N-Oxide derivatives of 3-(3-pyridyl)-2-phosphonopropanoic acids as potential inhibitors of Rab geranylgeranylation

Xiang Zhou[a], Ella J. Born[b], Cheryl Allen[c], Sarah A. Holstein[c],

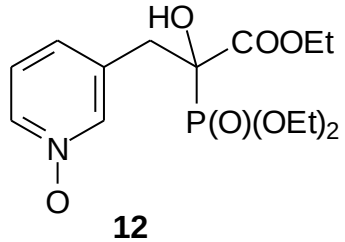
and David F. Wiemer[a]*

[a] *Department of Chemistry, University of Iowa, Iowa City, IA 52242-1294, USA*
[b] *Department of Internal Medicine, University of Iowa, Iowa City, IA 52242-1294, USA*
[c] *Department of Medicine, Roswell Park Cancer Institute, Buffalo, NY 14263, USA*

david-wiemer@uiowa.edu

General experimental procedures.

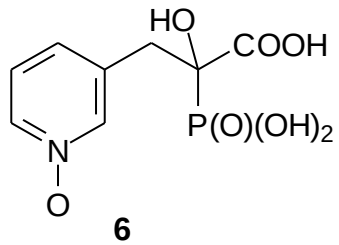
Tetrahydrofuran (THF) was freshly distilled from sodium/benzophenone. All other reagents and solvents were purchased from commercial sources and used without further purification. All reactions in nonaqueous solvents were conducted in flame-dried glassware under a positive pressure of argon and with magnetic stirring. The NMR spectra were obtained at 300 MHz for ^{1}H, and 75 MHz for ^{13}C, with internal standards of $(CH_3)_4Si$ (1H, 0.00) or $CDCl_3$ (^{1}H, 7.27; ^{13}C, 77.2 ppm) for non-aqueous samples, or D_2O (^{1}H, 4.80) and 1,4-dioxane (^{13}C, 66.7 ppm) for aqueous samples. The ^{31}P chemical shifts were reported in ppm relative to 85% H_3PO_4 (external standard). High resolution mass spectra were obtained at the University of Iowa Mass Spectrometry Facility.



12

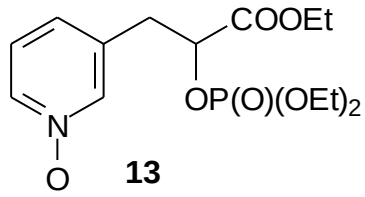
3-(2-(Diethoxyphosphoryl)-3-ethoxy-2-hydroxy-3-oxopropyl)pyridine 1-oxide (12). Solid *m*CPBA (210 mg, 0.930 mmol) was added to a solution of the 3-PEHPC ester **11** (206 mg, 0.620 mmol) in CH_2Cl_2 (3 mL) and the solution was allowed to react at room temperature overnight. The reaction solvent was then removed under vacuum and the resulting residue was purified by column chromatography (15% EtOH in CH_2Cl_2) to afford the desired product as a light yellow oil (215 mg, 74%): ^{1}H NMR ($CDCl_3$) δ 8.22 (s, 1H), 8.14 (d, J = 6.4 Hz, 1H), 7.29–7.16 (m, 2H), 4.93 (s, 1H), 4.33–4.18 (m, 6H), 3.38–3.16 (m, 2H), 1.41–1.24 (m, 9H); ^{13}C NMR ($CDCl_3$) δ 170.2 (d, J_{CP} = 3.7 Hz), 140.6, 137.8, 134.3, 128.7, 125.1, 77.3 (d, J_{CP} = 160.1 Hz), 64.2 (d, J_{CP} = 7.4 Hz), 64.1 (d, J_{CP} = 7.3 Hz), 63.1, 36.7 (d, J_{CP} = 2.3 Hz), 16.4 (d, J_{CP} = 6.3 Hz),

16.4 (d, J_{CP} = 5.9 Hz), 14.1; ^{31}P NMR ($CDCl_3$) δ 16.3; HRMS (ES^+, *m*/*z*) calcd for $(M+H)^+$ $C_{14}H_{23}NO_7P_2$: 348.1212; found: 348.1214.



6

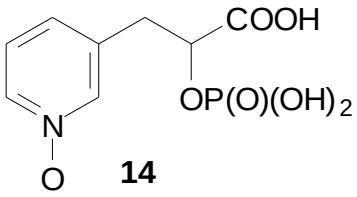
3-(2-Carboxy-2-hydroxy-2-phosphonoethyl)pyridine 1-oxide *(*6). The *N*-oxide **12** (160 mg, 0.460 mmol) was dissolved in concentrated HCl (1.60 mL) and the solution was heated at reflux overnight. After concentration under a stream of air, the resulting residue was dissolved in a minimum amount of hot water followed by the addition of acetone. This solution turned cloudy and a white solid was formed. After standing at -4 °C overnight, the resulting solid was isolated by filtration and dissolved in hot water. A white solid salt was formed upon removal of water with a lyophilizer (121 mg, 14%): ^{1}H NMR (D_2O): δ 8.39 (s, 1H), 8.36 (d, *J* = 6.6 Hz, 1H), 7.85 (d, *J* = 7.8 Hz, 1H), 7.65 (dd, *J* = 7.9 Hz, 6.7 Hz, 1H), 3.57 (dd, *J* = 14.0 Hz, J_{HP} = 4.3 Hz, 1H), 3.26 (dd, *J* = 13.7 Hz, J_{HP} = 7.4 Hz, 1H); ^{13}C NMR (D_2O) δ 174.2, 139.7, 137.5, 136.5, 136.3, 126.7, 77.5 (d, J_{CP} = 149.5 Hz), 37.0; ^{31}P NMR (D_2O) δ 13.3; HRMS (ES^-, *m*/*z*) calcd for $(M-H)^-$ $C_8H_9NO_7P$: 262.0117; found: 262.0112.



13

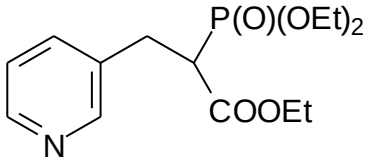
3-(2-((Diethoxyphosphoryl)oxy)-3-ethoxy-3-oxopropyl)pyridine 1-oxide (13). A solution of the *N*-oxide **12** (13.8 mg, 0.0396 mmol) in EtOAc was washed with saturated Na_2CO_3

and the aqueous layer was extracted with EtOAc three times. The combined organic layer was dried and concentrated to obtain the desired product as a colorless oil (13.8 mg, 100%); ^{1}H NMR ($CDCl_3$) δ 8.16–8.10 (m, 2H), 7.24–7.20 (m, 2H), 5.07–4.98 (m, 1H), 4.24 (q, J = 7.0 Hz, 2H), 4.21–3.96 (m, 4H), 3.25–3.04 (m, 2H), 1.36–1.25 (m, 9H); ^{13}C NMR ($CDCl_3$) δ 168.8 (d, J_{CP} = 3.7 Hz), 140.3, 138.2, 135.2, 127.3, 125.8, 74.6 (d, J_{CP} = 5.6 Hz), 64.7 (d, J_{CP} = 6.1 Hz), 64.5 (d, J_{CP} = 5.9 Hz), 62.3, 36.2 (d, J_{CP} = 6.0 Hz), 16.3 (d, J_{CP} = 1.7 Hz), 16.2 (d, J_{CP} = 2.4 Hz), 14.3; ^{31}P NMR ($CDCl_3$) δ -1.9.



14

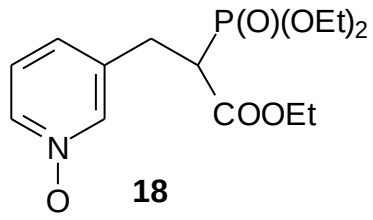
3-(2-Carboxy-2-(phosphonooxy)ethyl)pyridine 1-oxide (14). This compound was prepared according to the general procedure given for compound **6**: ^{31}P NMR (D_2O) δ 0.35.



17

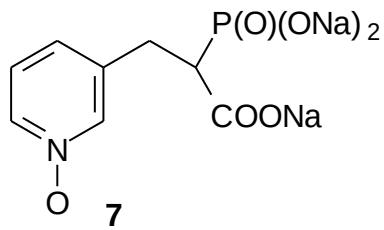
3-(2-(Diethoxyphosphoryl)-3-ethoxy-3-oxopropyl)pyridine (17). Solid 3-picolylchloride hydrochloride (1.0 g, 5.9 mmol) was dissolved in water and $NaHCO_3$ was added. The resulting solution was extracted with ether, and the combined organic extracts were dried and concentrated in vacuo to obtain the free base. Triethyl phosphonoacetate (1 mL, 4.9 mmol) was added to an ice cold suspension of NaH (235 mg, 5.9 mmol) in THF (5 mL), and after 30 min the picolyl chloride was added. The resulting solution was stirred at rt overnight, quenched by addition of water, and the aqueous layer was extracted with ethyl acetate. The organic layer

was dried and concentrated. Final purification by column chromatography (4:1, EtOAc:acetone) gave compound **17** (637 mg, 41%) with spectral data in agreement with literature values.[1]



18

3-(2-(Diethoxyphosphoryl)-3-ethoxy-3-oxopropyl)pyridine 1-oxide (18). Solid *m*CPBA (0.21 g, 0.95 mmol) was added to a solution of compound **17** (200 mg, 0.63 mmol) in CH_2Cl_2 (3.0 mL) and the resulting solution was allowed to stir overnight at room temperature. Once the reaction was complete based on TLC analysis, the solvent was removed under vacuum. The resulting residue was purified by column chromatography (15% EtOH in CH_2Cl_2) to afford the desired product as a yellow oil in 71% yield (150 mg): ^{1}H NMR ($CDCl_3$) δ 7.97–7.89 (m, 2H), 7.08–6.96 (m, 2H), 4.06–3.89 (m, 6H), 3.12–2.88 (m, 3H), 1.16 (t, J = 7 Hz, 3H), 1.15 (t, J = 6.6 Hz, 3H), 1.02 (t, J = 7.5 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 167.4 (d, J_{CP} = 5.0 Hz), 139.2, 137.9 (d, J_{CP} = 15.5 Hz), 137.5, 126.4, 125.6, 63.0 (d, J_{CP} = 6.8 Hz), 62.9 (d, J_{CP} = 6.4 Hz), 61.6, 46.3 (d, J_{CP} = 129.7 Hz), 29.5 (d, J_{CP} = 3.8 Hz), 16.2 (d, J_{CP} = 1.4 Hz), 16.1 (d, J_{CP} = 2.3 Hz), 13.8; ^{31}P NMR ($CDCl_3$) δ 20.1 ppm; HRMS (ES^+, *m*/*z*) calcd for $(M+H)^+$ $C_{14}H_{23}NO_6P$: 332.1263; found: 332.1269.



7

Sodium 3-(1-oxidopyridin-3-yl)-2-phosphonatopropanoate (7). The ethyl ester **18** (100 mg, 3.02 mmol) was heated at reflux with concentrated HCl (1 mL) overnight. Once the reaction was complete based on analysis of the ^{31}P NMR spectrum of the reaction mixture, the volatiles were removed under a stream of air. The resulting residue was dissolved in a minimum amount of NaOH (0.36 g, 9.06 mmol), acetone was added and the cloudy solution was cooled in a freezer overnight. The precipitate was then isolated by filtration, the solid was dissolved in water, treated with charcoal and the charcoal was further removed by filtration. The resulting clear solution was dried on a lyophilizer to afford the desire product as a white solid in 97% yield (90 mg): ^{1}H NMR (D_2O) δ 8.23 (s, 1H), 8.15 (d, J = 6.2 Hz, 1H), 7.67 (d, J = 7.9 Hz, 1H), 7.51 (dd, J = 7.8, 6.7 Hz, 1H), 3.18–3.02 (m, 2H), 2.89–2.73 (m, 1H); ^{13}C NMR (D_2O) δ 179.8, 142.9 (d, J_{CP} = 17.1 Hz), 138.5, 136.5, 132.9, 126.7, 53.3 (d, J_{CP} = 112.4 Hz), 32.4 (d, J_{CP} = 3.1 Hz); ^{31}P NMR (D_2O) δ 15.1 ppm; HRMS (ES^-, m/z) calcd for $(M-H)^-$ $C_8H_9NO_6P$: 246.0168; found: 246.0170.

Immunoblot analysis. RPMI-8226 cells were incubated with drugs for 48 hrs. Whole cell lysate was obtained using RIPA buffer (0.15M NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton (v/v) X-100, 0.05 M Tris HCl) containing protease and phosphatase inhibitors. Aqueous and detergent fractionations were obtained using Triton X-114. Protein content was determined using the bicinchoninic acid (BCA) method (Pierce Chemical, Rockford, IL). Equivalent amounts of cell lysate were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane, probed with the appropriate primary antibodies, and detected using HRP-linked secondary antibodies and Amersham Pharmacia Biotech ECL Western blotting reagents per manufacturer's protocols.

FDPS enzyme assay. Recombinant FDPS was kindly provided by Dr. Raymond Hohl, Penn State Cancer Center. Recombinant FDPS (10 nM) was incubated with assay buffer (50 mM Tris-HCl, pH 7.7, 20 mM $MgCl_2$, 5 mM TCEP, 5 μg/mL BSA) and test compounds for 10 minutes at room temperature. The reaction was initiated by the addition of 10 μM geranyl pyrophosphate (Sigma) and 10 μM [^{14}C]-IPP (American Radiolabeled Chemicals) and was carried out at 37 °C for 30 minutes. The reaction was stopped by the addition of saturated NaCl. Radiolabeled FPP was extracted with *n*-butanol and counted via liquid scintillation counting.

GGTase II enzyme assay. Reactions were initiated with the addition of 5 μM [^{3}H]-GGPP (American Radiolabeled Chemicals) to reaction buffer (50 mM sodium HEPES, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM NP-40) containing 30 nM recombinant GGTase II (Jena Biosciences), 2 μM recombinant Rab1A, and 1 μM recombinant REP-2 (Jena Biosciences) with or without the test compounds. After a 20 minute incubation at 37 °C, reactions were terminated by the addition of 200 μL 10% HCl/EtOH. Samples were filtered onto GF/P30 filtermats (Perkin Elmer). Filters were then washed in EtOH, dried, and counted using liquid scintillation counting.

Lambda light chain ELISA. Cells were incubated in the presence or absence of test compounds for 48 hrs. The cells were lysed in RIPA buffer containing protease and phosphatase inhibitors. Protein content was determined using the BCA method. Human lambda light chain kit (Bethyl Laboratories, Montgomery, TX) was used to quantify intracellular monoclonal protein levels.

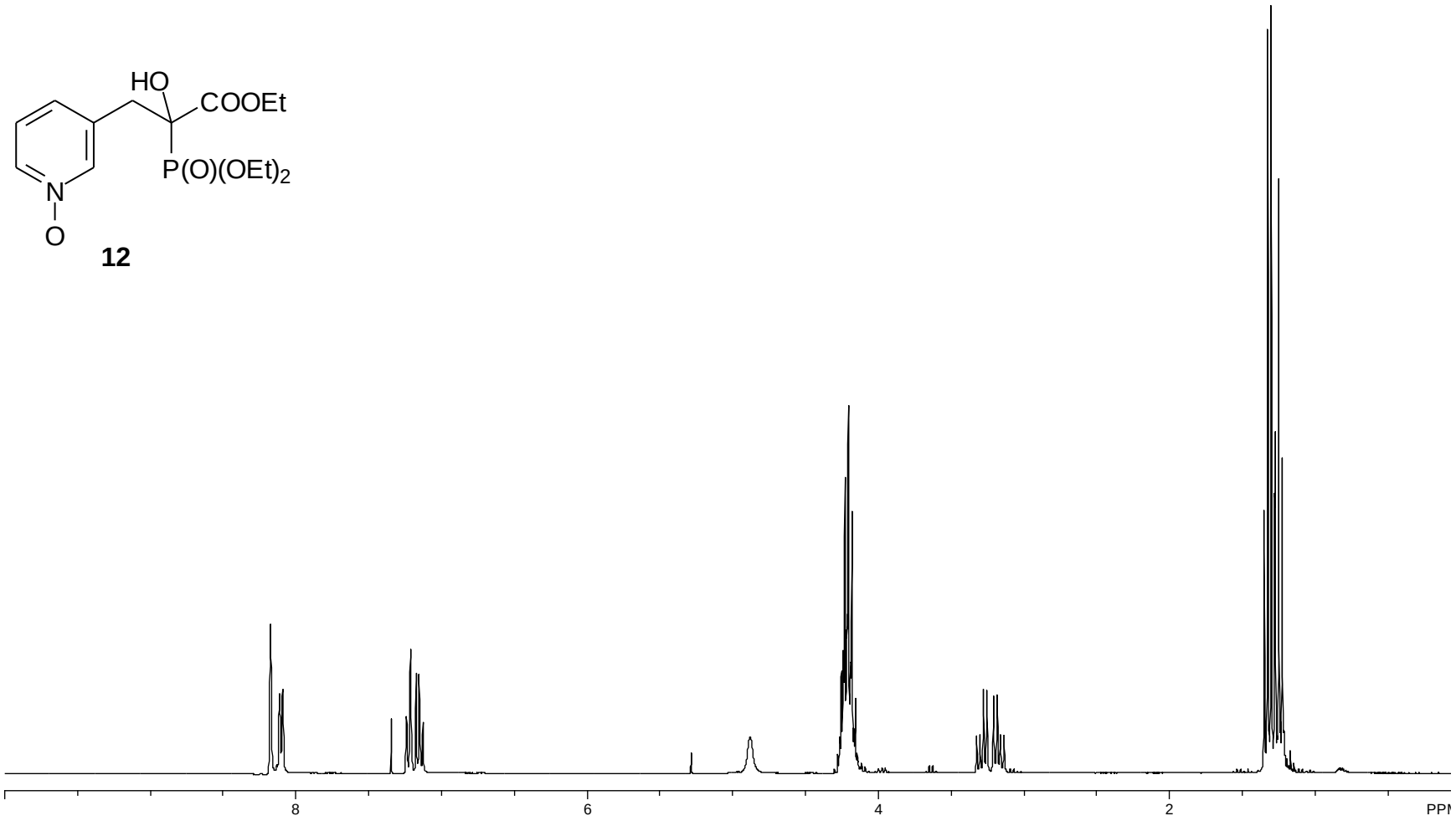
Statistics. Two-tailed *t*-testing was used to calculate statistical significance. An α of 0.05 was set as the level of significance. Concentration response curves for the enzyme assays were analyzed via CalcuSyn software (Biosoft, Cambridge, UK) to determine IC_{50} values.

References.

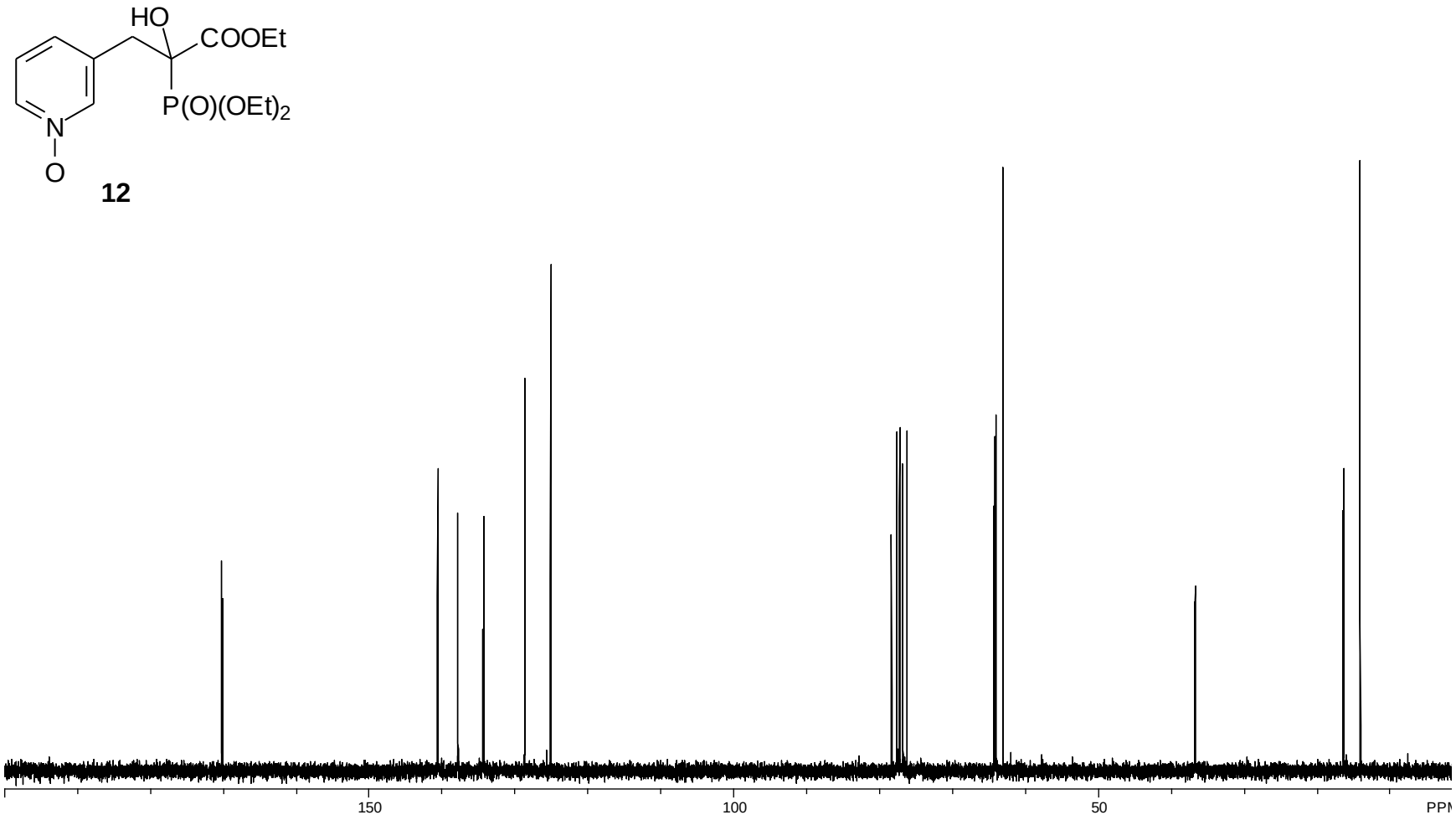
(1) Marma, M. S.; Xia, Z.; Stewart, C.; Coxon, F.; Dunford, J. E.; Baron, R.; Kashemirov, B. A.; Ebetino, F. H.; Triffitt, J. T.; Russell, R. G. G.; McKenna, C. E. *Journal of Medicinal Chemistry* **2007**, *50*, 5967.

(2) Bordier, C. *J Biol Chem* **1981**, *256*, 1604.

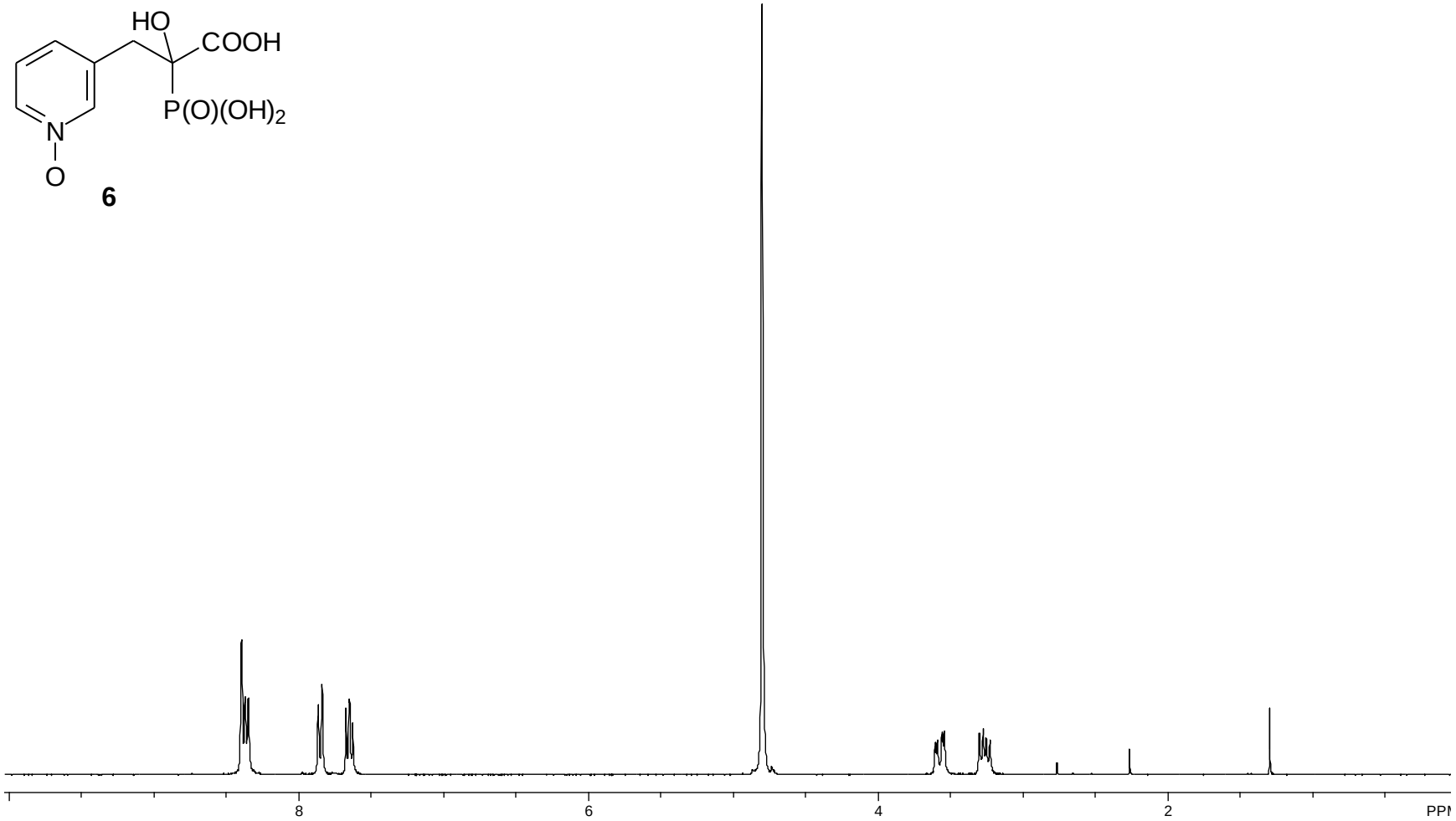
(3) Wasko, B. M.; Dudakovic, A.; Hohl, R. J. *J Pharmacol Exp Ther* **2011**, *337*, 540.



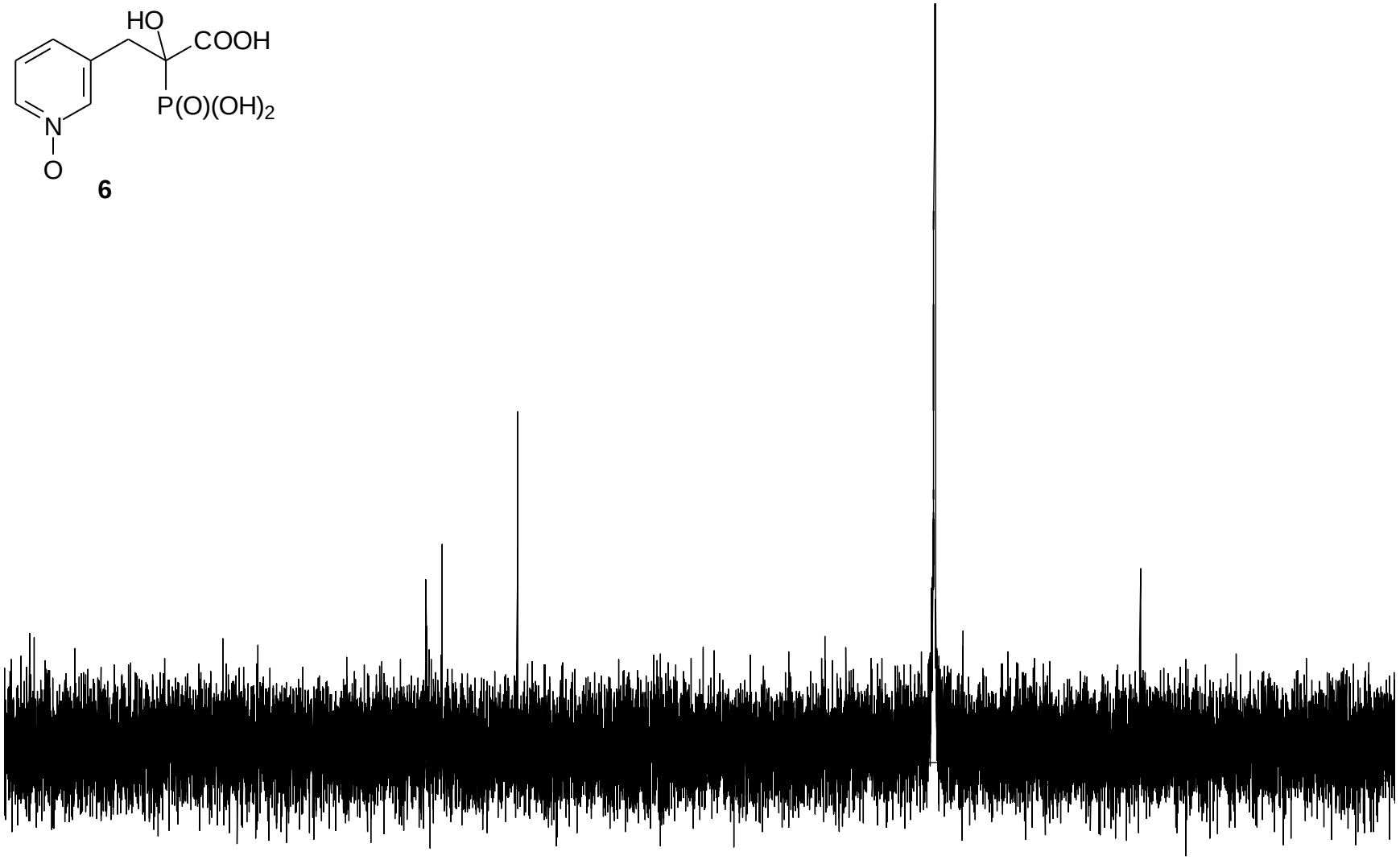
300 MHz ^{1}H NMR Spectrum of Compound **12** ($CDCl_3$).



75 MHz ^{13}C NMR Spectrum of Compound **12** ($CDCl_3$).

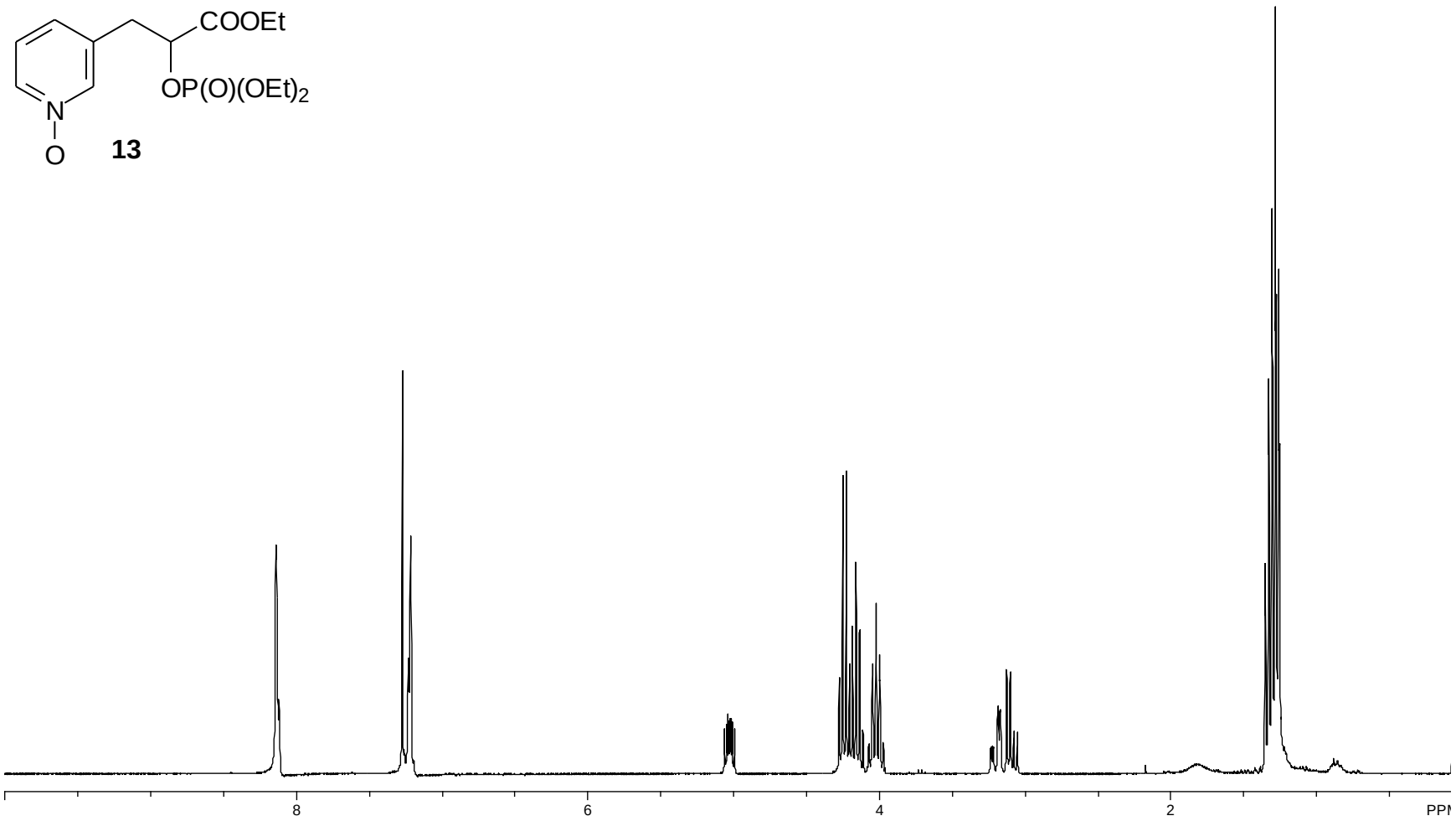


300 MHz ^{1}H NMR Spectrum of Compound **6** (D_2O).

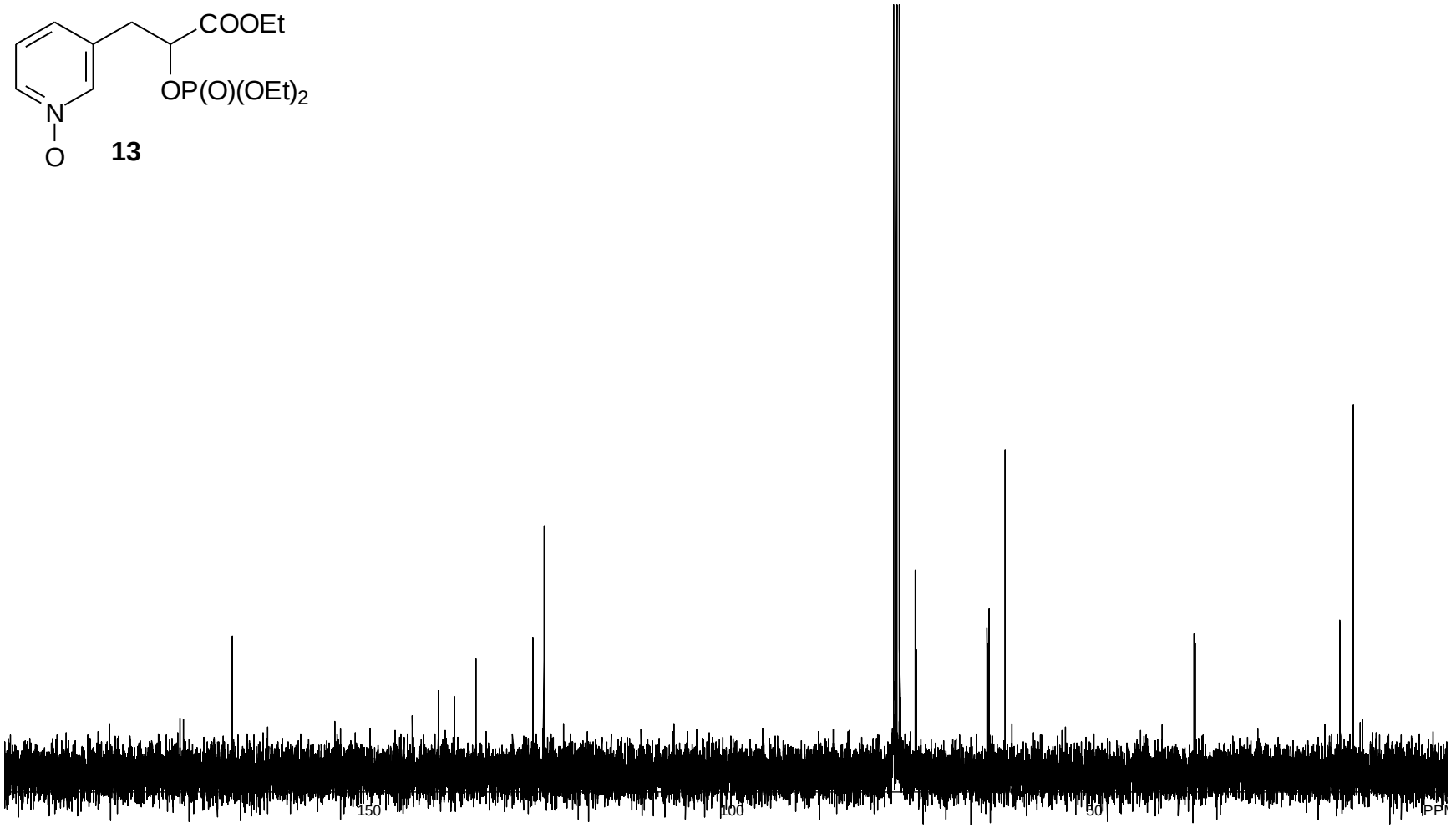


6

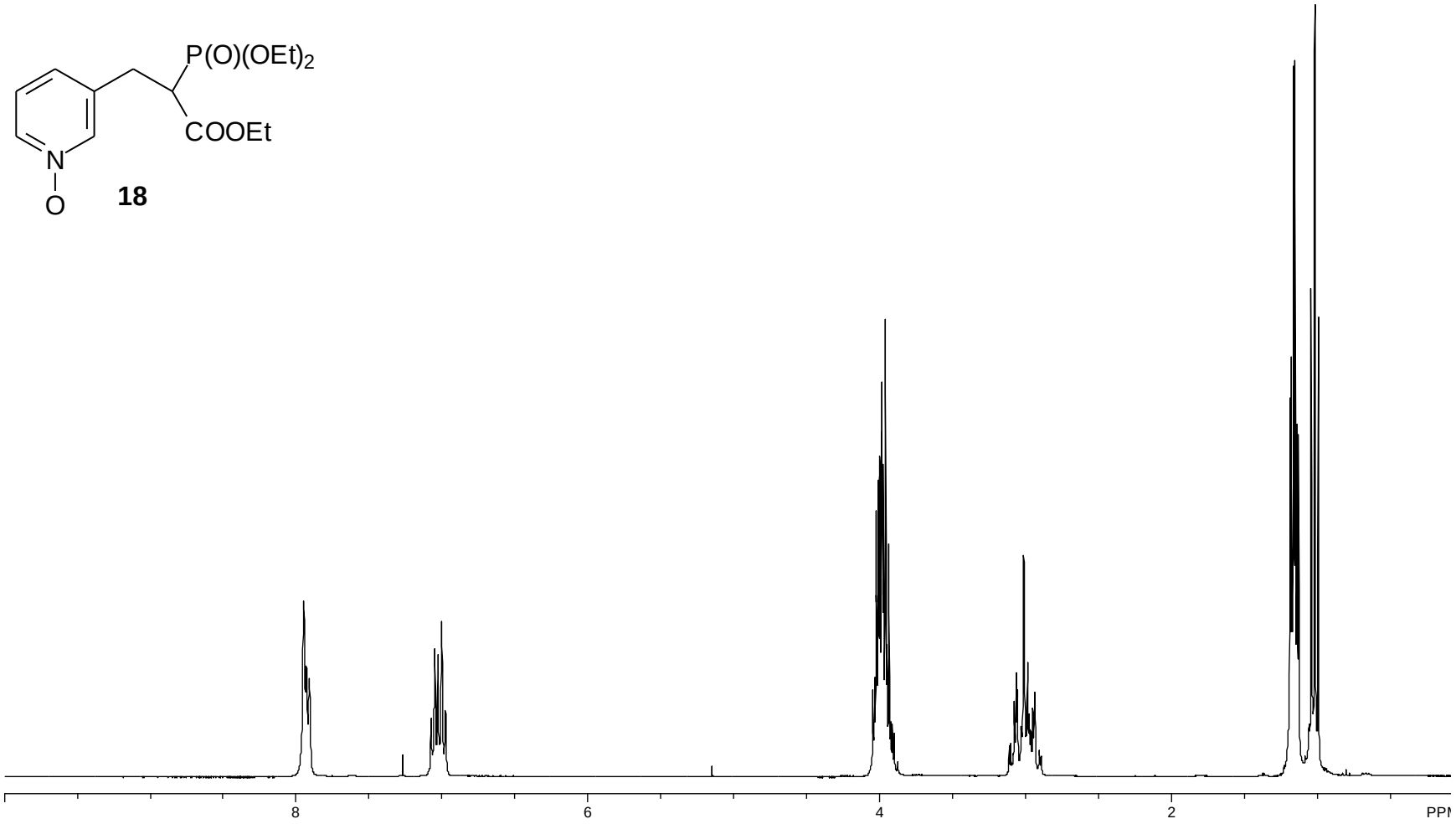
75 MHz ^{13}C NMR Spectrum of Compound **6** (D_2O).



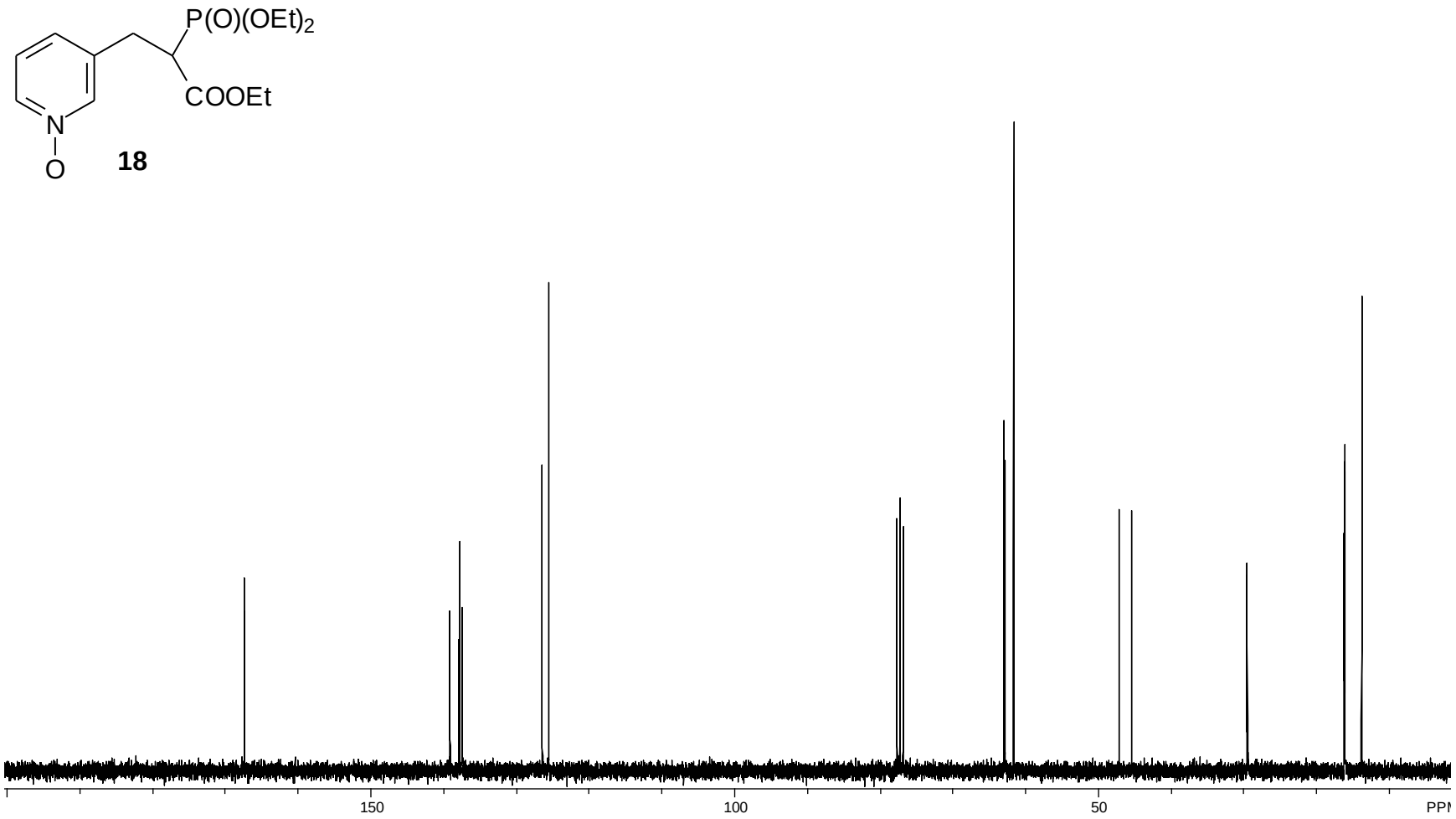
300 MHz ^{1}H NMR Spectrum of Compound **13** ($CDCl_3$).



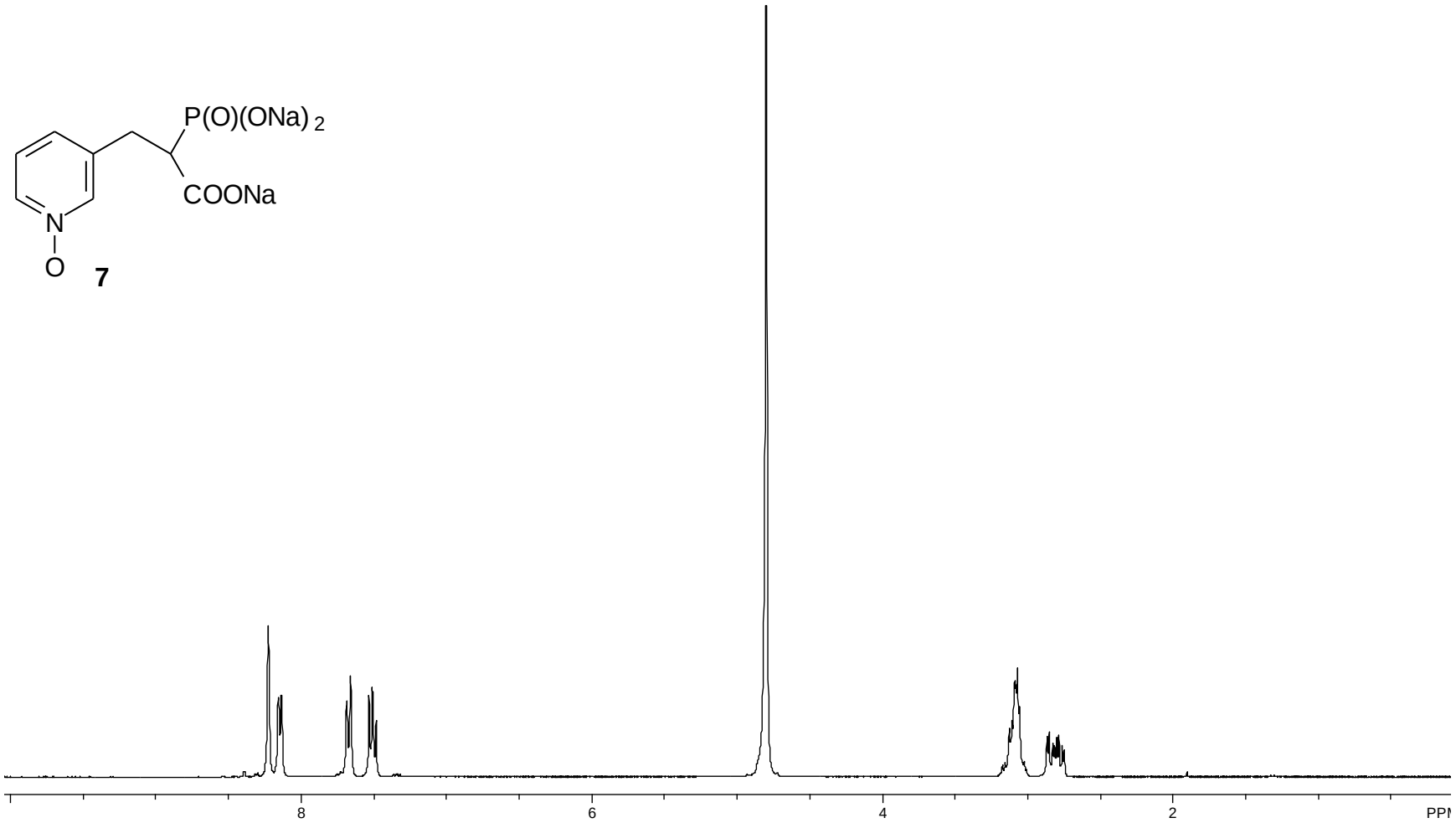
75 MHz ^{13}C NMR Spectrum of Compound **13** ($CDCl_3$).



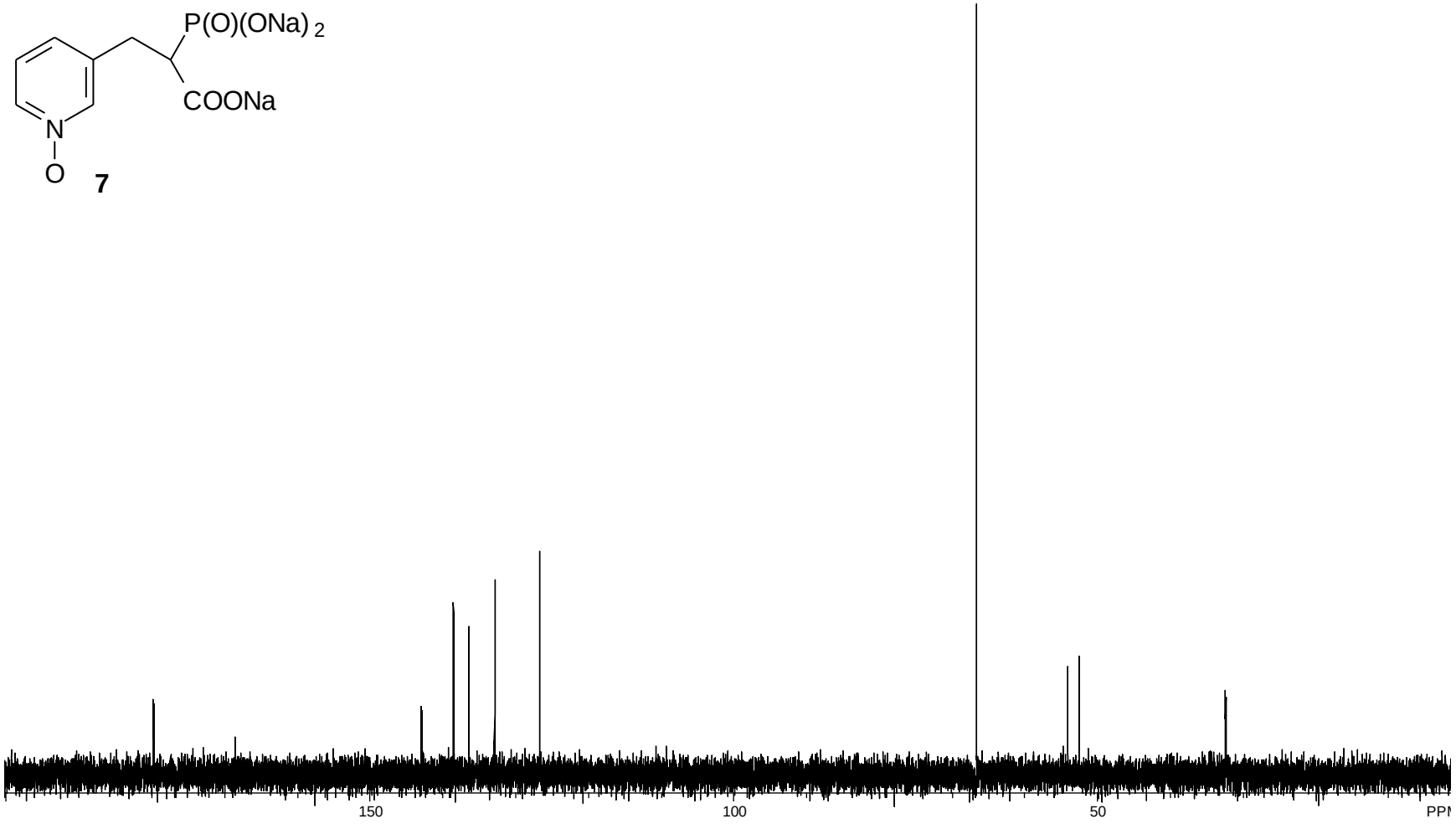
300 MHz ^{1}H NMR Spectrum of Compound **18** ($CDCl_3$).



75 MHz ^{13}C NMR Spectrum of Compound **18** ($CDCl_3$).



300 MHz ^{1}H NMR Spectrum of Compound **7** (D_2O).



75 MHz ^{13}C NMR Spectrum of Compound **7** (D_2O).