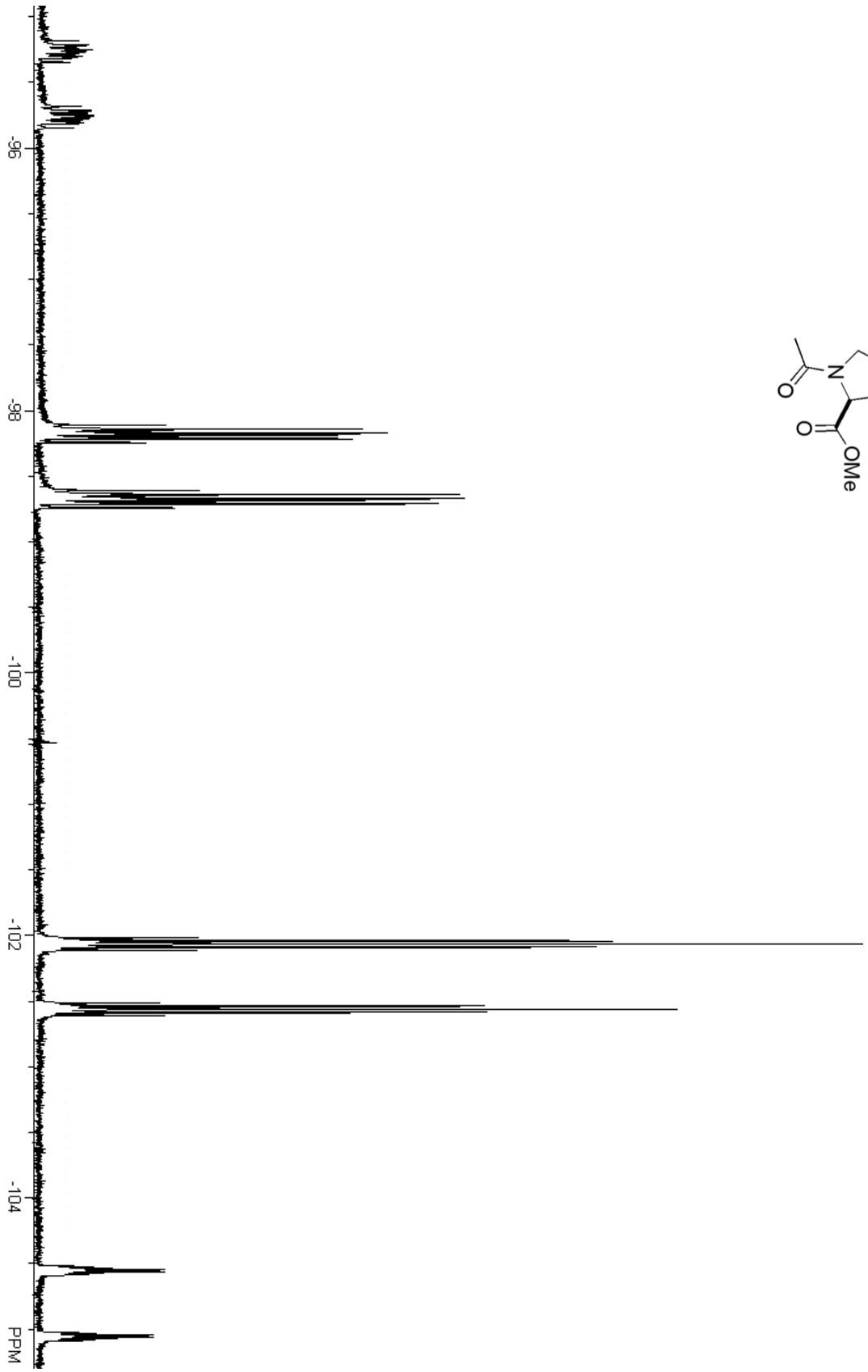


500 MHz $^{1}H\{^{19}F\}$ NMR Spectrum of Ac-Dfp-OMe in D_2O

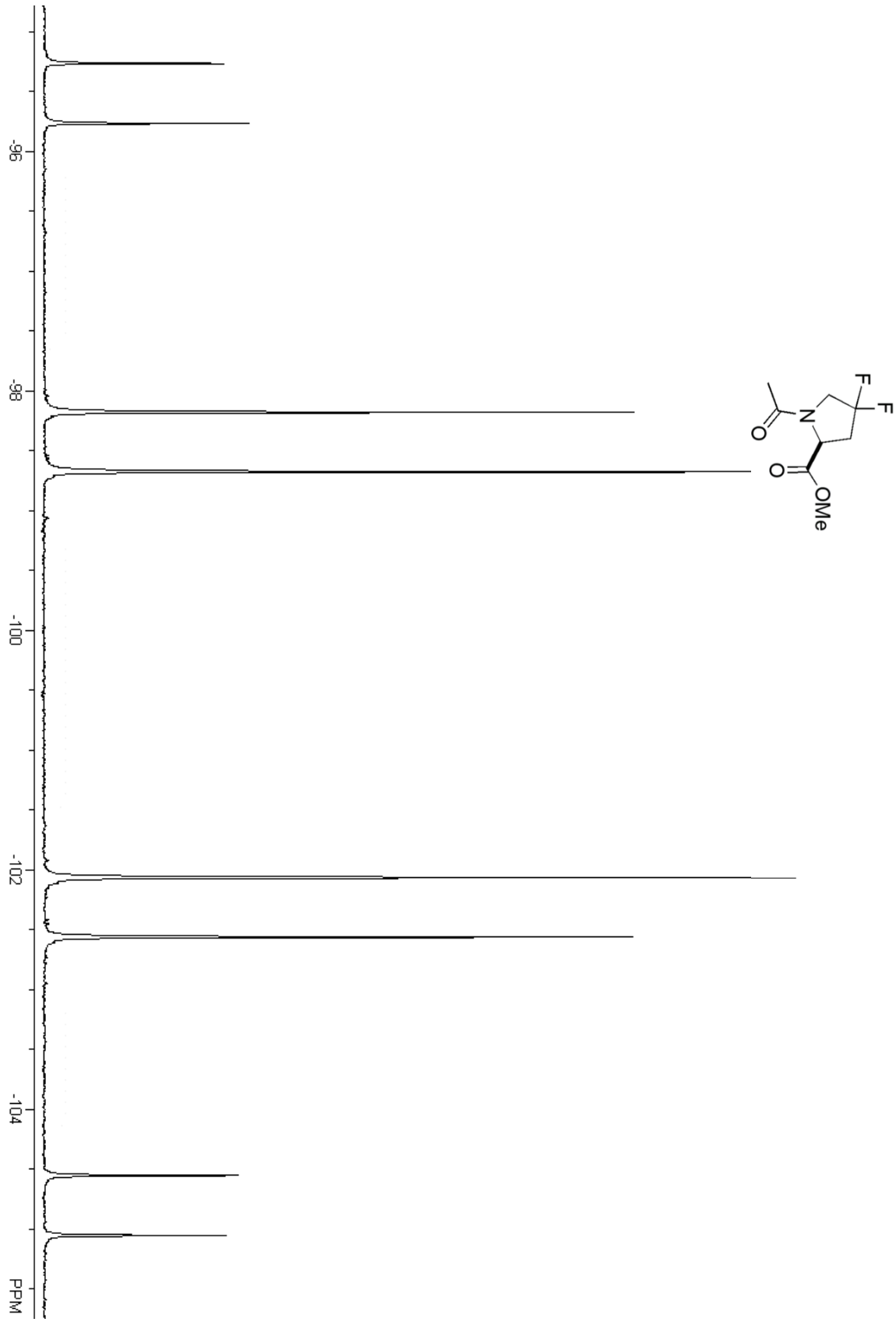


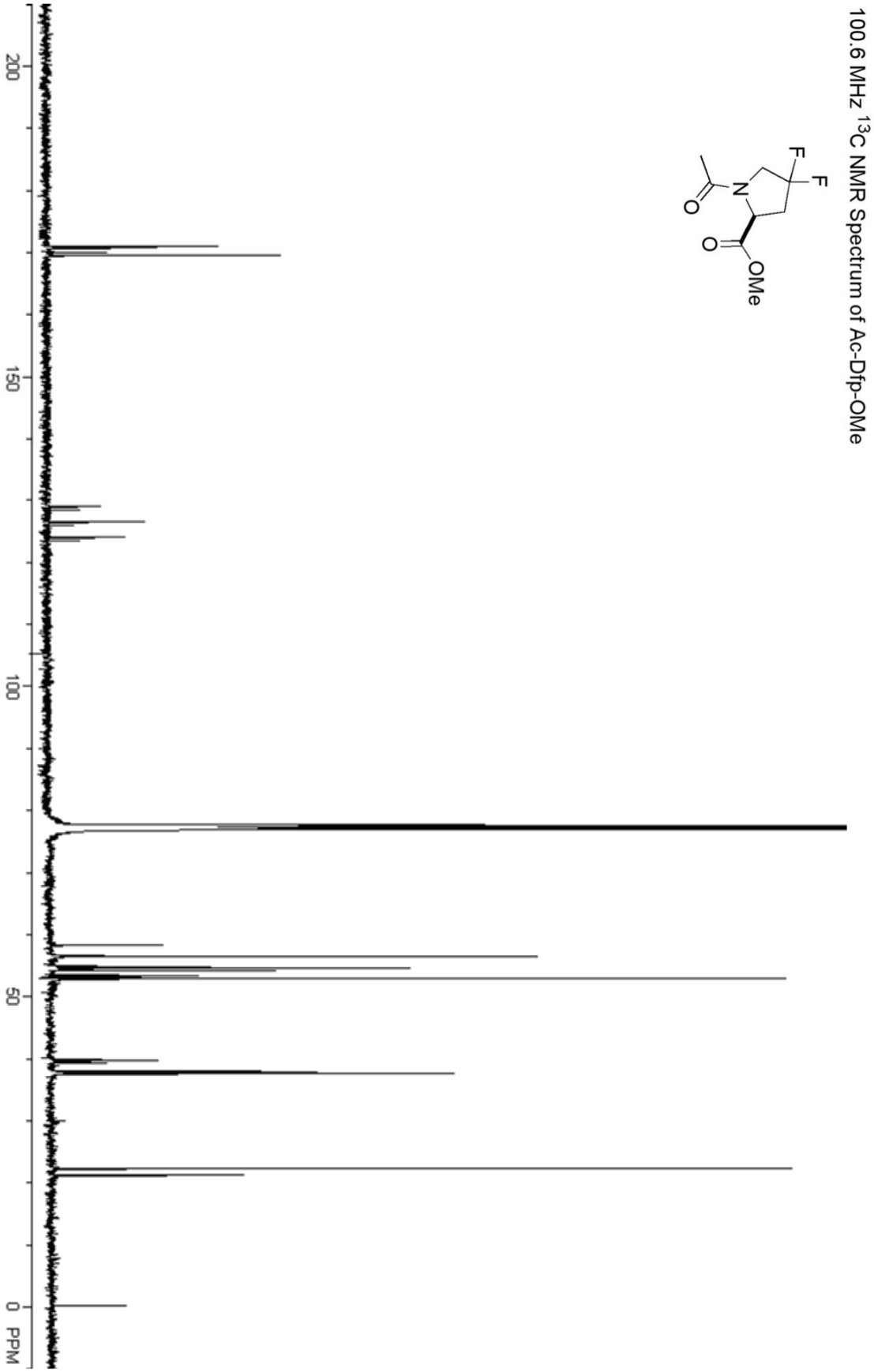
470.6 MHz ^{19}F NMR Spectrum of Ac-Dfp-OMe Referenced to ^{1}H NMR Spectrum in D_2O





470.6 MHz $^{19}F\{^{1}H\}$ NMR Spectrum of Ac-Dfp-OMe Referenced to ^{1}H NMR Spectrum in D_2O




100.6 MHz 13C NMR Spectrum of Ac-Dfp-OMe
F
F
N
O
O
OMe
200
150
100
50
0 PPM