Kinetic Isotope Effects Support the Twisted Amide Mechanism of Pin1 Peptidyl-Prolyl Isomerase

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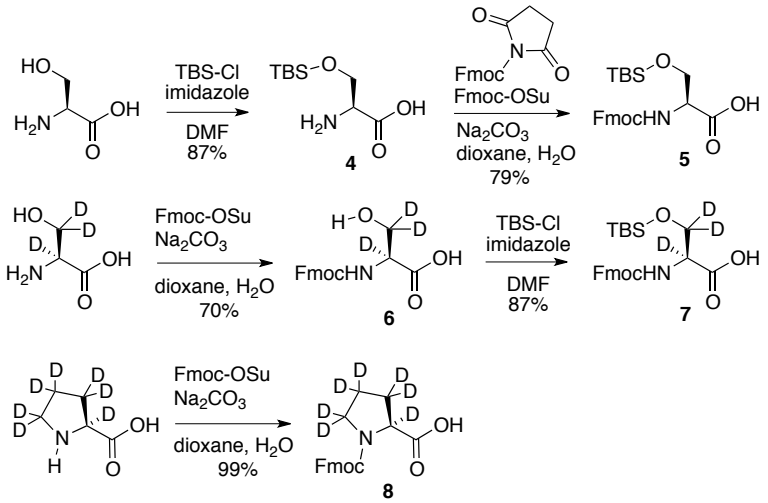
Supporting Information

RESULTS AND DISCUSSION

Synthesis of substrates. We synthesized the chromogenic substrates **1**–**3** via a combination of solid phase and solution phase peptide synthesis. The synthesis of the labeled substrates required incorporation of Fmoc–Ser-(TBS)–OH **5**, Fmoc–Ser-d_3(TBS)–OH **7**, and Fmoc–Pro-d_7–OH **8**. Protection of H–Ser–OH, H–Ser-d_3–OH, and H–Pro-d_7–OH were performed in good yields (Scheme S1). The serine hydroxyl was orthogonally protected with *tert*-butyldimethylsilyl (TBS) for peptide synthesis; either order of protection with TBS and Fmoc worked well (Scheme S1). The synthesis, but not the characterization, of Fmoc–Ser(TBS)–OH was reported previously in a

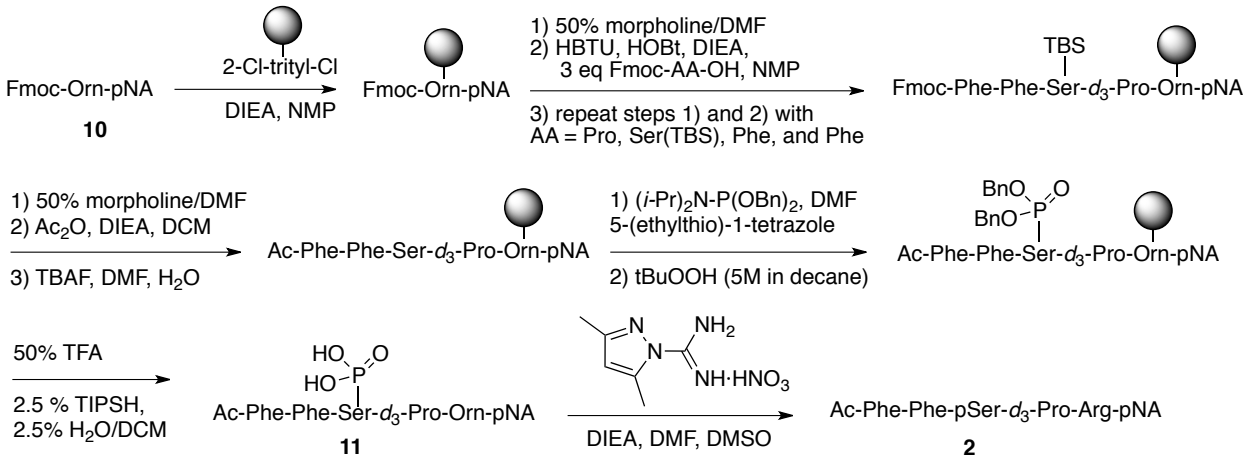
Scheme S1. Synthesis of protected, labeled amino acids: Fmoc–Ser-(TBS)–OH **5**, Fmoc–Ser-d_3(TBS)–OH **7**, and Fmoc–Pro-d_7–OH **8**.



patent.(*1*) *p*-Nitroaniline was incorporated as the chromophore at the *C*-terminus of Fmoc–Orn(Boc)–OH **10** for each substrate in the kinetic assay as previously reported.(*2, 3*)

During solid-phase peptide synthesis, morpholine (50% in NMP) was used for Fmoc deprotections to prevent cleavage of the chromophore from the *C*-termini of the peptides (Scheme S2).(*2*) After peptide elongation, tetra-*n*-butyl ammonium fluoride (TBAF), with 0.4% water to prevent epimerization, was used for TBS deprotection,(*4*) followed by phosphorylation of the peptide on-resin using (*N,N*-diisopropyl)-dibenzyl phosphoramidite.(*5*) After cleavage of peptides from the resin with 50% TFA using tri-*iso*-propylsilane (TIPSH) and water as cation scavengers, the Orn side chain of **11** was converted to Arg of **2** in solution using dimethyl-1H-pyrazole carboxamidine nitrate (Scheme S2).(*6*) The same scheme was used for **1** and **3**. CH_3CN precipitation was used to remove excess impurities from the final products to facilitate RP-HPLC substrate purification for substrates **1** and **2**. Peptides were purified by HPLC as described.

Scheme S2. Solid-phase synthesis and solution guanidinylation of peptide substrate **2**.



EXPERIMENTAL PROCEDURES

General. Deuterium-labeled amino acids were purchased from Cambridge Isotopes, anhydrous *N,N*'-dimethylformamide (DMF) and redistilled diisopropylethylamine (DIEA) were obtained commercially and used directly from sealed bottles. Brine (NaCl), $NaHCO_3$, and NH_4Cl refer to

saturated aqueous solutions. Flash chromatography was performed on 32-63 or 230-400 mesh. The substrates were characterized by ^{1}H NMR at 400 or 500 MHz, and by liquid chromatography–mass spectrometry (LC-MS) with electrospray ionization (ESI^+) or fast-atom bombardment (FAB^+) for molecular ion identification and purity.

Fmoc–Ser(TBS)–OH, 5. Unlabeled Fmoc–Ser(TBS)–OH has been reported previously by a different method.(*1*) H–Ser–OH (1.00 g, 9.44 mmol) and imidazole (1.29 g, 18.9 mmol) were dissolved in dry DMF (9.5 mL) under Ar, *t*-butyldimethylsilyl (TBS) chloride (1.55 g, 10.3 mmol) was added, and the reaction was stirred at room temperature for 20 h. The DMF was evaporated under high vacuum for 4 h. Hexanes:water (1:1, 10 mL) was added and stirred for 1 h, then the mixture was filtered, and the resulting white solid was washed with hexanes. The solid was dried under vacuum to yield 1.79 g (87%). The crude H–Ser(OTBS)–OH **4** (1.67 g, 7.60 mmol) and Na_2CO_3 (1.15 g, 9-10% in H_2O) were dissolved in H_2O (11.5 mL). Dioxane (5 mL) was added, and the mixture was heated to 37 ºC to dissolve the solids. Fmoc-OSu (2.69 g, 7.98 mmol) was dissolved in dioxane (11 mL) and transferred to the aqueous amino acid solution *via cannula* (pH 9). After stirring for 18 h, another 1 g of Na_2CO_3 was added to ensure deprotonation of the amino acid carboxylic acid (pH 10). The reaction mixture was diluted with H_2O (120 mL), washed with Et_2O (2 × 30 mL), and acidified to pH 2 with ice-cold 1M HCl (60 mL) to precipitate the amino acid. EtOAc (6 × 30 mL) was used to extract the precipitate, and the combined EtOAc layers were washed with brine (3 × 25 mL), water (25 mL), dried over Na_2SO_4, and concentrated to give a clear oil. Ether (3 × 25 mL) was added to the oil and concentrated in vacuo (3 ×), and dried under high vacuum, yielding 2.63 g (79%) of Fmoc–Ser(TBS)–OH **5** as a foamy, white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.2 (br s, 1H), 7.76 (d, J = 7.6, 2H), 7.61 (t, J = 7.6, 2H), 7.40 (t, J = 7.6, 2H), 7.31 (t, J = 7.6, 2H), 5.63 (d, J = 6.5, 1H), 4.48 (m, 1H), 4.42

(app quint, J = 8.6, 2H), 4.26 (t, J = 7.6, 1H), 4.16 (d, J = 10.8, 1H), 3.88 (d, J = 10.8, 1H), 0.93 (9H, s). 0.08 (6H, s).

Fmoc–Ser-*d*$_3$(TBS)–OH, 7. (2,3,3-^{2}H)-Serine (56 mg, 0.53 mmol) was dissolved in dioxane (1.5 mL), to which Na_2CO_3 (1.5 mL) was added and cooled to 0 °C. Fmoc-OSu (165 mg, 0.64 mmol) was dissolved in dioxane (1.5 mL), added dropwise via syringe, and the mixture was stirred at 0 °C for 0.5 h. After stirring at rt for 1 h, the mixture was diluted with H_2O (40 mL), washed with ether (3 × 30 mL), cooled to 0 °C, and acidified with 1 M HCl to pH 3. The resulting precipitate was extracted with DCM (4 × 30 mL), and the organic layer was dried over Na_2SO_4, and the solvent was evaporated. The resulting clear oil was purified by flash chromatography with EtOAc:AcOH (100:2) on SiO_2, and dried under vacuum to yield 122 mg (70%) of Fmoc–Ser-d_3–OH **6**. ^{1}H NMR (400 MHz, CD_3OD) δ 7.83 (2H, d, J = 7.6), 7.72 (2H, d, J = 6.6), 4.34 (2H, d, J = 5.2), 4.14 (1H, t, J = 6.8). The crude Fmoc–Ser-d_3–OH **6** (120 mg, 0.37 mmol) and imidazole (228 g, 3.35 mmol) were dissolved in dry THF (2 mL). *t*-Butyldimethylsilyl chloride (TBS-Cl) (160 mg, 1.06 mmol) was added, and the reaction was stirred at rt. After 4 h, the reaction was diluted with DCM (70 mL), washed with saturated aq. NH_4Cl (3 × 20 mL) and water (10 mL), dried over Na_2SO_4, and the solvent was evaporated. The solid was purified by flash chromatography with hexanes, then EtOAc:AcOH (100:3) on SiO_2. The resulting solid was dried under vacuum to yield 143 mg (87%) of Fmoc–Ser(TBS)-d_3–OH **7**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, J = 7.6, 2H), 7.61 (t, J = 7.2, 2H), 7.40 (t, J = 7.2, 2H), 7.31 (t, J = 7.6, 2H), 5.61 (1H, br s), 4.40 (td, J = 15, 8.9, 2H), 4.24 (1H, t, J = 6.2), 1.8 (1H, br s), 0.89 (9H, s), 0.08 (6H, s). ^{13}C NMR (100 MHz, $CDCl_3$) δ 175, 156.2,144,0, 141.4, 127.9, 127.3, 125.4, 120.2, 67.5, 62 (br), 56 (br), 47.3, 25.9, 18.5, –5.2.

Fmoc–Pro-*d*$_7$–OH, 8. (2,3,3,4,4,5,5-^{2}H)-Proline (H–Pro-d_7–OH, 250 mg, 2.05 mmol) was

dissolved in water (3 mL), to which Na_2CO_3 (300 mg, 10% in water) was added. Fmoc-OSu (725 mg, 2.15 mmol) was dissolved in dioxane (3 mL) and transferred to the aqueous amino acid solution dropwise via cannula (pH 9). After stirring 16 h, another 200 mg of Na_2CO_3 was added to ensure deprotonation of the amino acid carboxylic acid (pH 10). The reaction mixture was diluted with H_2O (60 mL), washed with Et_2O (2 × 10 mL), and acidified to pH 2 with ice-cold 1 M HCl (12 mL) to precipitate the amino acid. EtOAc (6 × 10 mL) was used to extract the precipitate, and the combined EtOAc layers were washed with water (7 mL), brine (3 × 7 mL), dried over Na_2SO_4, and concentrated to a clear oil. Ether was added to the oil and concentrated *in vacuo* (3 × 10 mL), and dried *in vacuo* to yield 698 mg (99%) of Fmoc–Pro-d_7–OH **8** as a white foamy solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, J = 7.6, 2H), 7.60 (t, J = 7.4, 2H), 7.42 (t, J = 7.4, 2H), 7.33 (t, J = 7.6, 2H), 4.53 (t, J = 8.4, 1H), 4.45 (t, J = 8.4, 1H), 4.28 (t, J = 6.9, 0.74H), 4.20 (br s, 0.26H).

Fmoc–Orn(·TFA)–pNA, 10. Prepared by the method of Bernhardt et al.(*2*) *p*-Nitroaniline (912 mg, 6.60 mmol) was dissolved in freshly distilled pyridine (16 mL) under Ar, and cooled to –10 °C in an NH_4Cl ice bath. Fmoc–Orn(Boc)–OH (3.00 g, 6.60 mmol), and $POCl_3$ (677 μL, 7.26 mmol) were added. The reaction was monitored by TLC with 5% MeOH in DCM. After completion in 35 min, the reaction was quenched with iced water (20 mL), and extracted with EtOAc (3 × 60 mL). The combined organic layers were washed with saturated aqueous $NaHCO_3$ (2 × 60 mL), brine (60 mL), 0.1 N HCl (2 × 60 mL), brine (60 mL), dried over $MgSO_4$, and concentrated *in vacuo*. Toluene (3 x 30 mL) was added and concentrated to remove the pyridine, followed by DCM (3 × 30 mL) to the remove toluene. The resulting solid was suspended in DCM, collected by vacuum filtration, and washed with DCM to yield 3.67 g (97% crude yield) of Fmoc-Orn(Boc)–*p*NA **9**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.35 (br s, 1H), 8.24 (d, J = 9.2, 2H),

7.83 (t, *J* = 9.2, 2H), 7.80 (d, *J* = 8.3, 2H), 7.64 (br s, 2H), 7.44 (t, *J* = 7.4, 2H), 7.34 (t, *J* = 8.3, 2H), 7.21 (d, *J* = 7.3, 1H), 5.71 (d, *J* = 7.7, 1H), 4.88 (br s, 1H), 4.70 (br s, 1H), 4.45 (d, *J* = 7.2, 2H), 4.25 (t, *J* = 7.2, 1H), 3.6 (br s, 1H), 3.1 (m, 1H), 1.97 (m, 1H), 1.67 (m, 3H), 1.48 (s, 9H). Without further purification, Fmoc-Orn(Boc)–*p*NA **9 (**2.65 g, 4.62 mmol) was suspended in DCM (24 mL), to which TFA (15 mL) was added via syringe, and the mixture was stirred for 60 min. The solution was concentrated to remove TFA, redissolved in MeOH, concentrated, and triturated in DCM (3 × 60 mL), ether (3 × 60 mL), and the solvents were evaporated to remove any remaining TFA. The resulting white solid was then washed with ether under vacuum filtration, and dried *in vacuo* to yield 2.2 g (100%) of The Fmoc–Orn(·TFA)–*p*NA salt **10**, which was used in peptide synthesis without further purification. ^{1}H NMR (DMSO-d_6) δ 10.72 (s, 1H), 8.23 (d, *J* = 8.4, 2H), 7.85 (m, 4H), 7.71 (t, *J* = 6.6, 2H), 7.64 (m, 2H), 7.40 (t, *J* = 7.6, 2H), 7.31 (m, 2H), 4.30 (m, 2H), 4.21 (m, 2H), 2.78 (br s, 2H), 1.75 (m, 1H), 1.63 (m, 3H).

Solid phase synthesis of Ac–Phe–Phe–pSer–Pro–Orn–pNA peptides. All peptides were synthesized under identical conditions unless otherwise noted. Peptides were manually synthesized with 3 equivalents of Fmoc-protected amino acids, coupling with HBTU, HOBt, and DIEA in NMP (2 × 20 min), and deprotecting with 50% morpholine/NMP solution. The unlabeled substrate, Ac–Phe–Phe–pSer–Pro–Arg–*p*NA **1**, and Ac–Phe–Phe–pSer-d_3–Pro–Arg–*p*NA **2** for kinetic comparison, were synthesized on a 2-chlorotritylchloride resin (0.5 g, 0.8-1.6 mmol/g) using a disposable polypropylene column. Similarly **1** and Ac–Phe–Phe–pSer–Pro-d_7–Arg–*p*NA **3**, for kinetic comparison, were synthesized in parallel on a 5.8-fold larger scale on 2-chlorotritylchloride resin (2.89 g, 1.5 mmol/g) using a glass reaction vessel with sintered glass filter. The resin was swollen for 20 min in DCM, the Fmoc–Orn(·TFA)–*p*NA salt **10** (552 mg, 1.2 mmol) and DIEA (730 μL 4.2 mmol) were dissolved in NMP (2 mL). The mixture was

added to the 2-chlorotritylchloride resin and shaken for 4 h. The Fmoc protecting group was removed with 50% morpholine in NMP (2 × 5 mL). The resin was washed with NMP (2 × 8 mL). The Fmoc-Pro-OH (700 mg, 2.1 mmol) was coupled to the resin using HBTU (797 mg, 2.1 mmol), HOBt (284 mg, 2.1 mmol), and DIEA (730 μL, 4.2 mmol) in NMP (5 mL), and double coupled (2 × 20 min). The Fmoc-Pro-Orn(resin)-*p*NA was dried under high vacuum overnight, and split in two batches of 282 mg. The peptide on resin (282 mg) was elongated by adding HOBt (142 mg, 1.1 mmol), HBTU (399 mg, 1.1 mmol), and DIEA (365 μL, 2.1 mmol) and coupled for 20 min with the following amino acids sequentially: Fmoc–Ser(TBS)–OH **5** (150 mg, 0.35 mmol), and twice with Fmoc–Phe–OH (400 mg, 1.05 mmol), to give Fmoc-Phe-Phe-Ser(TBS)-Pro-Orn(resin)-*p*NA. For the labeled substrates, Fmoc–Ser-d_3(TBS)–OH **7** (150 mg, 0.35 mmol) was coupled for 20 min once; while Fmoc–Pro-d_7–OH **8**, was double coupled (2 × 45 min). The final Fmoc group was removed using 50% morpholine in NMP (5 mL, 2 × 20 min), and the free *N*-terminus was acetylated using a mixture of 10% DIEA and 10% Ac_2O in DCM for 10 min (3 mL). The peptide was dried under vacuum overnight. The resin was suspended in DMF (6 mL), to which TBAF (0.15 M, 2.5 mL) and H_2O (0.4%) were added,(*4*) and the mixture was shaken for 4 h. The resin was then washed with DMF (5 mL, 3 × 30 sec), DCM (5 mL, 3 × 30 sec), MeOH (5 mL, 3 × 30 sec), and dried *in vacuo* overnight.

Ac–Phe–Phe–pSer-d_3–Pro–Orn(·TFA)–*p*NA, 11. (*N,N*-Diisopropyl)dibenzyl-phosphoramidite (345 μL, 1.05 mmol) was dissolved in anhydrous DMF (5 mL) containing 5-ethylthio-1-tetrazole (365 mg, 2.8 mmol). This solution was transferred to a dry 10 mL conical bottom flask containing the dried peptide under Ar, and reacted for 1 h or 6 h, as noted below. The resin was then covered with DMF (1 mL), *t*-BuOOH (5 M in decane, 3 mL) was added under Ar, and the mixture was shaken for 45 min. The resin was transferred to a disposable polypropylene column,

and washed with DMF (5 mL, 3 × 30 sec), DCM (5 mL, 3 × 30 sec), MeOH (5 mL, 3 × 30 sec), and dried *in vacuo* overnight. The dried resin was then treated with 95% TFA, with 2.5% tri-*iso*-propylsilane (TIPSH) and 2.5% H_2O as cation scavengers, for 4 h. The resin was removed by filtration, and the peptide was collected in the filtrate. The resin beads were washed with MeOH (5 mL, 3 × 1 min), and DCM (5 mL, 3 × 1 min), and the filtrates were combined and concentrated. The solids were triturated with diethyl ether (15 mL) to remove any remaining TFA. The other peptides were phosphorylated as above for the times and crude yields obtained, as listed below. Ac–Phe–Phe–pSer-d_3–Pro–Orn(·TFA)–*p*NA **11**, phosphorylated for 6 h (24.3 mg). LC-ESI$^+$-MS: calcd. for $C_{39}H_{46}D_3N_8O_{12}P$ $[MH]^+$ m/z = 857.2, found m/z = 857.2

Ac–Phe–Phe–pSer–Pro–Orn–*p*NA, phosphorylated for 6 h (31.7 mg). LC-ESI$^+$-MS: calcd. for $C_{39}H_{49}N_8O_{12}P$ $[MH]^+$ m/z = 853.3, found m/z = 853.4.

Ac–Phe–Phe–pSer–Pro-d_7–Orn–*p*NA, phosphorylated for 1 h (330 mg). FAB$^+$-MS: calcd. for $C_{39}H_{42}D_7N_8O_{12}P$ $[MH]^+$ m/z = 859.5, found m/z = 859.5.

Ac–Phe–Phe–pSer–Pro–Arg–pNA, 1. The crude Ac–Phe–Phe–pSer–Pro–Orn(·TFA)–*p*NA (40 mg, 0.047 mmol) was dissolved in DMF (2.0 mL) and DMSO (2.0 mL). Dimethyl-1H-pyrazole carboxamidine nitrate (66 mg, 0.33 mmol) and DIEA (0.095 mL, 0.56 mmol) was added, and the mixture was heated to 45 ºC for 24 h. The reaction was quenched with ice water (ca. 20 mL), frozen with liquid nitrogen, and lyophilized overnight to remove the DMF:DMSO solvent mixture. The crude peptide was precipitated with cold CH_3CN (3 × 12 mL), and purified by RP-HPLC, using a Waters C18 XBridge 5 μm, 19 × 100 mm semi-preparative column on a Varian Pro-Star 218 HPLC with linear gradient of 0 to 40% CH_3CN/H_2O over 16.5 min, flow rate 10 mL/min, λ = 210 nm. The desired peak was collected repetitively at 12.6 min, CH_3CN was removed under vacuum, and the water was removed by lyophilization to give Ac–Phe–Phe–

pSer–Pro–Arg–*p*NA **1** (3 mg, 0.84% yield). ^{1}H NMR (500 MHz, CD_3OD) δ 8.15 (d, *J* = 9.1 Hz, 2H), 7.98 (d, *J* = 9.1, 2H), 7.2-7.0 (m, 10H), 5.00 (dd, *J* = 8.8, 4.7, 1H), 4.67 (dd, *J* = 8.3, 6.0, 2H), 4.54 (dd, *J* = 9.3, 5.5, 2H), 4.38 (m, 2H), 4.23 (dt, *J* = 9.8, 4.7 1H), 4.13 (dd, *J* = 16.6, 6.7, 1H), 4.04 (m, 1H), 3.95 (dd, *J* = 20.7 10.4, 1H), 3.79 (m, 1H), 3.44 (m, 0.5H), 3.24 - 3.16 (m, 2.5H), 3.02 (dd, *J* = 14.0, 5.5, 1H), 2.95 (dd, *J* = 13.9, 8.4, 1H), 2.78 (dd, *J* = 14.0, 9.3, 1H), 2.48 (dd, J = 15.4, 7.8, 0.2H), 2.35 (m, 0.8H), 2.10 (m, 2H), 2.00 (m, 3H), 1.85 (s, 2.7H), 1.84 (s, 0.3), 1.79 (m, 1H), 1.67 (m, 1H). ^{13}C NMR (126 MHz, CD_3OD) δ 175.26, 173.41, 173.40, 173.31, 173.28, 173.01, 158.78, 145.87, 144.72, 138.41, 138.23, 130.50, 130.15, 129.47, 129.41, 127.80, 127.71, 125.54, 121.21, 65.41, 63.55, 57.48, 56.76, 56.21, 55.35, 53.16, 42.14, 38.96, 38.56, 30.98, 29.46, 26.43, 26.19, 22.38. ^{31}P NMR (DMSO-d_6) δ –0.82. LC-HRMS (FAB^+): 12.55 min, calcd. for $C_{40}H_{52}N_{10}O_{12}P$ $[M+H]^+$ *m/z* = 895.3504, found *m/z* = 895.3523, calcd. for $C_{40}H_{51}N_{10}O_{12}P{\cdot}Na$ $[M+Na]^+$ *m/z* = 917.3323, found *m/z* = 917.3330.

Ac–Phe–Phe–pSer-*d*$_3$–Pro–Arg–*p*NA, 2. The crude Ac–Phe–Phe–pSer-d_3–Pro–Orn(·TFA)–*p*NA **12** (24 mg, 0.028 mmol) was dissolved in DMF (2.0 mL) and DMSO (2.0 mL). Dimethyl-1H-pyrazole carboxamidine nitrate (41.0 mg, 0.20 mmol) and DIEA (0.058 mL, 0.34 mmol) was added, and the mixture was heated to 45 ºC for 24 h. The reaction was quenched with ice water (20 mL), frozen with liquid nitrogen, and lyophilized overnight to remove the DMF:DMSO solvent mixture. The crude peptide Ac–Phe–Phe–pSer-d_3–Pro–Arg–*p*NA **2** was precipitated with cold CH_3CN (3 × 12 mL), and purified by RP-HPLC, using a Waters C18 XBridge 5 μm, 19 × 100 mm semi-preparative column on a Varian Pro-Star 218 HPLC with linear gradient of 0 to 40% CH_3CN/H_2O over 16.5 min, flow rate 10 mL/min, λ = 210 nm. The desired peak was collected repetitively at 12.6 min. CH_3CN was removed under vacuum, and the water was removed by lyophilization to give Ac–Phe–Phe–pSer-d_3–Pro–Arg–*p*NA **2** (1.9 mg, 0.53% yield).

^{1}H NMR (500 MHz, CD_3OD) δ 8.16 (d, *J* = 9.4, 2H), 8.08 (d, *J* = 9.4, 2H), 7.3-7.1 (m, 10H), 4.66 (dd *J* = 8.3, 5.9, 1H), 4.60 (m, 1H), 4.54 (dd *J* = 9.3, 5.5, 1H), 4.38 (m, 2H), 4.14 (m, 1H), 3.89 (m, 1H), 3.16 (m, 2H), 3.02 (dd, *J* = 13.9, 5.5, 1H), 2.94 (dd, *J* =13.8, 8.3, 1H), 2.78 (dd, *J* = 13.9, 9.3, 1H), 2.35 (m, 1H), 2.11 (m, 2H), 2.01 (m, 3H), 1.85 (s, 3H), 1.78 (m, 1H), 1.67 (m, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.0, 171.8, 171.5, 171.0, 169.4, 157.0, 145.2, 142.5, 138.2, 137.7, 129.4, 129.32, 129.25, 128.20, 128.17, 126.5, 126.3, 125.1, 119.3, 65.4, 62.0, 55.4, 54.1, 53.7, 40.5, 37.6, 37.4, 29.8, 27.7, 25.2, 24.7, 22.5. LC-MS (ESI^+): 12.55 min, calcd. for $C_{40}H_{48}{}^{2}H_3N_{10}O_{12}P$ $[M+H]^+$ *m/z* = 898.4, found *m/z* = 898.3.

Ac–Phe–Phe–pSer–Pro-d_7–Arg–*p*NA, 3. The crude Ac–Phe–Phe–pSer–Pro-d_7–Orn(·TFA)–*p*NA (330 mg, 0.38 mmol) was dissolved in DMF (1.6 mL) and DMSO (1.6 mL). Dimethyl-1H-pyrazole carboxamidine nitrate (540 mg, 2.7 mmol) and DIEA (0.80 mL, 4.6 mmol) was added, and the mixture was heated to 45 ºC for 24 h. The reaction was quenched with water, frozen with liquid nitrogen, and lyophilized overnight to remove the DMF:DMSO solvent mixture. The crude peptide was purified by RP-HPLC, using a Varian Polaris 10 μm, C18 45 × 100 mm preparative column, with 0.1% TFA in 4 to 42% CH_3CN/H_2O gradient over 18 min, increased to 100 % CH_3CN over 1 min, and held for 10 min, flow rate 50 mL/min, λ = 254 nm. The desired peak was collected repetitively at 19.5 min, the CH_3CN was removed under vacuum, and the water was removed by lyophilization to give Ac–Phe–Phe–pSer–Pro-d_7–Arg–*p*NA **3**. (400 MHz, DMSO-d_6) δ 10.43 (s, 1H), 8.51 (br s, 2H), 8.23 (d, *J* = 9.2, 2H), 8.08 (d, *J* = 8.0, 2H), 7.98 (d, *J* = 8.8, 2H), 7.25 (m, 10H), 4.83 (m, 1H), 4.61 (m, 1H), 4.46 (m, 1H), 4.34 (m, 1H), 4.15 (m, 1H), 3.91 (m, 1H), 3.63 (m, 1H), 3.14 (m, 3H), 3.06 (dd, *J* = 14.0, 4.8, 1H), 2.94 (dd, *J* = 14.0, 4.5, 1H), 2.83 (dd, *J* = 13.6, 8.7, 1H), 2.67 (dd, *J* = 13.6, 10.1, 1H), 1.84 (m, 1H), 1.74/1.72 (two s, 3H), 1.57 (m, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.0, 171.8, 171.5, 171.0, 169.4, 157.0,

145.2, 142.5, 138.2, 137.7, 129.4, 129.32, 129.25, 128.20, 128.17, 126.5, 126.3, 125.1, 119.3, 62.0, 54.4, 54.1, 53.7, 51.5, 42.0, 37.6, 37.4, 28.0, 25.3, 22.6. ^{31}P NMR (DMSO-d_6) δ –1.14. HRMS (ESI$^+$): calcd. for $C_{40}H_{45}{}^2H_7N_{10}O_{12}P$ $[M+H]^+$ *m/z* = 902.3938, found *m/z* = 902.3925, calcd. for $C_{40}H_{45}{}^2H_7N_{10}O_{12}P{\cdot}Na$ $[M+Na]^+$ *m/z* = 925.3835, found *m/z* = 925.3780.

Expression and Purification of Pin1. The plasmid pET28C that expresses a recombinant form of human His8–Pin1 was a generous gift from Professor Joseph Noel at the Salk Institute. The plasmid pET28C (2 μL) was transfected in competent *E. coli* BL21-DE3 cells in $CaCl_2$ 0.1 M (20 min on ice, heat-shocked 2 min at 42°C). The cells were then plated on LB kanamycin-50 agar plates (Sigma-Aldrich, L0543) for 15 h, after which they were cultivated in LB media (500 mL) containing 40 μg/mL kanamycin (Sigma-Aldrich, K0254) for 2 h until an OD of 0.8 was reached. The cells were induced with 1% lactose at 30 °C for 4 h, and centrifuged at 5,000 rpm at 4 °C for 10 min. The supernatant was decanted, and the cell pellet was stored at –20 °C for one week. The cell pellet (3.5 g) was resuspended in 10 mL of lysis buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris at pH 7.9) containing protease inhibitor cocktail (100 μL, Thermo Scientific, # 78410), after which it was sonicated (Branson Sonifier®, S-450D) at the 60% level (1 min in, 1 min out) for 8 min, with cooling in an ice bath. The supernatant was collected after centrifuging the cells at 20,000 rpm at 4 °C for 20 min. The resulting protein was purified on a cobalt (Co^{2+}) metal-chelating 6% agarose resin slurry (1.5 mL, Thermo Scientific, # 89964) in a disposable polypropylene tube (Thermo Scientific). The eluted fractions (3 of 5 mL) were dialyzed overnight in dialysis tubing with a 10,000 MWCO (Fisher Scientific, 08667B) in 20 mM HEPES, 100 mM NaCl at pH 7.5, 1L at 4 °C, and concentrated via membrane filtration (Vivascience, VS0601). The protein was analyzed by SDS-PAGE, and a single band of ca. 22 kDa was identified. A protein concentration of 1.2 mg/mL was determined by Bradford assay.

Statistical Analysis of KIE Data. The k_{obs} values for direct comparison were measured on the same day, and all points were used in calculating the slope of each line using both Kaleidagraph 3.6.2 and JMP Pro10. The standard deviations for each line were calculated by JMP Pro10. To calculate the standard error of the k_H/k_D ratios reported, the error for Q (σ_Q) was derived by taking the square root of the sum of the partial derivatives of k_H and k_D with respect to each other as follows.(*7*)

$$Q = \frac{k_H}{k_D}$$

$$\sigma_Q = \sqrt{\sum_{i=1}^{N} \left(\frac{\partial Q}{\partial X_i} \right)_{X_j}^2 \sigma_{X_i}^2}$$

$$\sigma_{\frac{k_H}{k_D}} = \sqrt{\left(\frac{\partial \frac{k_H}{k_D}}{\partial k_H} \right)^2 \sigma_{k_H}^2 + \left(\frac{\partial \frac{k_H}{k_D}}{\partial k_D} \right)^2 \sigma_{k_D}^2}$$

$$\sigma_{\frac{k_H}{k_D}} = \sqrt{\left(\frac{1}{k_D} \right)^2 \sigma_{k_H}^2 + \left(\frac{-k_H}{k_D^2} \right)^2 \sigma_{k_D}^2}$$

For example, the error for k_H/k_D for Ac–Phe-Phe–pSer-d_3–Pro–Arg–NH_2 was calculated as follows.

$$\sigma_{\frac{k_H}{k_D}} = \sqrt{\left(\frac{1}{0.00271} \right)^2 (0.000368)^2 + \left(\frac{-0.00438}{0.00271^2} \right)^2 (0.000335)^2}$$

$$\sigma_{\frac{k_H}{k_D}} = 0.24$$

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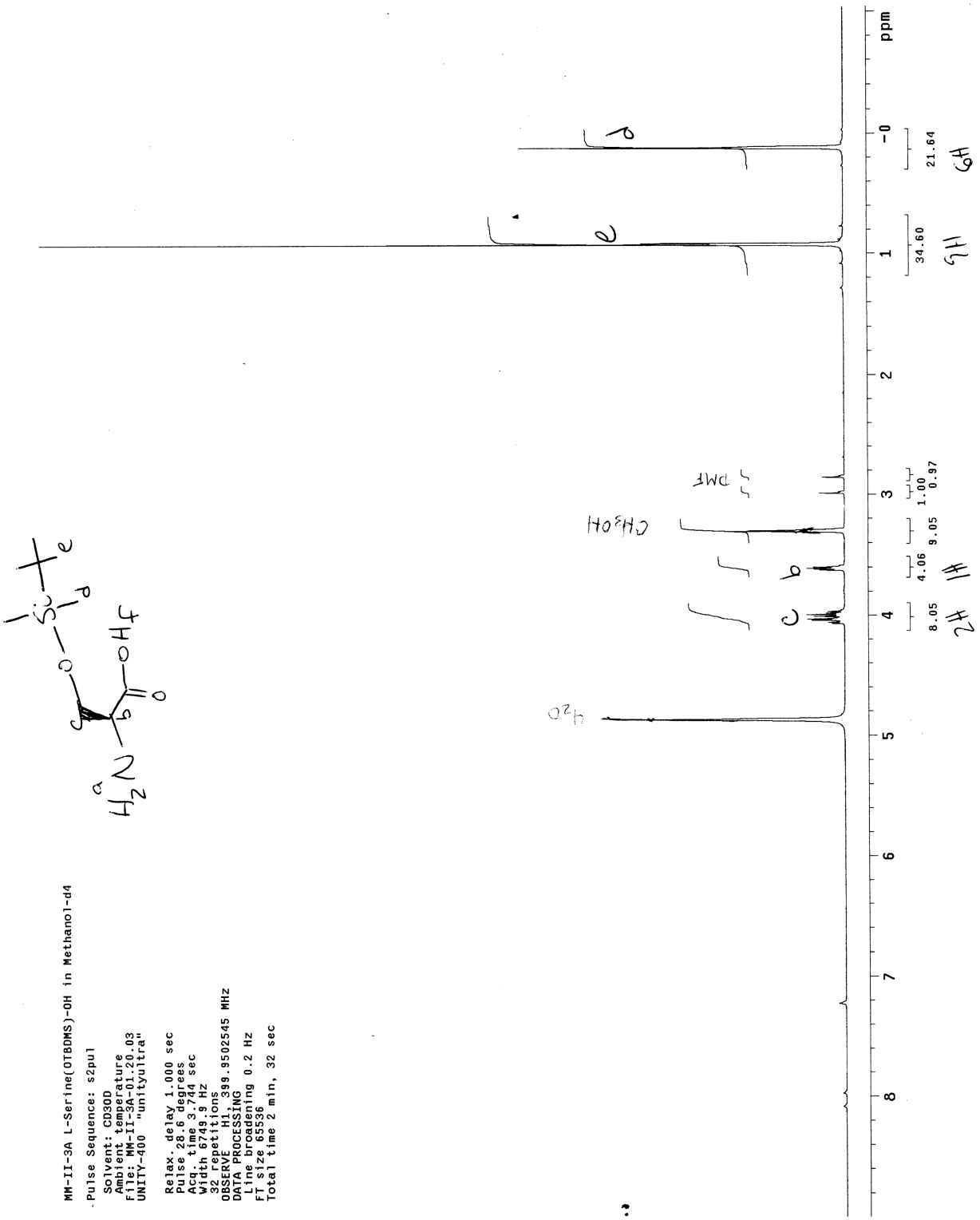

Figure S1. ^{1}H NMR in CD_3OD of H–Ser(TBS)–OH, **4**.

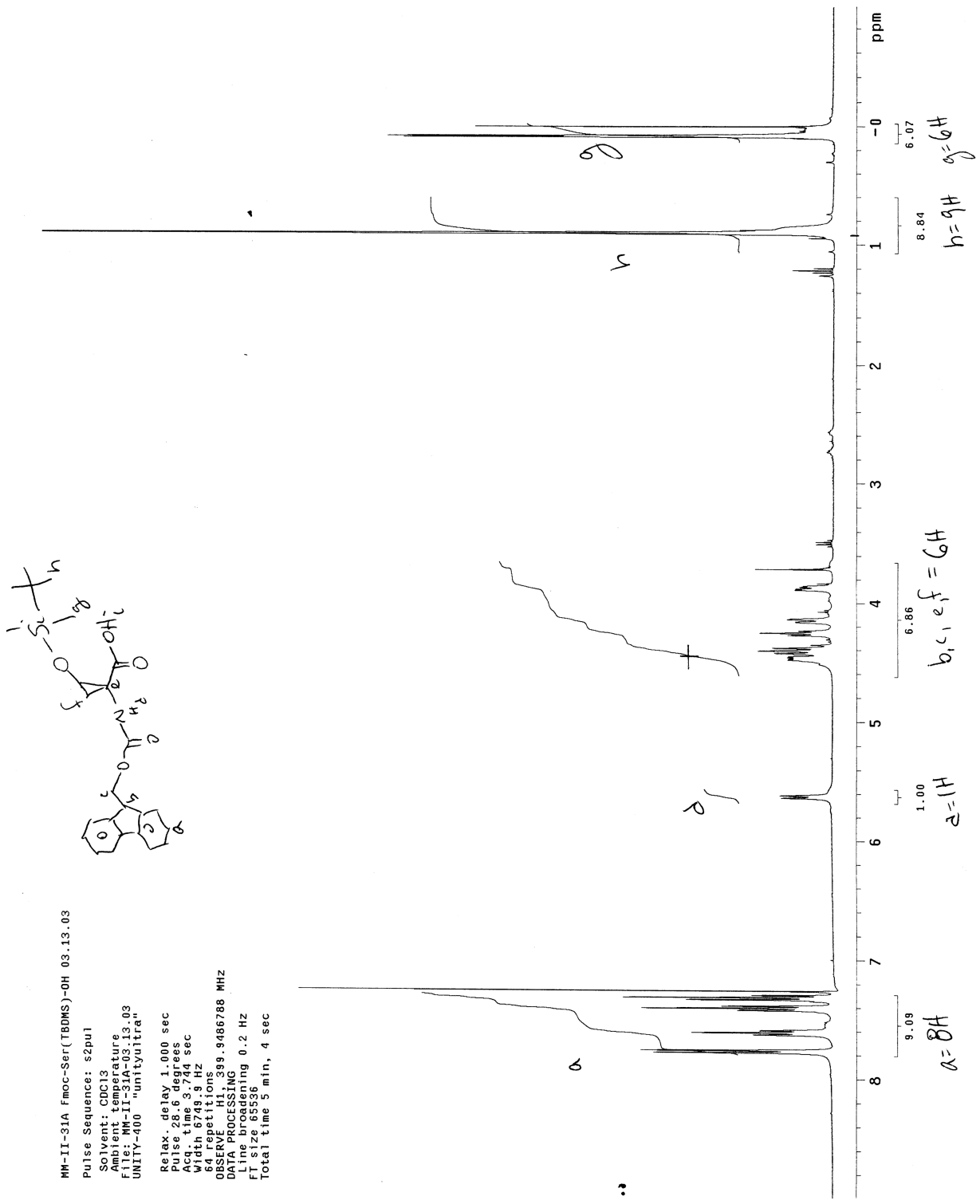

Figure S2. ^{1}H NMR in $CDCl_3$ of Fmoc–Ser(TBS)–OH, **5**.

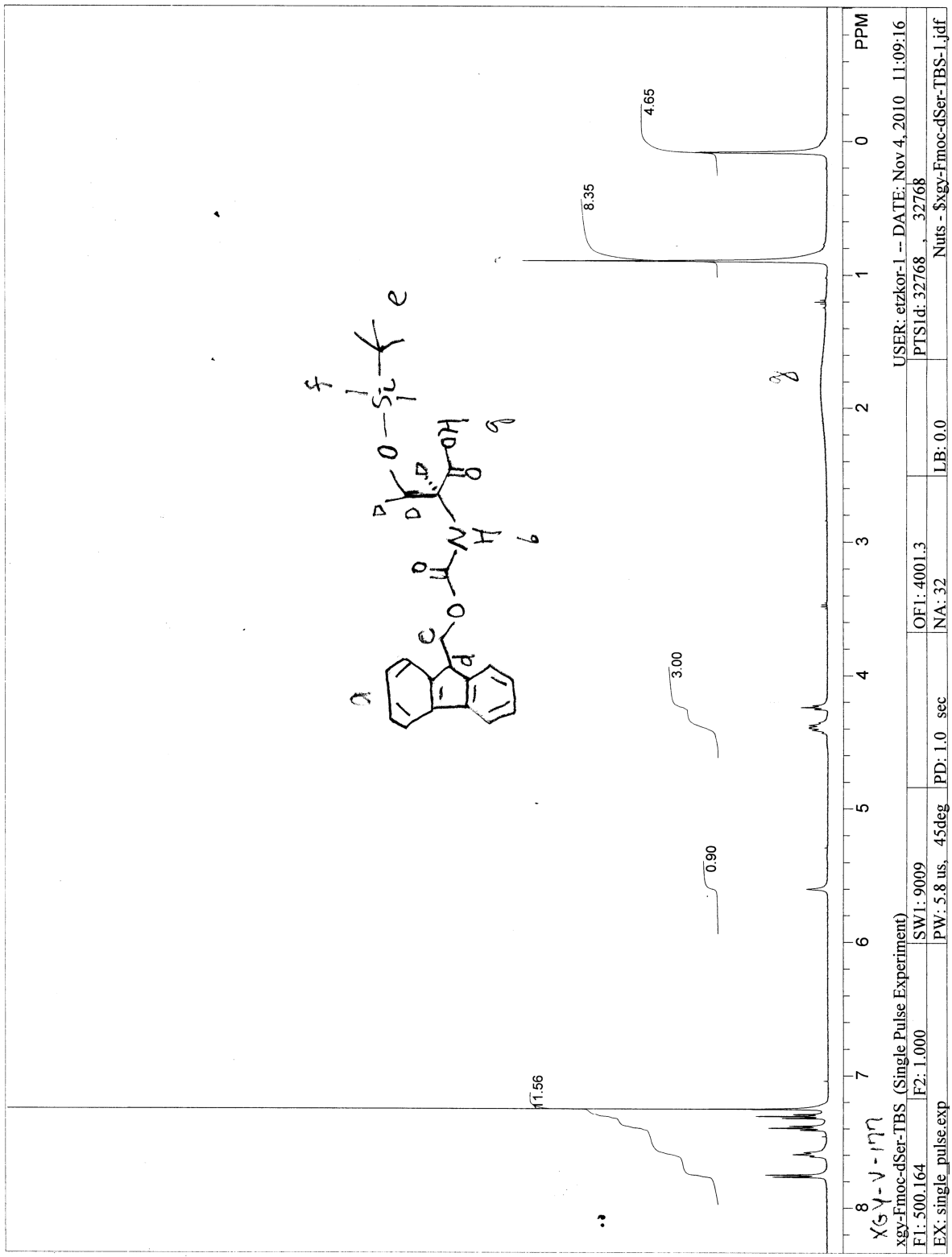

Figure S3. ^{1}H NMR in $CDCl_3$ of Fmoc–Ser(TBS)-d_3–OH, **7**.

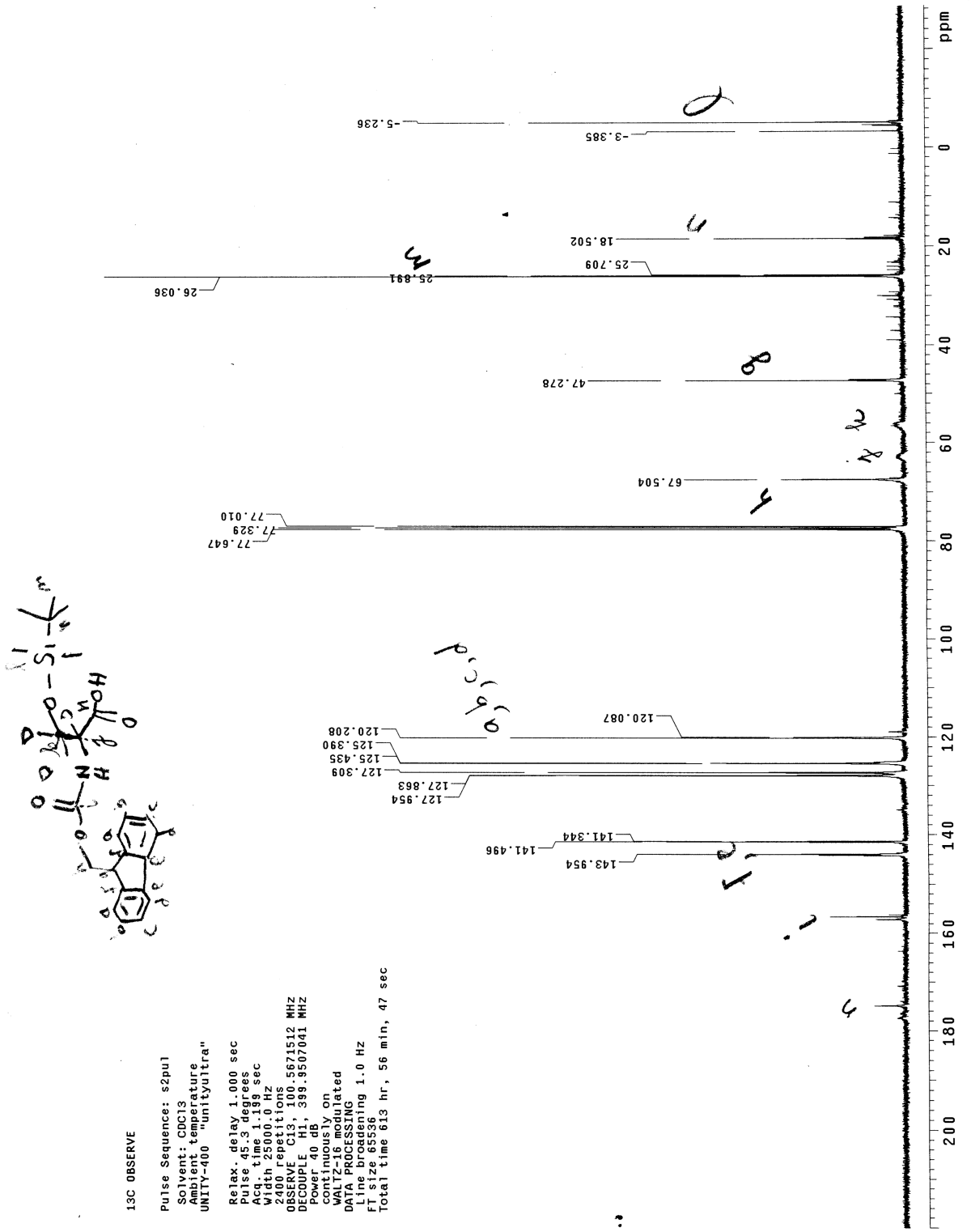

Figure S4. ^{13}C NMR of Fmoc–Ser(TBS)-d_3–OH, **7**.

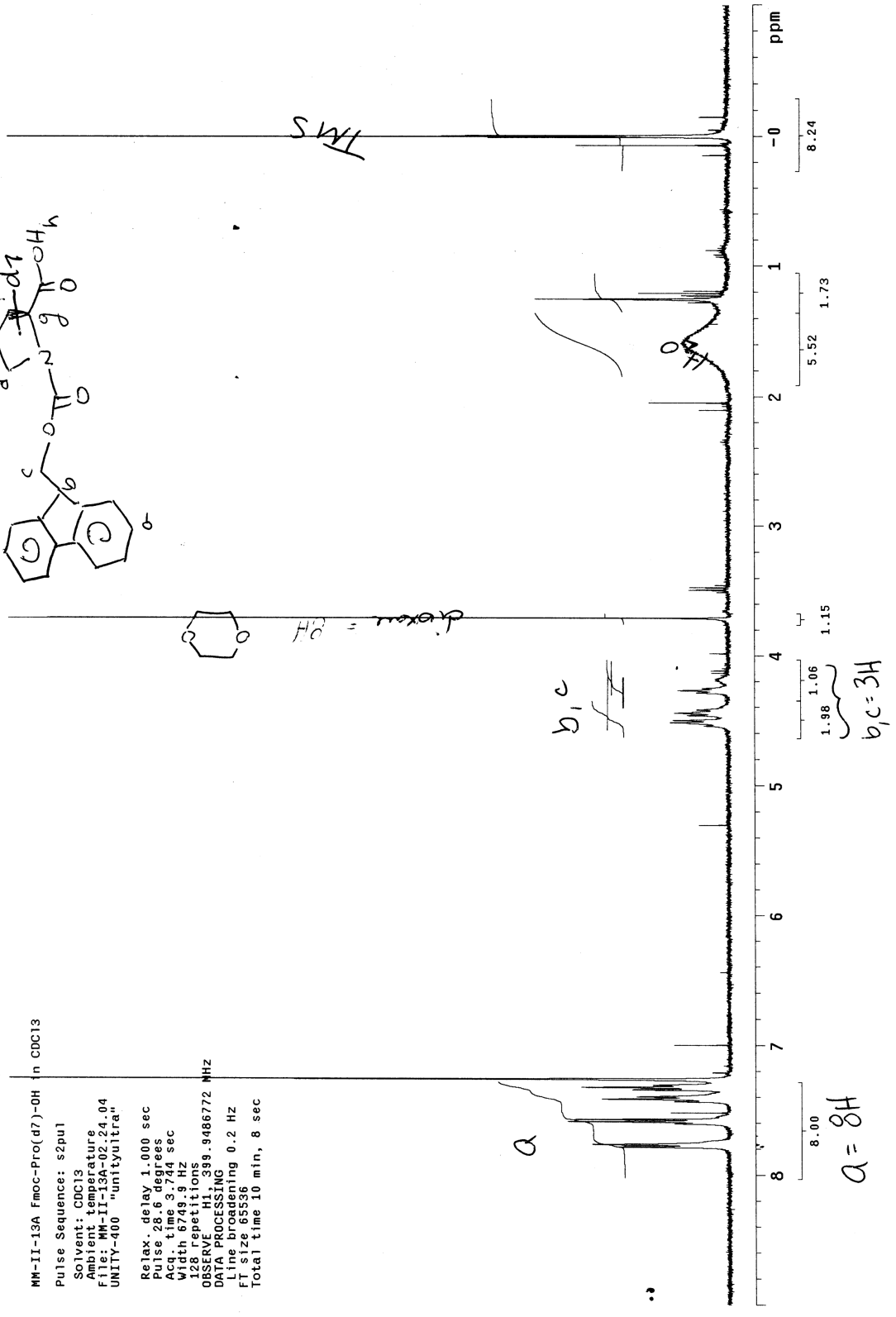

Figure S5. ^{1}H NMR of Fmoc–Pro-d_7–OH, **8**.

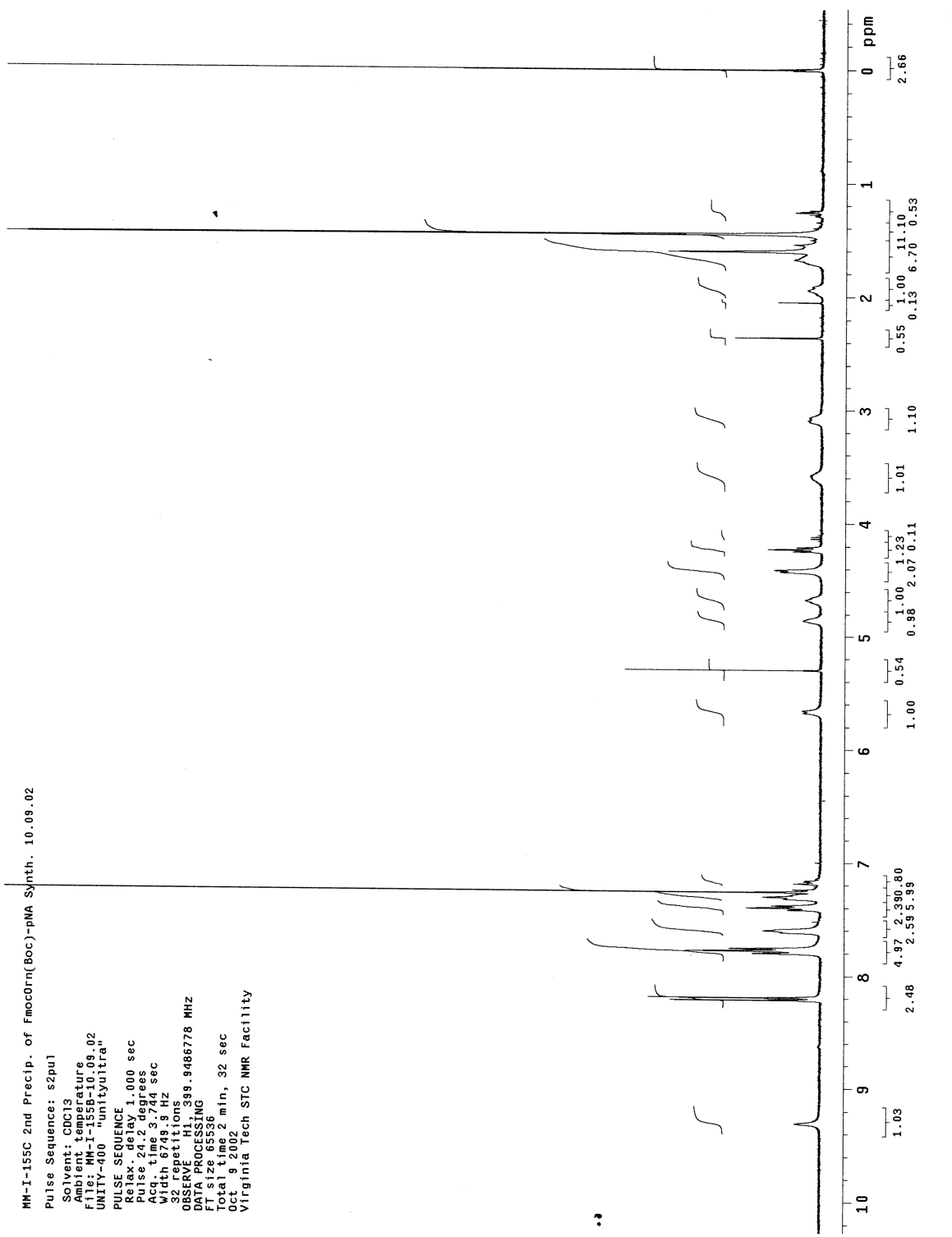

Figure S6. ^{1}H NMR of Fmoc–Orn(Boc)–*p*NA, **9**.

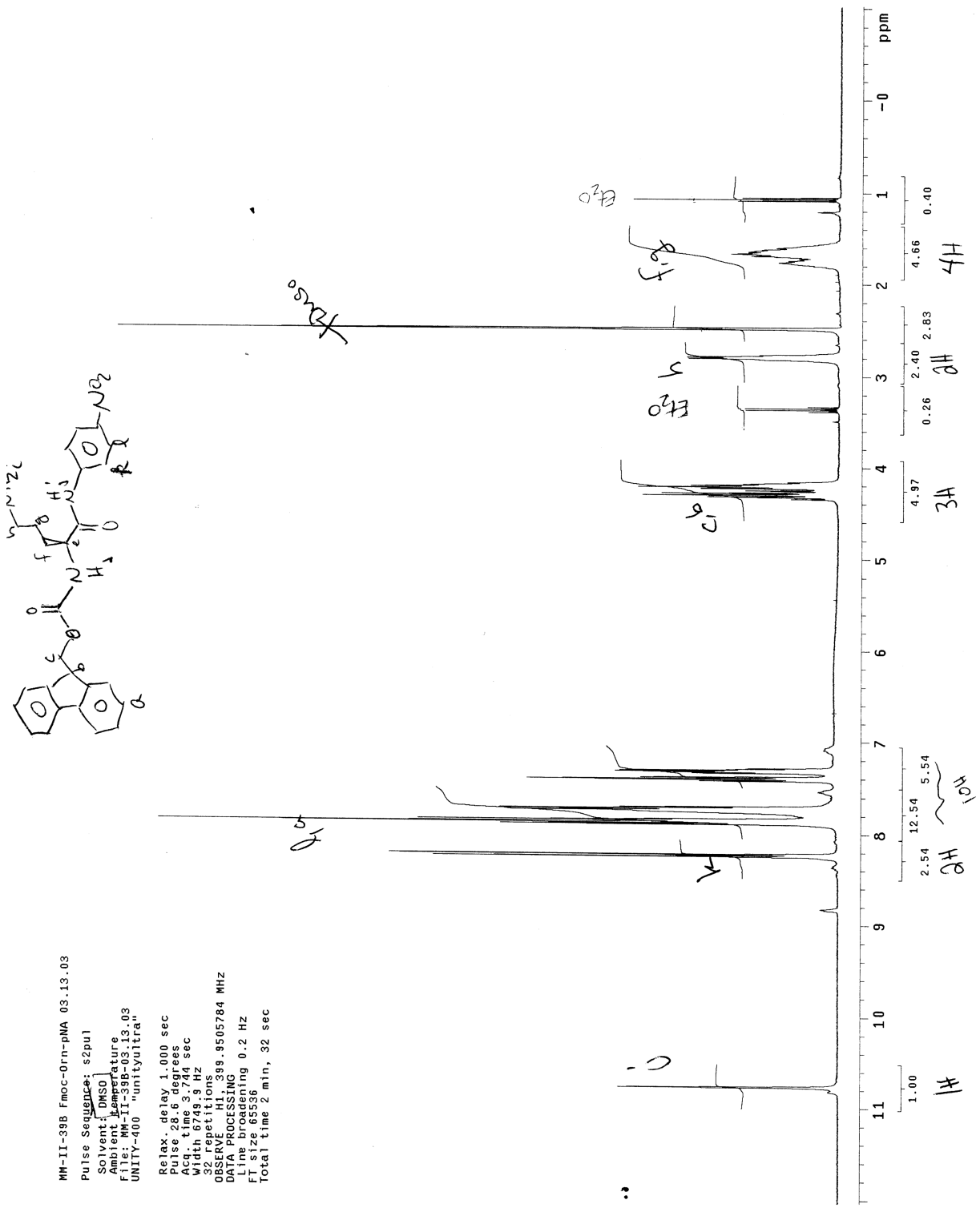

Figure S7. ^{1}H NMR of Fmoc–Orn(·TFA)–OH, **10**.

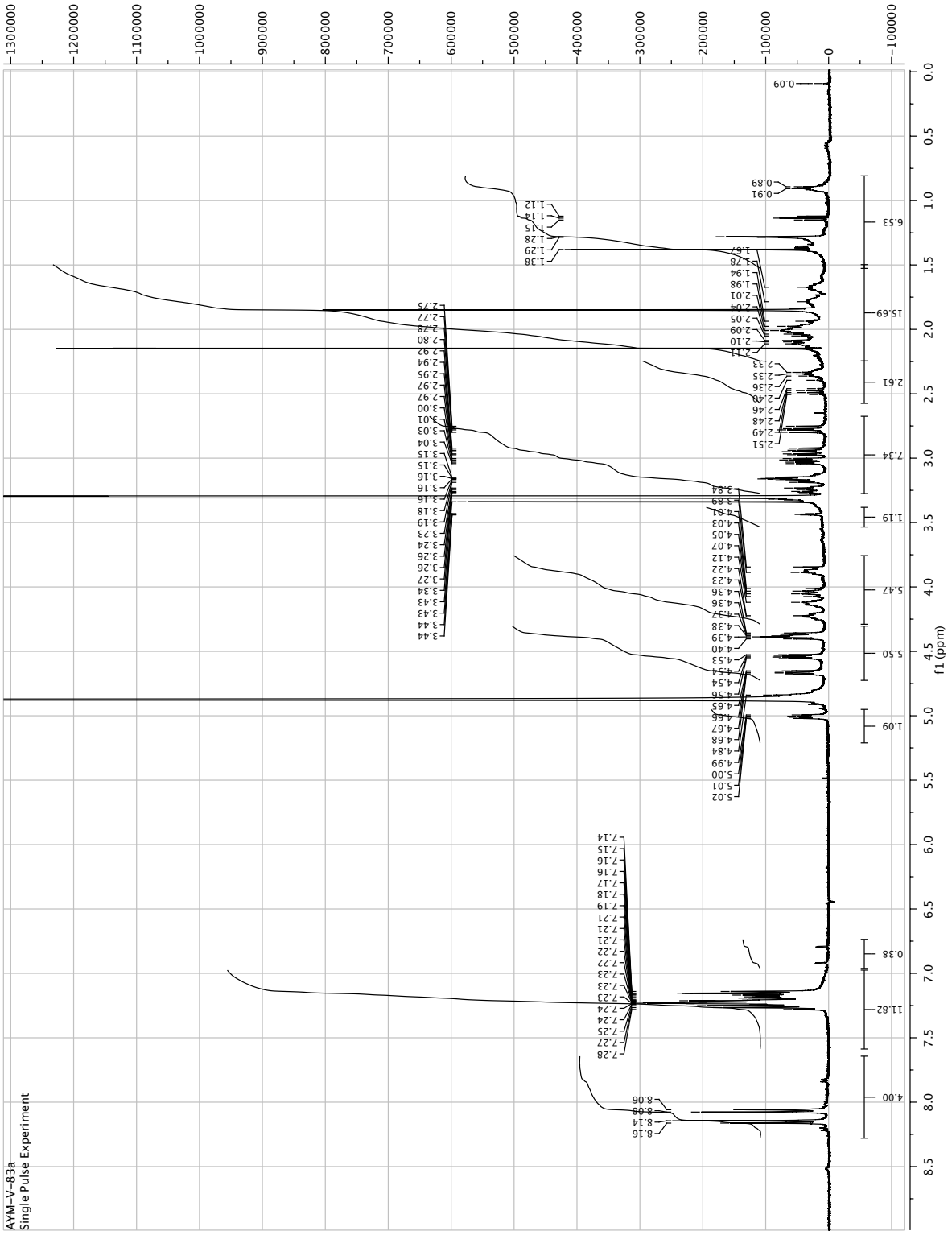

Figure S8. ^{1}H NMR in CD_3OD of unlabeled Ac–Phe–Phe–pSer–Pro–Arg–*p*NA, **1**.

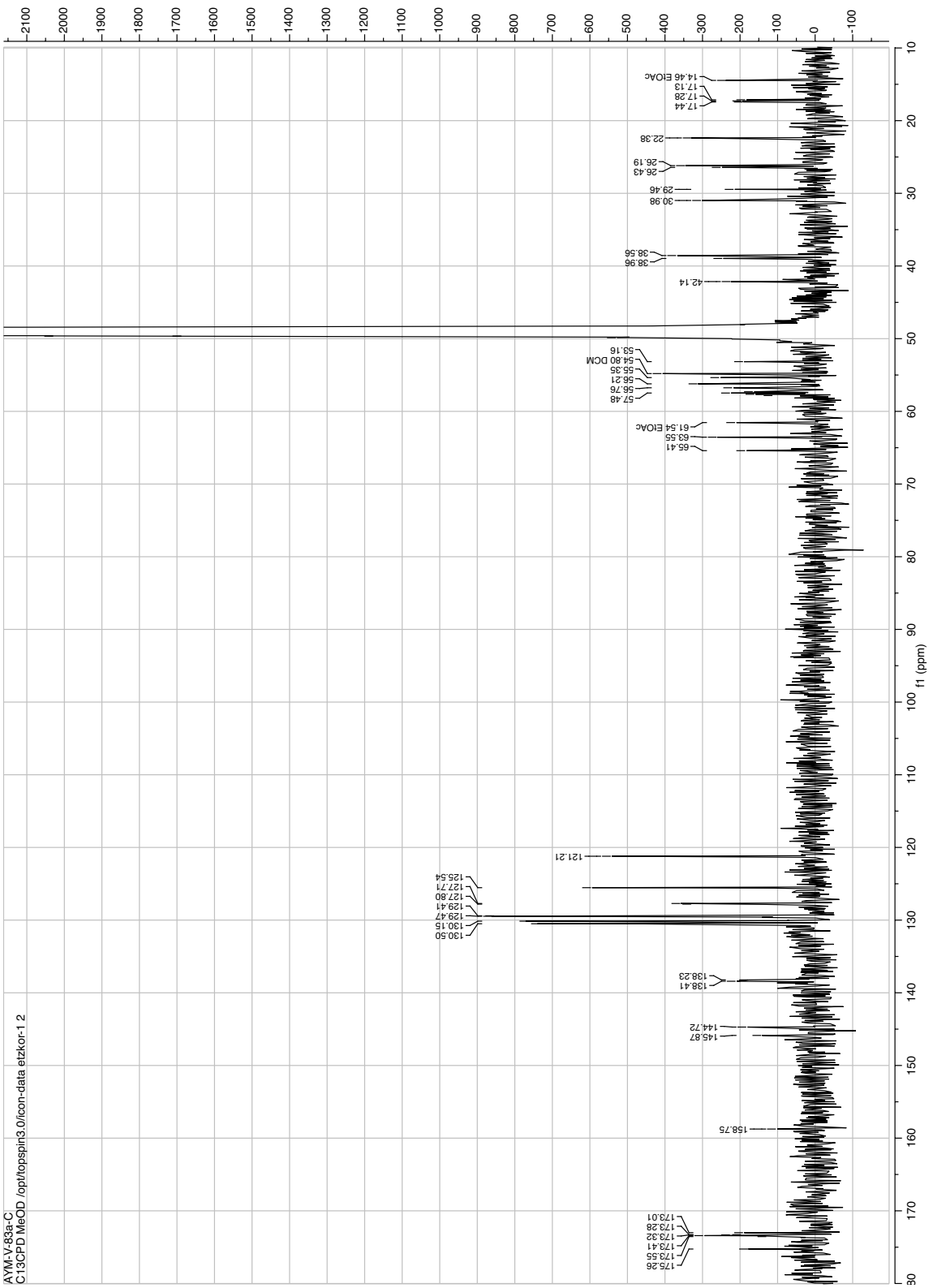

Figure S9. ^{13}C NMR in CD_3OD of unlabeled Ac–Phe–Phe–pSer–Pro–Arg–*p*NA, **1**.

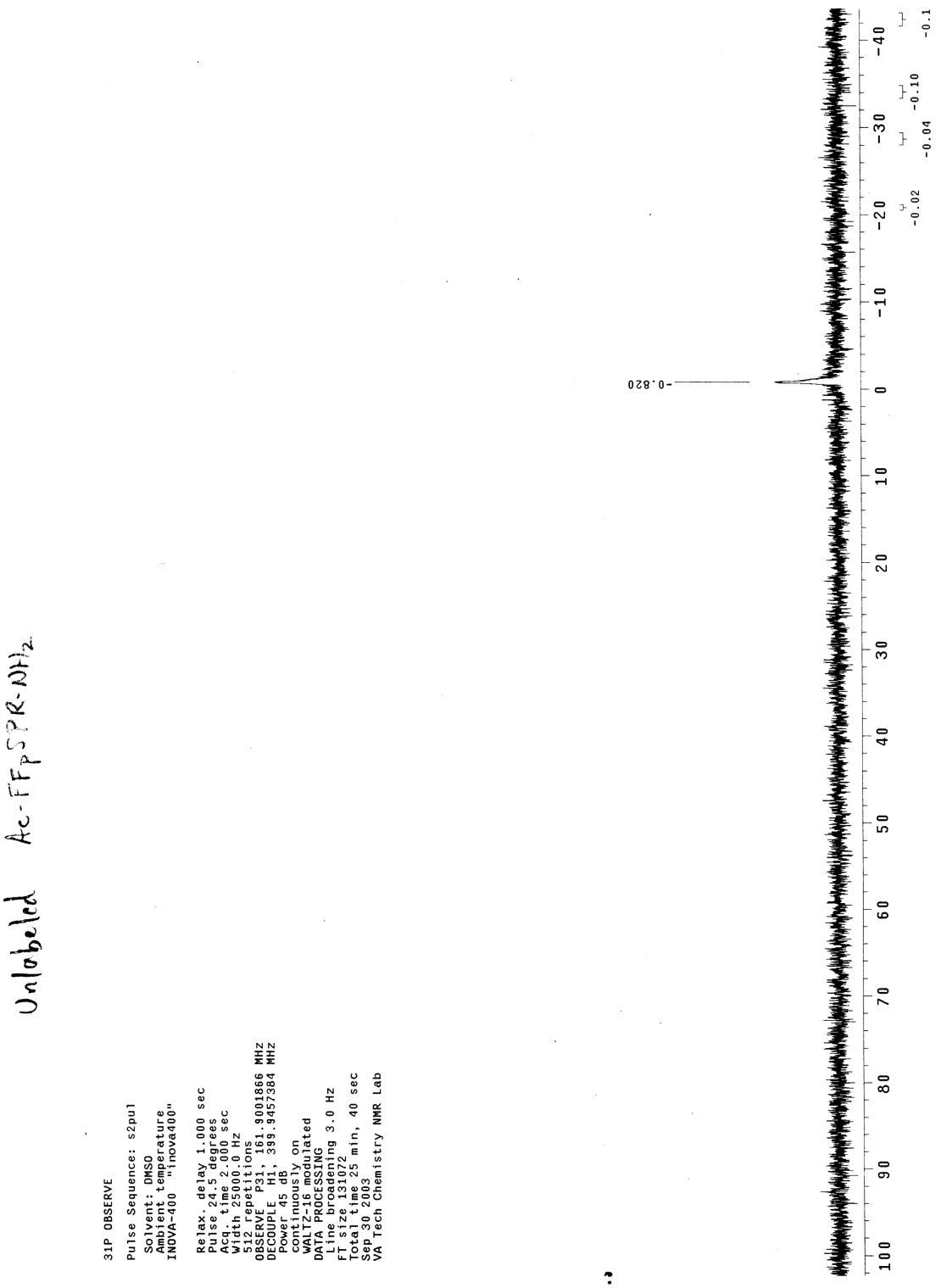

Figure S10. ^{31}P NMR in DMSO-d_6 of unlabeled Ac–Phe–Phe–pSer–Pro–Arg–*p*NA, **1**.

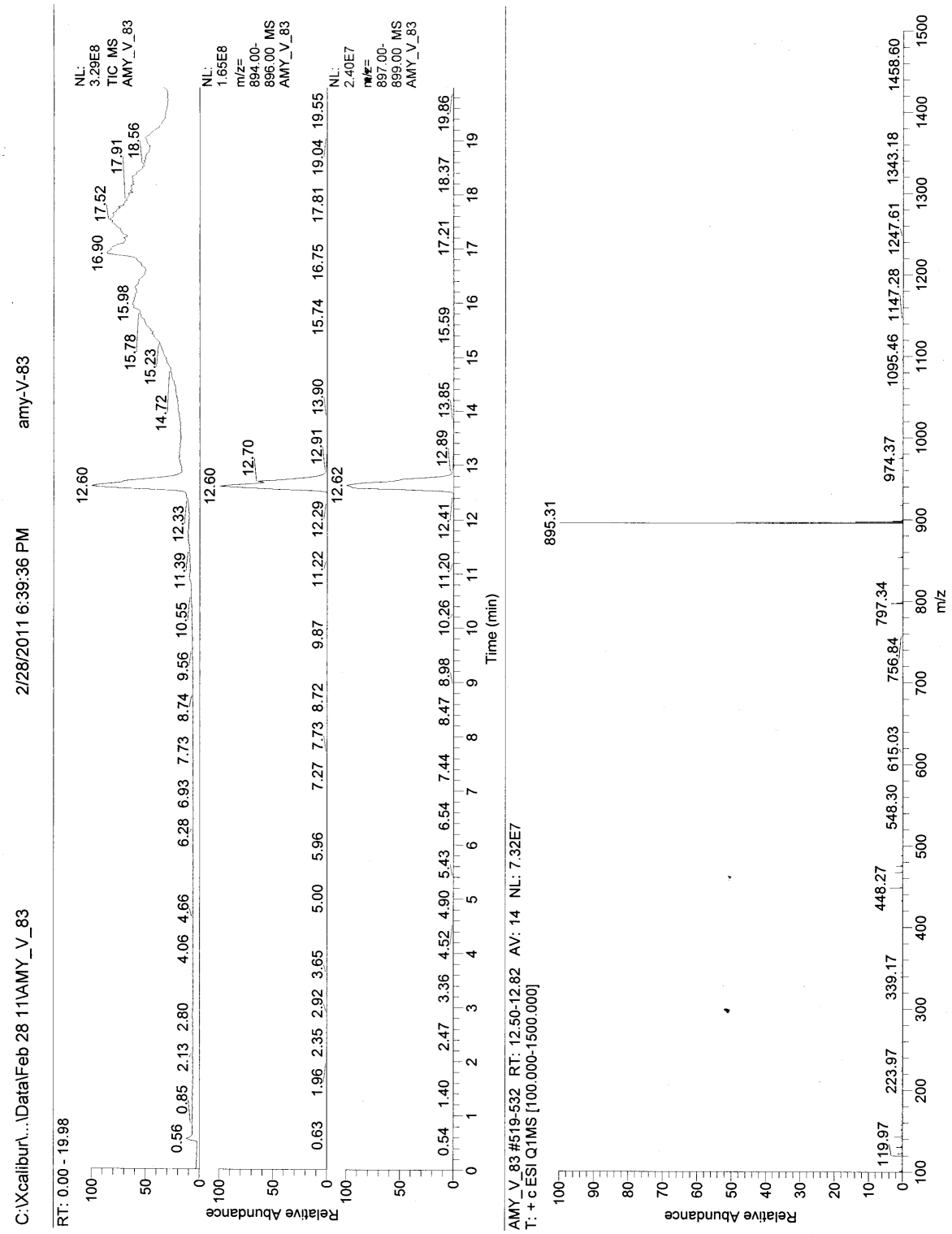

Figure S11. LC-MS total ion chromatogram Ac–Phe–Phe–pSer–Pro–Arg–*p*NA, **1**. MS with electrospray ionization (ESI^+) showing the molecular ion for **1**. The LC peak from 12.6 min was selected for molecular ion detection, identifying $[MH]^+ = 895.3$ *m/z*.

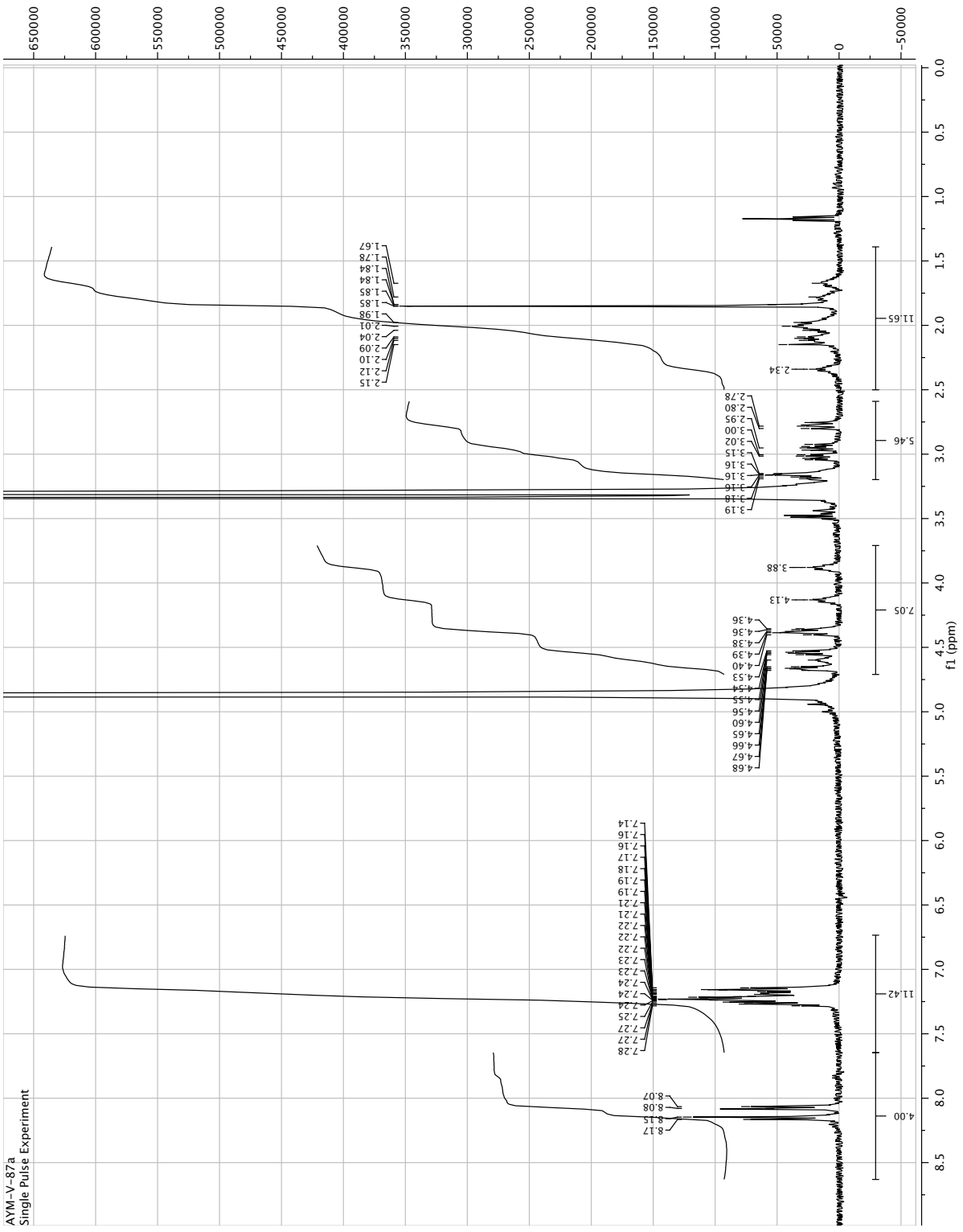

Figure S12. ^{1}H NMR in CD_3OD of Ac–Phe–Phe–pSer-d_3–Pro–Arg–*p*NA, **2**.

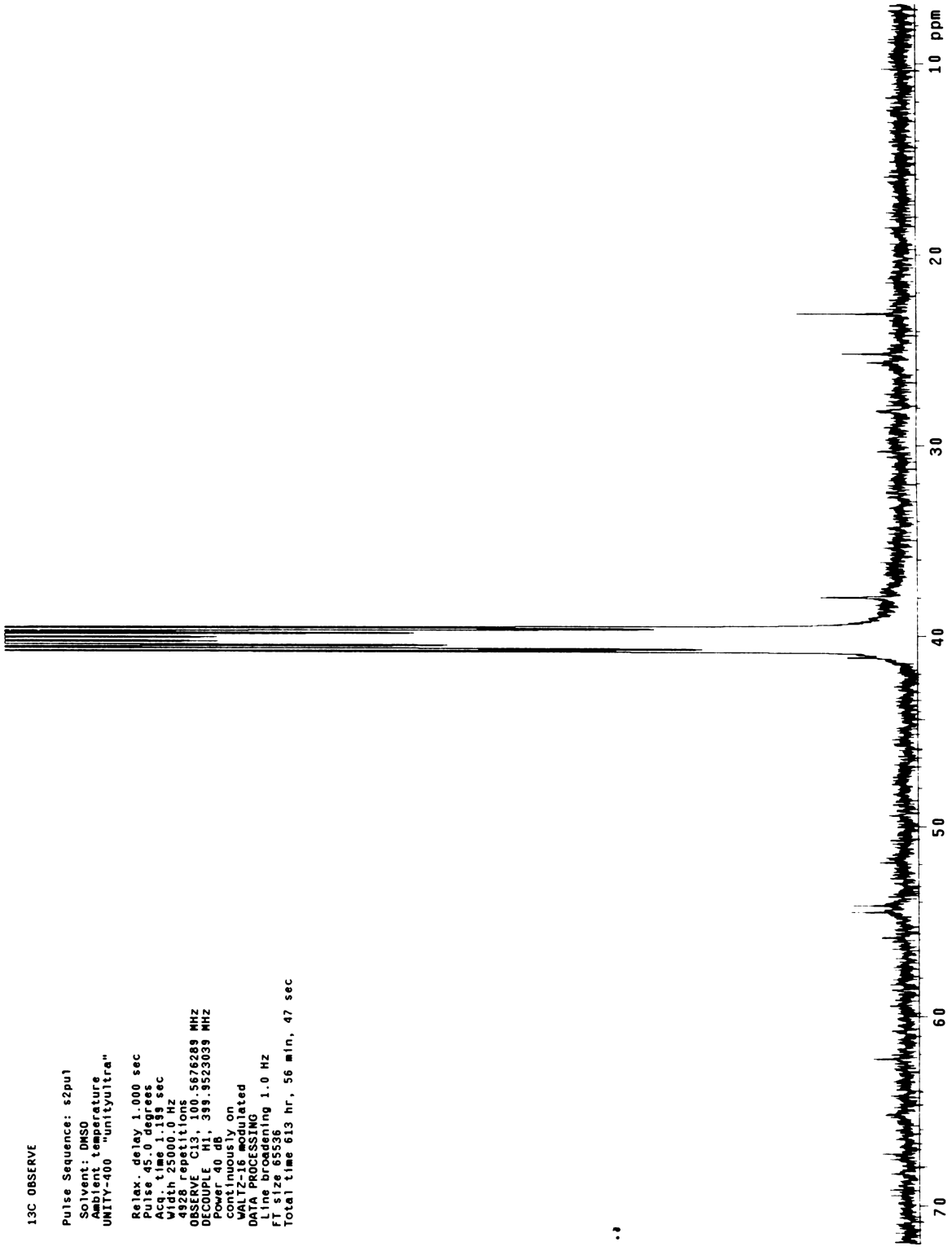

Figure S13A. ^{13}C NMR in DMSO-d_6 of Ac–Phe–Phe–pSer-d_3–Pro–Arg–*p*NA, **2**.

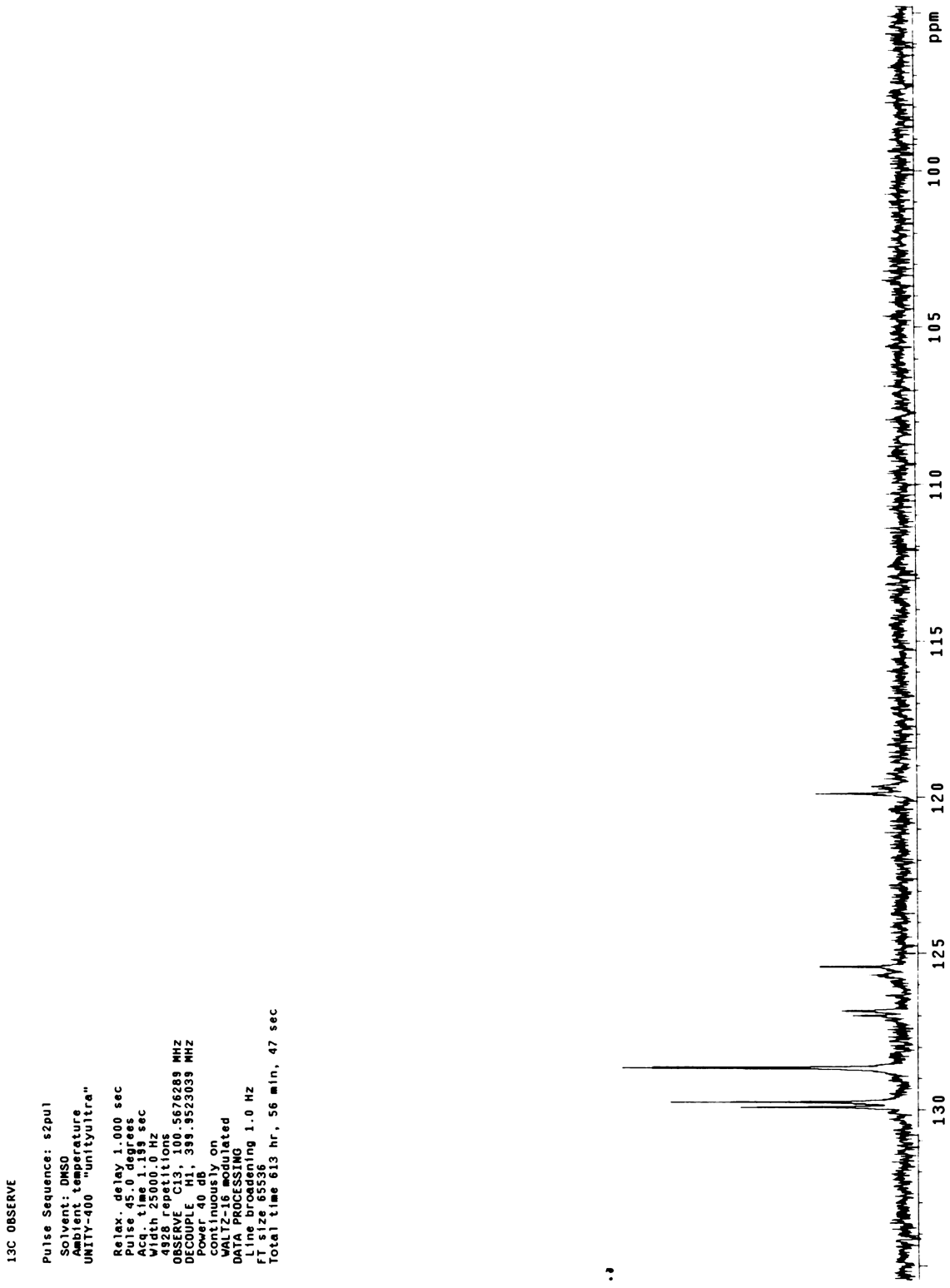

Figure S13B. ^{13}C NMR in DMSO-d_6 of Ac–Phe–Phe–pSer-d_3–Pro–Arg–*p*NA, **2**.

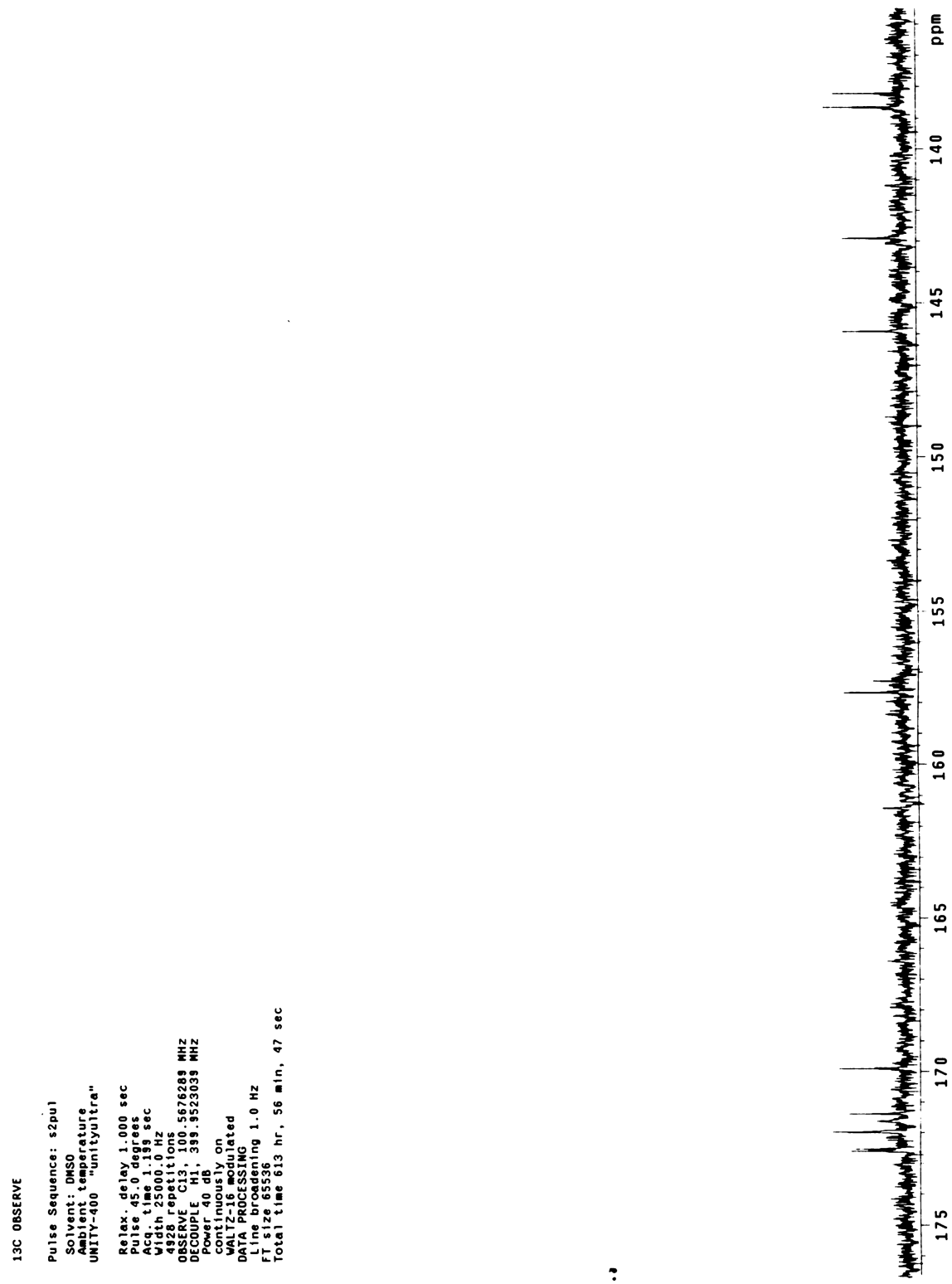

Figure S13C. ^{13}C NMR in DMSO-d_6 of Ac–Phe–Phe–pSer-d_3–Pro–Arg–*p*NA, **2**.

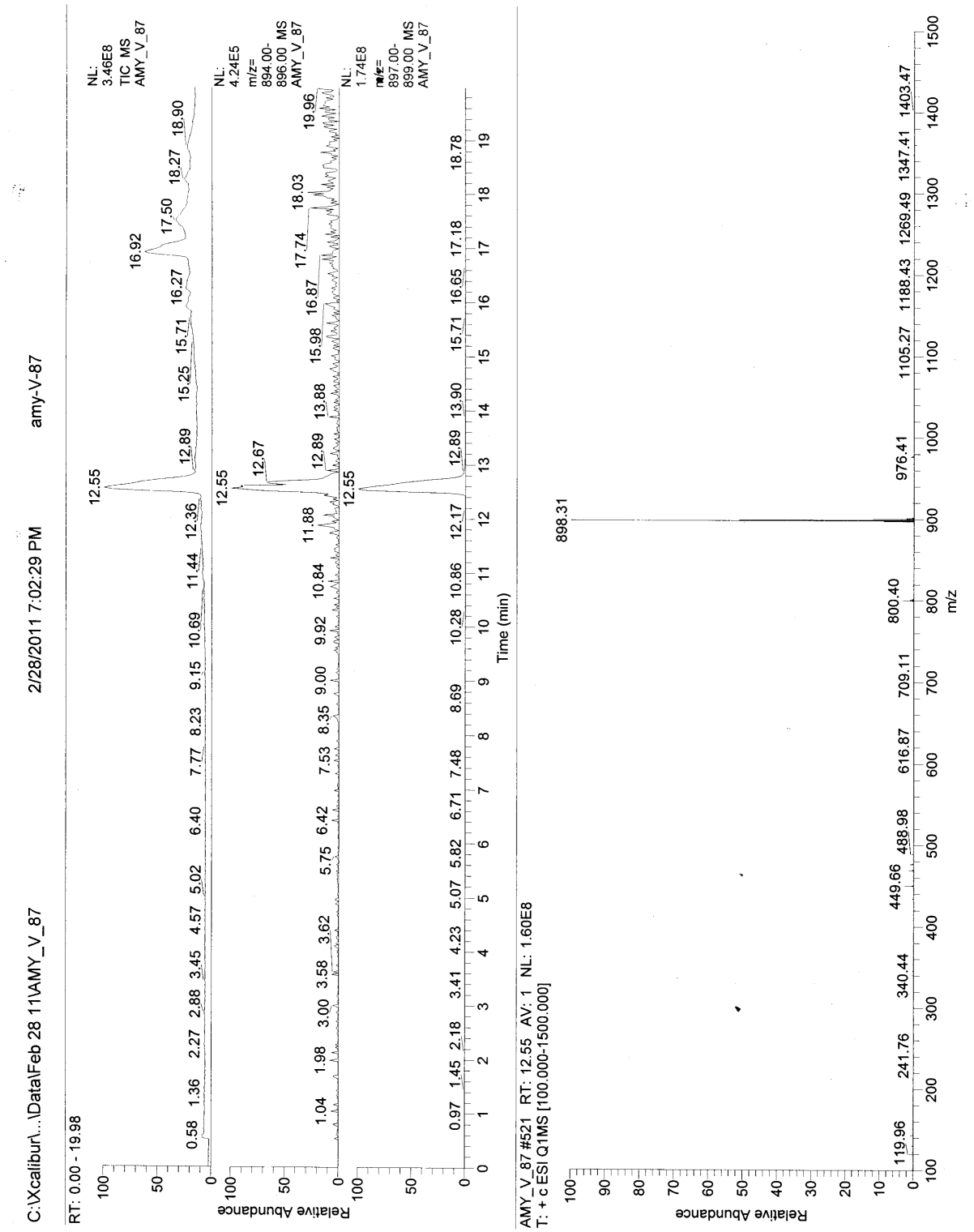

Figure S14. LC-MS total ion chromatogram Ac–Phe–Phe–pSer-d_3–Pro–Arg–*p*NA **2**. MS with electrospray ionization (ESI^+) showing the molecular ion for **2**. The LC peak from 12.55 min was selected for molecular ion detection, identifying $[MH]^+$ = 898.3 *m/z*.

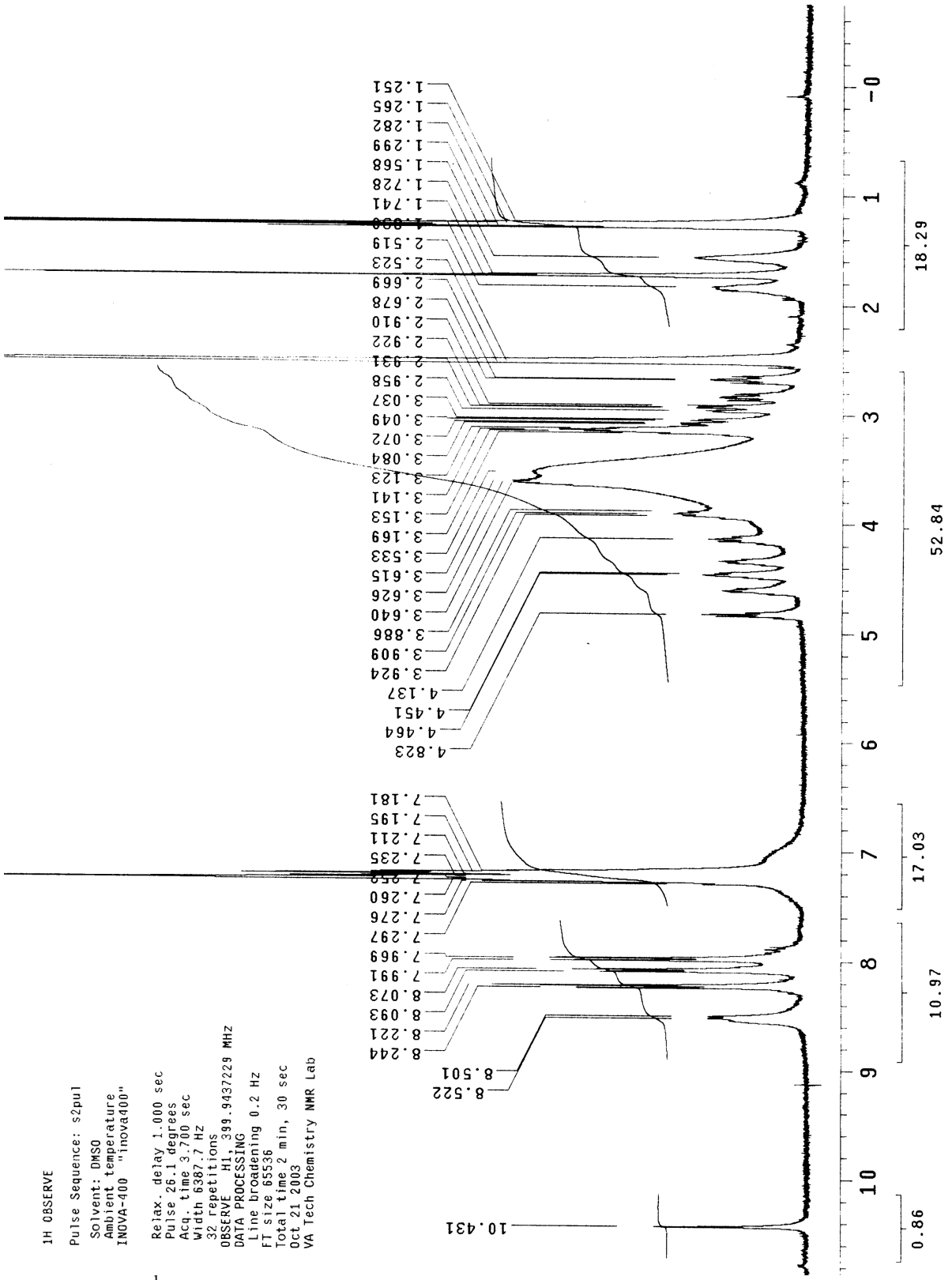

Figure S15A. ^{1}H NMR of Ac–Phe–Phe–pSer–Pro-d_7–Arg–*p*NA, **3**.

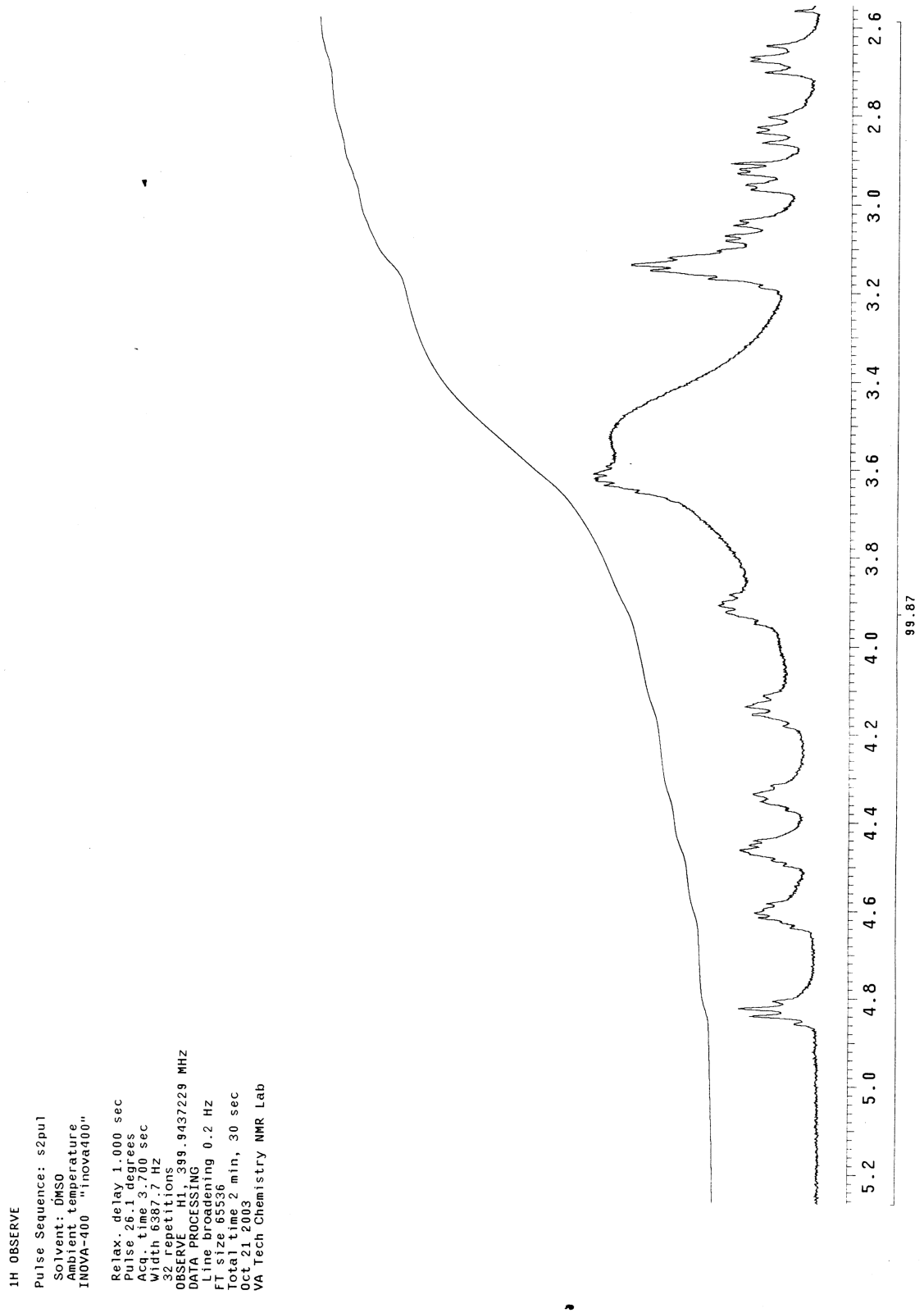

Figure S15B. Expanded αH-region of ^{1}H NMR of Ac–Phe–Phe–pSer–Pro-d_7–Arg–*p*NA, **3**.

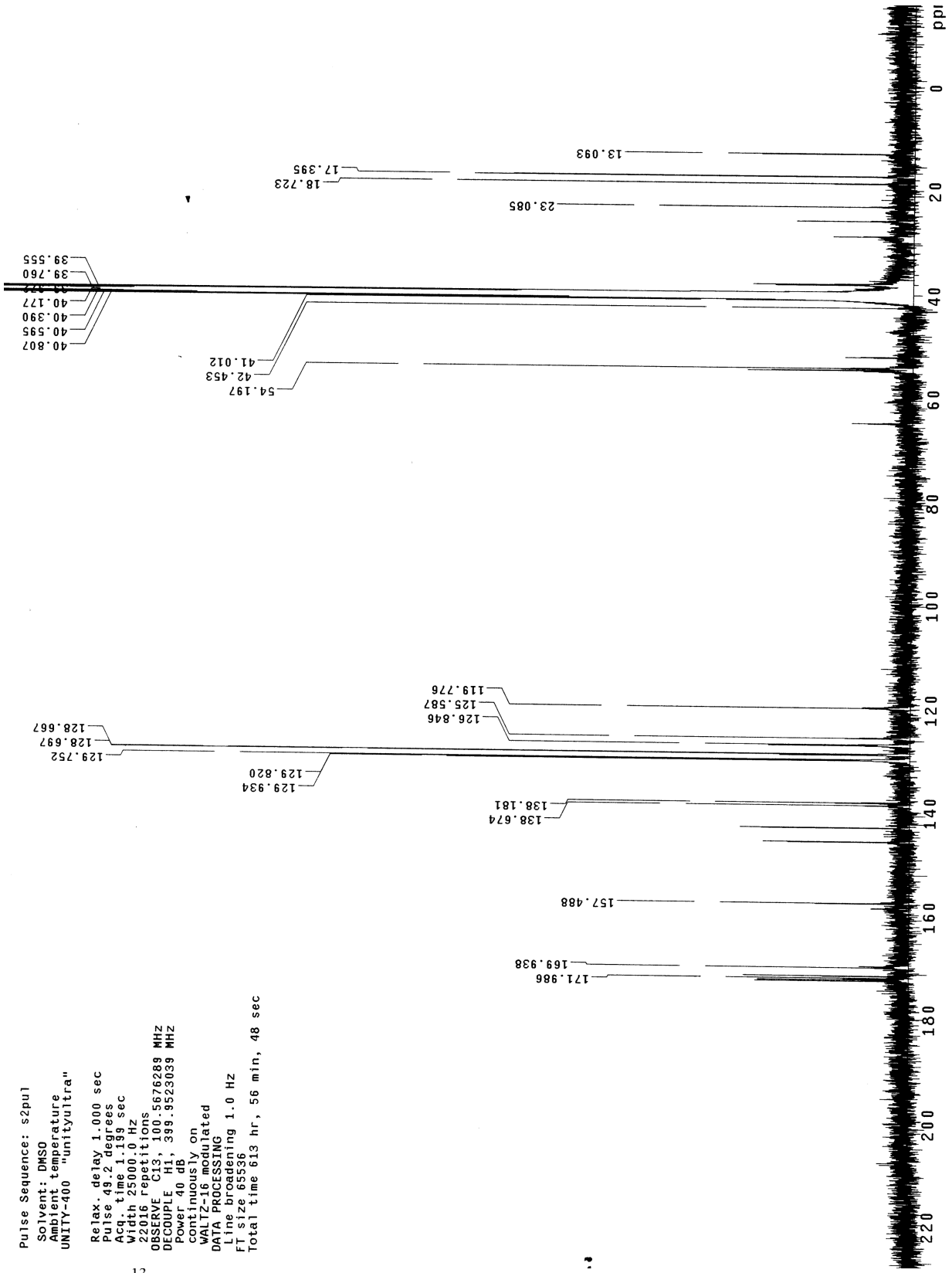

Figure S16. ^{13}C NMR of Ac–Phe–Phe–pSer–Pro-d_7–Arg–*p*NA, **3**.

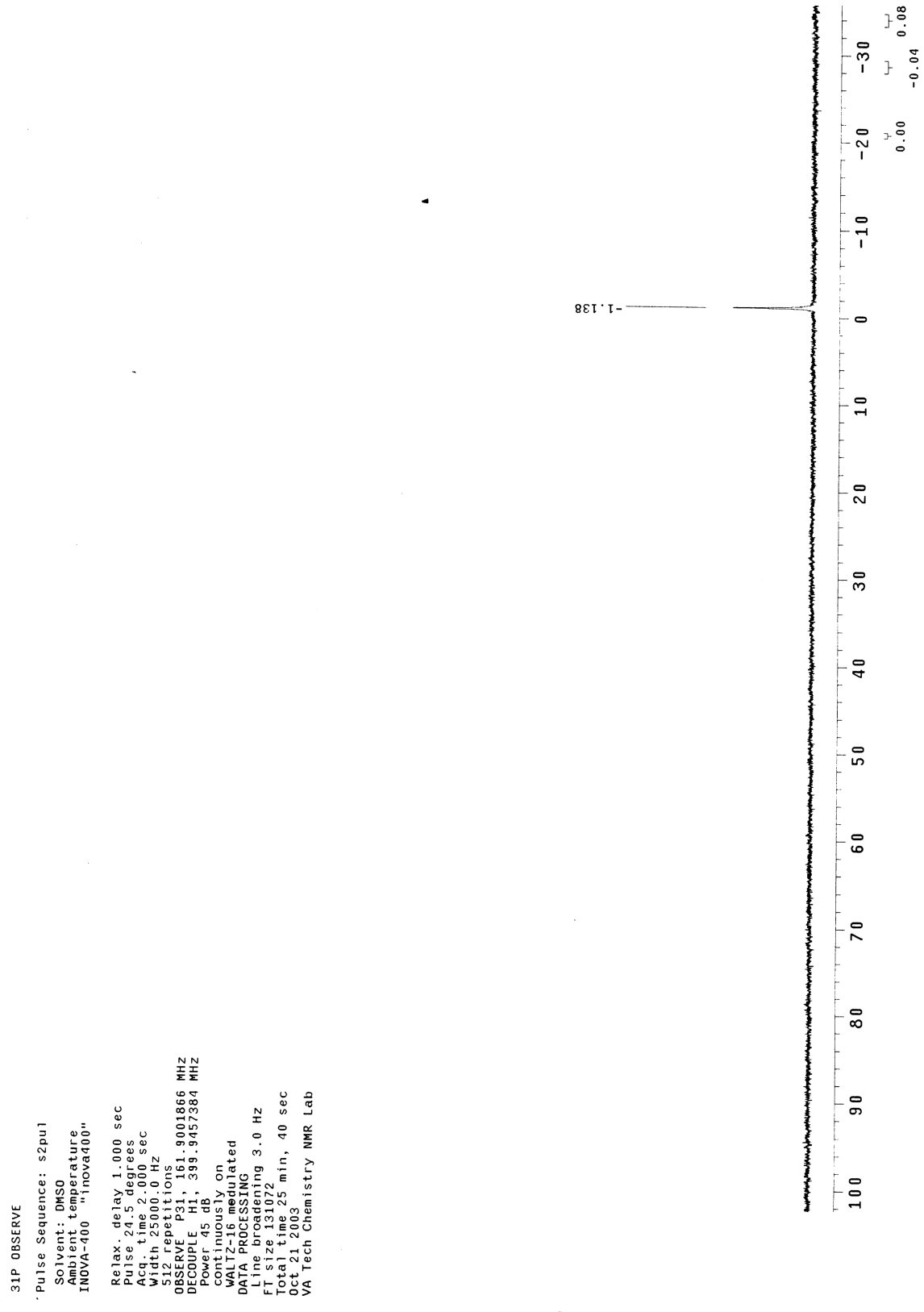

Figure S17. ^{31}P NMR of Ac–Phe–Phe–pSer–Pro-d_7–Arg–*p*NA, **3.**

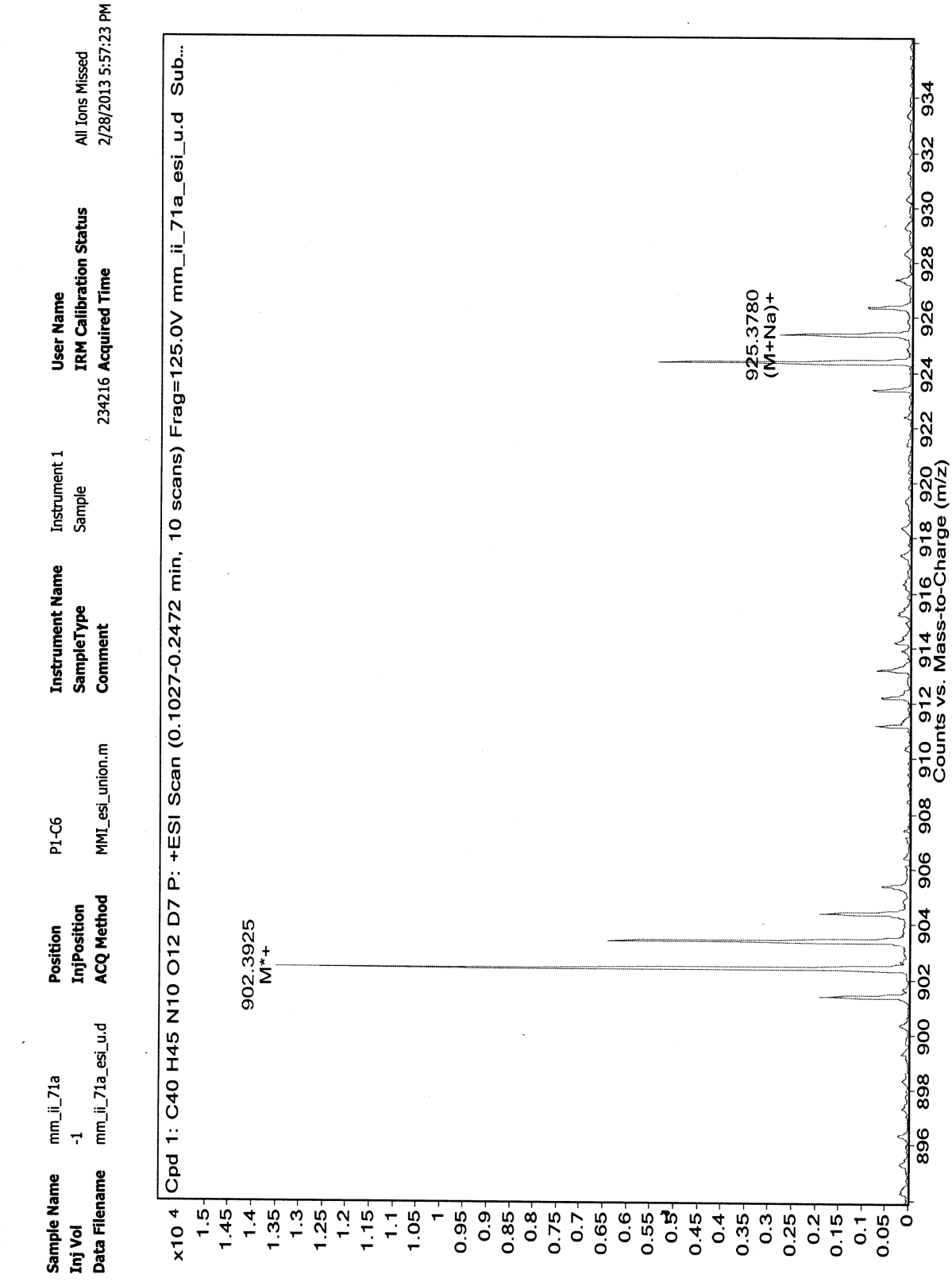

Figure S18. ESI$^+$ HRMS of Ac–Phe–Phe–pSer–Pro-d_7–Arg–*p*NA, **3.**