Supporting Information for:

Synthesis and Metabolism of BTN3A1 Ligands: Studies on Modifications of the Allylic Alcohol

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General experimental conditions. Tetrahydrofuran was freshly distilled from sodium/benzophenone, while acetonitrile was distilled from calcium hydride prior to use and dimethylformamide, pyridine, and triethylamine were dried over 4 Å molecular sieves (5% w/v). All other reagents and solvents were purchased from commercial sources and used without further purification. All reactions in non–aqueous solvents were conducted in flame–dried glassware under a positive pressure of argon and with magnetic stirring. All NMR spectra were obtained at 300, 400, or 500 MHz for ^{1}H, 75, 100, or 125 MHz for ^{13}C, and 121, 161, or 202 MHz for ^{31}P with internal standards of $(CH_3)_4Si$ (^{1}H, 0.00 ppm), $CDCl_3$ (^{1}H, 7.27; ^{13}C, 77.2 ppm), CD_3OD (^{1}H, 3.31; ^{13}C, 49.0 ppm), $CD_3C(O)CD_3$ (^{1}H, 2.05; ^{13}C, 206.3 ppm), or CD_3CN (^{1}H, 1.94; ^{13}C, 118.3 ppm) for non–aqueous samples or D_2O (^{1}H, 4.80 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. Silica gel (60 Å, 0.040–0.063 mm) was used for flash chromatography. The purity of compounds submitted for biological assay was analyzed by HPLC on an Agilent 1120 infinity LC solvent delivery system with a variable wavelength UV detector, and compounds for bioassay were ≥95% pure based on their UV absorption. Verification was obtained by inspection of the ^{31}P NMR spectra for the assayed compounds.

4-Methylpent-3-enyl phosphonic acid (8). To dimethyl phosphonate **7** (624 mg, 3.3 mmol) in CH_2Cl_2 (5 mL) at 0 °C was added 2,4,6-trimethylpyridine (3.1 mL, 22.8 mmol) followed by a dropwise addition of bromotrimethylsilane (3.5 mL, 26.1 mmol). The reaction was allowed to stir at 0 °C for 2.5 h and then was concentrated *in vacuo*. The residue was dissolved in benzene and concentrated (2 x 2 mL). The concentrate was dissolved in a minimal amount of H_2O and the

pH was adjusted to 10 with 1 N NaOH. The reaction was allowed to stir for 18 h. The organic compounds were extracted into CH_2Cl_2 (3 x 15 mL) and the aqueous layer was concentrated to afford the sodium salt **8** (294 mg, 43%) as a pink solid: ^{1}H NMR (300 MHz, D_2O) δ 5.14 (t, J = 7.9 Hz, 1H), 2.07–1.97 (m, 2H), 1.57 (s, 3H), 1.51 (s, 3H), 1.32–1.18 (m, 2H); ^{13}C NMR (75 MHz, D_2O) δ 132.6, 126.1 (d, J_{PC} = 15.3), 29.5 (d, J_{PC} = 130.8 Hz), 24.9, 22.9, 17.0; ^{31}P NMR (121 MHz, D_2O) δ +22.3; HRMS (ES^+, *m/z*) calcd for $[M+H]^+$ $C_6H_{12}O_3P$: 163.0524, found: 163.0534.

Ethyl 2-(((4-methylpent-3-en-1-yl)(1-naphthyloxy)phosphoryl)amino)acetate (10). The mixed ester **9**[1] (932 mg, 3.1 mmol) was dissolved in freshly distilled acetonitrile (16 mL) and added as a solution to solid, flame-dried sodium iodide (518 mg). The resultant solution was heated at reflux overnight, allowed to cool to room temperature, and then concentrated *in vacuo* to reveal a pale yellow to white solid. Glycine ethyl ester HCl (782 mg, 5.6 mmol) was added followed by anhydrous pyridine (15 mL) and then triethylamine (4.6 mL, 32.9 mmol) and the resulting solution was stirred at rt. In a separate flask, 2,2'-dithiodipyridine (5.0 g) and PPh_3 (4.2 g) were dissolved in anhydrous pyridine (15 mL) and the resultant solution was stirred for 20 minutes. This solution was added to the solution of monosodium salt and the mixture was stirred overnight at 60 °C. The reaction mixture was concentrated *in vacuo* and the residue was dissolved in EtOAc and filtered. The filtrate was concentrated *in vacuo* and the residue was subjected to silica gel chromatography (0-10% EtOAc in Et_2O) to provide the desired monoamidate **10** (612 mg, 53% over two steps) as a clear to pale yellow oil: ^{1}H NMR (400 MHz, CD_3OD) δ 8.17–8.14 (m, 1H), 7.89–7.87 (m, 1H), 7.69 (d, J = 8.3 Hz, 1H), 7.57–7.50 (m, 2H), 7.49 (d, J = 7.6 Hz, 1H), 7.42 (d, J = 7.9 Hz, 1H), 5.52 (td, J = 7.2, 1.4 Hz, 1H), 4.08 (q, J = 7.1 Hz, 2H), 3.81–3.60 (m, 2H), 2.51–2.44 (m, 2H), 2.16–2.08 (m, 2H), 1.69 (s, 3H), 1.63 (s, 3H), 1.18 (t, J = 7.1 Hz, 3H); ^{13}C NMR (100 MHz,

CD_3OD) δ 172.8 (d, J_{PC} = 4.4 Hz), 147.8 (d, J_{PC} = 9.8 Hz), 136.4, 133.9, 128.9, 128.2 (d, J_{PC} = 4.4 Hz), 127.7, 127.4, 126.6, 125.6, 124.3 (d, J_{PC} = 17.3 Hz), 122.8, 116.6 (d, J_{PC} = 4.1 Hz), 62.1, 43.2, 29.3 (d, J_{PC} = 128.7 Hz), 25.8, 22.1 (d, J_{PC} = 4.3 Hz), 17.7, 14.4; ^{31}P NMR (161 MHz, CD_3OD) δ +35.9; HRMS (ES+, m/z) calcd. for $[M+Na]^+$ $C_{20}H_{26}NNaO_4P$: 398.1497; found: 398.1496. A portion of this isolated material was further purified by semi-preparative HPLC to afford material suitable for biological assay.

Methyl 2-[[[(*E*)-4-methyl-5-oxo-pent-3-enyl]-phenoxyphosphoryl]-amino]-acetate (**17**). Selenium dioxide (244 mg, 2.2 mmol) and pyridine (0.2 mL) were stirred for 30 minutes at room temperature and then cooled to 0 °C. The monoamidate **11**[2] (101 mg, 0.3 mmol) was dissolved in MeOH (5 mL), added to the solution of oxidant, and the reaction mixture was stirred overnight at rt. The solution was concentrated *in vacuo* and the residue was dissolved in EtOAc, washed with aqueous potassium carbonate (2x) and then brine, dried ($MgSO_4$), and filtered. The filtrate was concentrated *in vacuo* and the residue was subjected to silica gel chromatography (0–20% acetone in CH_2Cl_2) to provide both the alcohol (21 mg) and its corresponding aldehyde **17** (83 mg, 85%) as a yellow oil: ^{1}H NMR (400 MHz, CD_3OD) δ 9.39 (s, 1H), 7.38–7.34 (m, 2H), 7.22–7.16 (m, 3H), 6.70 (tq, J = 7.3, 1.3 Hz, 1H), 3.82–3.71 (m, 2H), 3.68 (s, 3H), 2.86–2.76 (m, 2H), 2.24–2.16 (m, 2H), 1.77 (s, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 196.9, 173.4 (d, J_{PC} = 4.0 Hz), 154.5 (d, J_{PC} = 15.5 Hz), 151.7, 141.0, 130.8 (2C), 126.0, 121.9 (d, J_{PC} = 4.6 Hz, 2C), 52.5, 43.0, 27.6 (d, J_{PC} = 132.0 Hz), 23.1 (d, J_{PC} = 4.2 Hz), 9.1; ^{31}P NMR (161 MHz, CD_3OD) δ +33.9; HRMS (ES+, *m/z*) calcd. for $[M+Na]^+$ $C_{15}H_{20}NO_5PNa$: 348.0977; found: 348.0983.

Ethyl 2-[[[(*E*)-4-methyl-5-oxo-pent-3-enyl]-phenoxyphosphoryl]-amino]-acetate (18). Selenium dioxide (26 mg, 0.2 mmol) and pyridine (0.1 mL, 0.6 mmol) were dissolved in 70% aqueous *tert*-butyl hydroperoxide solution (0.2 mL), stirred for 30 minutes at room temperature

and cooled to 0 °C.[3] The monoamidate **12**[2] (245 mg, 0.8 mmol) was dissolved in MeOH (0.7 mL), added to the solution of oxidant and the reaction mixture was stirred overnight. The solution was concentrated *in vacuo* and the residue was dissolved in EtOAc, washed with aqueous potassium carbonate (2x) and then brine, dried ($MgSO_4$), and filtered. The filtrate was concentrated *in vacuo* and the residue was subjected to silica gel chromatography (0–20% acetone in CH_2Cl_2) to provide both the alcohol (42 mg) and its corresponding aldehyde **18** (171 mg, 63%) as a yellow oil: ^{1}H NMR (400 MHz, CD_3OD) δ 9.39 (s, 1H), 7.37–7.33 (m, 2H), 7.22–7.17 (m, 3H), 6.70 (tq, J = 7.2, 1.1 Hz, 1H), 4.15 (q, J = 7.2 Hz, 2H), 3.81–3.62 (m, 2H), 2.86–2.76 (m, 2H), 2.25–2.16 (m, 2H), 1.77 (s, 3H), 1.24 (t, J = 7.1 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 196.9, 172.9 (d, J_{PC} = 3.5 Hz), 154.6 (d, J_{PC} = 15.9 Hz), 151.7, 141.0, 130.8 (2C), 126.0, 121.9 (d, J_{PC} = 4.4 Hz, 2C), 62.2, 43.2, 27.6 (d, J_{PC} = 132.2 Hz), 23.1 (d, J_{PC} = 4.3 Hz), 14.5, 9.1; ^{31}P NMR (161 MHz, CD_3OD) δ +33.9; HRMS (ES+, *m/z*) calcd. for $[M+H]^+$ $C_{16}H_{23}NO_5P$: 340.1314; found: 340.1319.

Isopropyl 2-[[[(*E*)-4-methyl-5-oxo-pent-3-enyl]-phenoxyphosphoryl]-amino]-acetate (19). Selenium dioxide (54 mg, 0.5 mmol) and pyridine (0.2 mL, 2.4 mmol) were dissolved in 70% aqueous *tert*-butyl hydroperoxide (0.5 mL), stirred for 30 minutes at rt and cooled to 0 °C.[3] The monoamidate **13** (121 mg, 0.3 mmol) was dissolved in MeOH (1.5 mL), added to the solution of oxidant, and the reaction mixture was stirred overnight. The solution was concentrated *in vacuo* and the residue was dissolved in EtOAc, washed with aqueous potassium carbonate (2x) and then brine, dried ($MgSO_4$), and filtered. The filtrate was concentrated *in vacuo* and the residue was subjected to silica gel chromatography (0–20% acetone in CH_2Cl_2) to provide both the alcohol (26 mg) and its corresponding aldehyde **19** (88 mg, 83%) as a yellow oil: ^{1}H NMR (400 MHz, $CD_3C(O)CD_3$) δ 9.42 (s, 1H), 7.36–7.32 (m, 2H), 7.28–7.25 (m, 2H), 7.15 (td, J = 7.3, 0.7 Hz, 1H), 6.71 (tq, J = 7.2, 1.0 Hz, 1H), 4.97 (sept, J = 6.20 Hz, 1H), 4.67–4.60 (m, 1H), 3.85–3.63 (m,

2H), 2.85–2.74 (m, 2H), 2.20–2.11 (m, 2H), 1.73 (s, 3H), 1.19 (dd, J = 6.3, 1.4 Hz, 6H); ^{13}C NMR (100 MHz, $CD_3C(O)CD_3$) δ 195.3, 171.7 (d, J_{PC} = 4.5 Hz), 153.9 (d, J_{PC} = 15.4 Hz), 152.1, 140.2, 130.4 (2C), 125.2, 121.7 (d, J_{PC} = 4.5 Hz, 2C), 69.2, 43.4, 27.6 (d, J_{PC} = 130.7 Hz), 23.0 (d, J_{PC} = 4.1 Hz), 22.1 (2C), 9.2; ^{31}P (202 MHz, CD_3CN) δ +31.8; HRMS (ES+, *m/z*) calcd. for $[M+H]^+$ $C_{17}H_{25}NO_5P$: 354.1470; found: 354.1471.

Ethyl 2-[[[(3*E*)-5-hydroxyimino-4-methylpent-3-enyl]-phenoxyphosphoryl]-amino]-acetate (20). Manganese dioxide (650 mg, 7.5 mmol) was added to freshly distilled CH_2Cl_2 (3 mL) at rt. Allylic alcohol **15**[2] (66 mg, 0.2 mmol) was added and the mixture was stirred overnight. The reaction mixture was filtered through Celite, which was subsequently washed with 40% acetone in CH_2Cl_2. The filtrate was concentrated *in vacuo* to afford aldehyde **18** (32 mg, 48%). To a solution of aldehyde **18** (16 mg, 0.05 mmol) in H_2O (0.5 mL) was added NH_2OH (1.7 µL, 50 wt% in H_2O). The reaction was allowed to stir for 15 min and then was quenched by addition of sat. NH_4Cl (5 mL). After extraction into EtOAc (3 x 2 mL), the combined extracts were concentrated to afford aldoxime **20** (17 mg, 100%) as a yellow solid: ^{1}H NMR (500 MHz, CD_3OD) δ 7.69 (s, 1H), 7.37 (dd, J = 7.6, 7.6 Hz, 2H), 7.23 (d, J = 7.6 Hz, 3H), 5.80 (t, J = 7.6 Hz, 1H), 4.59 (bs, 1H), 4.16 (q, J = 7.1 Hz, 2H), 3.80–3.63 (m, 2H), 2.71–2.56 (m, 2H), 2.13–2.06 (m, 2H), 1.86 (s, 3H), 1.25 (t, J = 7.2 Hz, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 171.8, 153.2, 150.3, 135.3, 132.3, 129.3 (2C), 124.5, 120.5 (2C), 60.8, 41.8, 26.9 (d, J_{PC} = 131 Hz), 20.7 (d, J_{PC} = 3.8 Hz), 13.0, 10.2; ^{31}P NMR (202 MHz, CD_3OD) δ +34.4; HRMS (ES+, *m/z*) calcd. for $[M + Na]^+$ $C_{16}H_{23}N_2O_5PNa$: 377.124; found: 377.124. Final purification was achieved by preparatory HPLC (C18 column; 60 to 100% acetonitrile in H_2O) to afford aldoxime (2 mg) suitable for bioassay.

[2,2-Dimethylpropanoyloxymethoxy-[(*E*)-4-methyl-5-oxo-pent-3-enyl]-phosphoryl]-oxymethyl 2,2-dimethylpropanoate (22). In an oven-dried flask, compound **21** (77 mg, 0.19

mmol) was dissolved in CH_2Cl_2 (3 mL) under argon and cooled to 0 °C using an ice-bath. Dess-Martin periodinane (120 mg, 0.28 mmol) was added in one portion and the reaction was allowed to stir for 1 h, at which point the ice-bath was removed and the solution was stirred at rt for 1 h until the starting material was consumed, as indicated by TLC (silica). The reaction was quenched by addition of a saturated aqueous solutions of $Na_2S_2O_3$ (2 mL) and $NaHCO_3$ (3 mL). Water was added and the organics were extracted with EtOAc (3 x 50 mL). The organic extracts were combined, washed with brine, dried (Na_2SO_4), and then concentrated *in vacuo*. The concentrate was subjected to silica gel chromatography (20% to 50% EtOAc in hexanes) to provide aldehyde **22** (53 mg, 67%) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.43 (s, 1H), 6.46 (t, J = 7.1 Hz, 1H), 5.77 – 5.57 (m, 4H), 2.71–2.63 (m, 2H), 2.09 – 2.01 (m, 2H), 1.78 (s, 3H), 1.26 (s, 18H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 194.7, 176.9 (2C), 150.7 (d, J = 15.9 Hz), 140.3, 81.5 (d, J = 6.0 Hz, 2C), 38.8 (2C), 26.9 (6C), 25.3 (d, J = 141.7 Hz), 21.7 (d, J = 4.7 Hz), 9.2; ^{31}P NMR (202 MHz, $CDCl_3$) δ +30.5; HRMS (TOF-LCMS) calcd. for $[M + H]^+$ $C_{18}H_{32}O_8P$: 407.1835; found 407.1814.

[[(*E*)-4-Methyl-5-oxo-pent-3-enyl]-(2-naphthyloxy)-phosphoryl]-oxymethyl 2,2-dimethylpropanoate (26). Selenium dioxide (60 mg, 0.6 mmol) was added to a solution of phosphonate **23** (230 mg, 0.6 mmol) in CH_2Cl_2 (3 mL) and allowed to react for 48 h. The reaction was diluted with CH_2Cl_2 (20 mL) and the inorganic components removed by addition of three portions of brine (5 mL). The organic portion was dried (Na_2SO_4), filtered through Celite, and concentrated *in vacuo*. The resulting oil was purified by HPLC (C_{18}, acetonitrile) to give the aldehyde **26** as a yellow oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.41 (s, 1H), 7.83 (dd, J = 11.6, 8.4 Hz, 3H), 7.53 (s, 1H), 7.49 (m, 2H), 7.33 (ddd, J = 8.8, 2.4, 0.8 Hz, 1H), 6.49 (td, J = 7.2, 1.2 Hz, 1H), 5.77 (dd, J = 13.4, 4.8 Hz, 1H), 5.67 (dd, J = 12.4, 4.8 Hz, 1H), 2.83–2.74 (m, 2H), 2.24–2.16 (m, 2H), 1.78 (s, 3H), 1.14 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 194.9, 177.1, 150.8, 140.5,

134.0, 131.3, 130.3, 127.9, 127.7, 127.1, 125.9 (d, J_{PC} = 17.3 Hz), 120.3 (d, J_{PC} = 5.3 Hz), 117.2 (d, J_{PC} = 5.2 Hz), 82.1 (d, J_{PC} = 6.0 Hz), 38.9, 31.2, 26.9 (3C), 25.2 (d, J_{PC} = 137.7 Hz), 22.1 (d, J_{PC} = 5.1 Hz), 9.4; ^{31}P NMR (161 MHz, $CDCl_3$) δ +27.3; HRMS (ES^+, *m*/*z*) calcd for $[M+Na]^+$ $C_{22}H_{27}NaO_6P$: 441.1443, found: 441.1441.

[[(*E*)-5-Hydroxy-4-methyl-pent-3-enyl]-(2-oxochromen-7-yl)-oxy-phosphoryl]-oxymethyl 2,2-dimethylpropanoate (27) and [[(*E*)-4-Methyl-5-oxo-pent-3-enyl]-(2-oxochromen-7-yl)-oxy-phosphoryl]-oxymethyl 2,2-dimethylpropanoate (28). To a solution of phosphonate **24** (1.36 g, 3.22 mmol), in CH_2Cl_2 (14 mL), selenium dioxide (2.50 g, 22.5 mmol), *p*-hydroxybenzoic acid (0.050 g, 0.45 mmol) and finally *tert*-butyl hydroperoxide (1.25 mL, 12.8 mmol) were added and the solution was left to stir for 14 h at rt. The reaction was quenched by addition of a saturated $NaHCO_3$ solution and extracted with CH_2Cl_2. The combined organic extracts were dried ($MgSO_4$) and then filtered. The filtrate was concentrated *in vacuo* and the residue was subjected to silica gel chromatography (15% acetone in CH_2Cl_2) to provide the alcohol **27** (160 mg, 11%) along with aldehyde **28** (158 mg, 11%) as pale yellow oils. Final purification was performed via HPLC. The HPLC conditions: wavelength: 250 nm for alcohol and 325 nm for aldehyde, flow rate: 2 mL/min, column: C18; column and sample temperature: ambient; Injection Volume: 100 µL; 100% HPLC grade ACN over 15 minutes.

For the alcohol **27**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.69 (d, *J* = 9.6 Hz, 1H), 7.48 (d, *J* = 9.1 Hz, 1H), 7.22 (br, 2H), 6.39 (d, *J* = 9.6 Hz, 1 H), 5.77 (dd, J_{PH} = 13.4, 5.1 Hz, 1H), 5.70 (dd, J_{PH} = 12.5, 5.0 Hz, 1H), 5.45 (td, *J* = 8.9, 2.1 Hz, 1H), 4.01 (s, 2H), 2.54–2.44 (m, 2H), 2.14–2.05 (m, 2H), 1.69 (s, 3H), 1.19 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.0, 160.2, 154.9, 152.6 (d, J_{PC} = 16.1 Hz), 142.7, 136.9, 129.1, 122.7 (d, J_{PC} = 16.1 Hz), 117.3 (d, J_{PC} = 4.4 Hz), 116.2, 115.9, 109.2 (d, J_{PC} = 5.1 Hz), 81.8 (d, J_{PC} = 6.2 Hz), 68.1, 38.7, 26.8 (3C), 26.2 (d, J_{PC} = 138.5 Hz) 20.4

(d, J_{PC} = 5.0 Hz), 13.7; ^{31}P NMR (161 MHz, $CDCl_3$) δ +29.6; HRMS (ES+, m/z) calcd. for $[M+H]^+$ $C_{21}H_{28}O_8P$: 439.1522; found: 439.1531.

For the aldehyde **28**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.42 (s, 1H), 7.69 (d, *J* = 9.5 Hz, 1H), 7.49 (d, *J* = 8.3 Hz, 1H), 7.21 (d, *J* = 1.4 Hz, 1H), 7.19 (d, *J* = 1.1 Hz, 1H), 6.48 (td, *J* = 7.7, 1.2 Hz, 1H), 6.40 (d, *J* = 9.6 Hz, 1H), 5.75 (dd, J_{PH} = 13.2, 6.8 Hz, 1H), 5.68 (dd, J_{PH} = 12.2, 5.1 Hz, 1H), 2.80–2.74 (m, 2H), 2.26–2.19 (m, 2H), 1.78 (s, 3H), 1.18 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 194.6, 176.9, 160.1, 155.0, 152.3 (d, J_{PC} = 9.0 Hz), 150.2 (d, J_{PC} = 15.9 Hz), 142.7, 140.5, 129.2, 117.1 (d, J_{PC} = 4.6 Hz), 116.4, 116.0, 109.1 (d, J_{PC} = 4.9 Hz), 82.0 (d, J_{PC} = 6.0 Hz), 38.7, 29.7, 26.9 (3C), 25.2 (d, J_{PC} = 141.2 Hz), 21.8 (d, J_{PC} = 5.0 Hz), 9.32; ^{31}P NMR (161 MHz, $CDCl_3$) δ +27.9; HRMS (ES+, m/z) calcd. for $[M+Na]^+$ $C_{21}H_{25}NaO_8P$: 459.1185; found: 459.1185.

(*E*)-4-Methyl-5-oxo-pent-3-enylphosphonic acid, dimethyl ester (29). To a solution of activated selenium dioxide (72 mg, 0.65 mmol) in CH_2Cl_2 at 0 °C was added *tert*-butyl hydroperoxide (0.49 mL, 5 – 6 M in decane) and the reaction was allowed to stir for 10 minutes at 0 °C. To the reaction was added compound **7** (236 mg, 1.23 mmol) and the reaction was allowed to stir for 24 h while it was allowed to warm to rt. To the residue was added CH_2Cl_2 and the solids were removed by filtration. The filtrate was concentrated by rotary evaporator to afford aldehyde **29** (197 mg, 78% yield): ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.18 (1H, s), 6.28 (1H, t, *J* = 7.0 Hz), 3.53 (6H, d, *J* = 10.8 Hz), 2.42 (2H, dt, *J* = 15.1, 7.4 Hz), 1.79 - 1.69 (2H, m), 1.79 - 1.67 (2H, m), 1.53 (3H, s); ^{31}P NMR (162 MHz, $CDCl_3$) δ +33.0 ppm; HRMS ESI$^+$ $[M+H]^+$ calculated: 207.0786 found: 207.0779.

Biological Methods

Cells and reagents. K562 cells were from Sigma Aldrich (St. Louis, MO). Buffy coat was obtained from Research Blood Components (Boston, MA). Interleukin 2 and the TCR γ/δ+ T Cell Isolation Kit were from Miltenyi (Bergisch Gladbach, Germany). The pooled human plasma and FITC-conjugated anti-γδ-TCR (5A6.E91) antibody were purchased from Fisher Scientific (Waltham, MA). The interferon γ enzyme-linked immunosorbent assay kit and phycoerythrin-conjugated anti-CD3 (UCHT1) antibody were purchased from Biolegend (San Diego, CA). The CellQuanti-Blue Cell Viability Assay Kit was purchased from BioAssay Systems (Hayward, CA).

K562 cell viability. K562 cells (5,000 cells in 100 μL of T cell media) were distributed into each well of a 96-well plate. Compounds at concentrations of 100 μM, 10 μM and 1 μM were added for 72 hours compared to media as a negative control. During the last 2 hours 10 μL of cell-QB reagent was added, following which signals were quantified with a fluorescence plate reader. Viable cells were expressed as a fraction of untreated control cells after subtraction of a media-only blank.

Test compound stimulation of T cell proliferation. Peripheral blood mononuclear cells (PBMCs) were purified from buffy coat using lymphoprep and were stimulated for 3 days in T cell media (RPMI media supplemented with 1.5 g/L sodium bicarbonate with 10% heat-inactivated fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, 1x MEM nonessential amino acids, 1x penicillin-streptomycin solution and 50 μM 2-mercaptoethanol) with test compounds at various doses (10-fold serial dilutions with concentration range determined in a pilot assay). Cells were cultured for another 11 days after compound removal. Cells were pelleted and suspended in 100 μL of FACS buffer (2% BSA in PBS). Cells were co-stained with γδ TCR and CD3 antibodies. Cells were stained at 4 °C for 30 min, washed twice, and then fixed in 3% paraformaldehyde. Data were obtained with a BD Fortessa. Data were analyzed with FlowJo. Dose response curves were

analyzed using a log (agonist) versus response - variable slope (four parameters) model where the top was determined from the positive controls of 100 nM HMBPP and 100 nM compound **21**, and the bottom was determined with the negative control of untreated PBMCs. The maximum efficacy of the test compounds did not differ significantly from the positive controls which is consistent with our prior study.[4]

Culture of human Vγ9Vδ2 T cells for ELISA assays. PBMCs were purified from buffy coat using lymphoprep and then cultured in T cell media for 14 days. PBMCs were stimulated with 10 nM HMBPP for the first 72 hours. IL-2 (5 ng/mL) was added every three days. On day 12, γδ T cells were purified via negative selection and resuspended in fresh media containing IL-2 to obtain Vγ9Vδ2 T cells. All compounds were evaluated for their ability to stimulate interferon γ production by these γδ T cells using ELISA.

Interferon γ ELISA. The compounds were evaluated for stimulation of human Vγ9Vδ2 T cell cytokine production, which is quantified by the release of interferon γ. K562 cells were treated with compounds at different concentrations for 4 hours, washed twice, then mixed with purified expanded γδ T cells. Each well contained 200 μL in duplicate, with a 3:1 ratio of γδ T cells: K562 cells (12,000 T cells and 4,000 K562 cells in each well). The cell co-culture was incubated for 20h, following which the concentration of interferon γ was determined by ELISA. In each experiment, the dose response of compounds was evaluated in comparison to negative controls that contained cells in the absence of compounds. EC_{50} values were determined as the concentration that induced 50% of the maximum effect.

LCMS for prodrug metabolism. K562 cells (5M cells in 500 μL of T cell media) were treated for 1 hour with 100 μM of each test compound. The cells were pelleted by centrifugation (600 rcf for 3 minutes) and media was aspirated. The metabolites were extracted by addition of

200 µL of extraction solvent (75% LCMS grade acetonitrile, 25% 75 mM NH_3OH)[5] and vigorous mixing for 30 seconds. Insoluble debris was pelleted by centrifugation at 10,000 rcf for 2 minutes. 10 µL of the extract was evaluated by LCMS with a Waters Synapt G2-Si Mass Spectrometer in negative mode using a C18 column and a gradient of 10 mM triethylammonium acetate (A) and methanol/10 mM triethylammonium acetate pH 7 (90/10, v/v) (B).[6] The gradient started at 10% B then increased to 80% B over 4 minutes and held there for 2.5 minutes before re-equilibration.

Identification of metabolites. Extracts were compared to authentic standards of C-HMBP and C-HMBPP. Mono-acid forms of the POM prodrugs were expected based on literature and the calculated m/z values of these aldehyde and alcohol form metabolites were searched. To identify additional unknown metabolites, the LCMS traces from extracts of K562 cells treated with compound **22** were compared to untreated K562 cell extracts using Progenesis software (Waters) and ranked according to fold-increase in the treated versus untreated cells. The masses and retention times for identified compounds were as follows (free acid alcohol, t = 1.47–1.55 min; free acid aldehyde, t = 1.37–1.57 min; mono-POM alcohol, t = 3.8–3.92 min; mono POM aldehyde, t = 3.91–4.05 min; mono-naphthyl alcohol, t = 4.09–4.19 min; mono-naphthyl aldehyde, t = 4.19–4.29 min). At pH 7 the phosphonate is expected to be mono-protonated and the mono-esters deprotonated such that all compounds detected carried a natural charge of -1. For all compounds tested, masses corresponding to the molecular ion $[M]^-$, were observed at the reported retention time.

Statistics. Cell based activity experiments were performed at least three times (n=3) with cells from at least two different donors. Metabolism studies were performed twice (n=2). Data was analyzed and graphed using GraphPad Prism 6. Dose response curves were analyzed using a log

(agonist) versus response - variable slope (four parameters) model. EC_{50} values with 95% confidence intervals are reported.

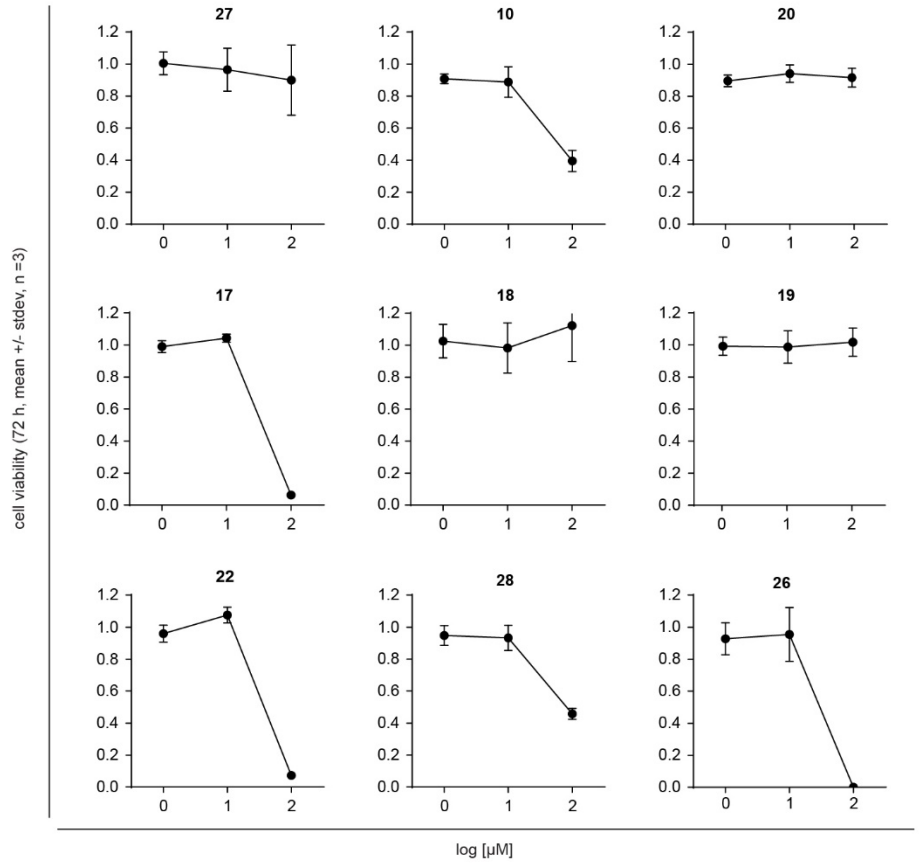


Figure S1. Viability of K562 cells. K562 cells were treated for 72 h with indicated test compounds. Results indicate fraction of untreated cells remaining after incubation. Data represents the mean and standard deviation of three independent experiments (n=3).

ELISA

Compound	4 h ELISA EC_{50} (nM)	Fold difference vs. corresponding C-HMBP analog
17	16	34
18	7.9	46
19	11	15
22	0.34	18
28	0.90	22

Table S1. Interferon γ EC_{50} values. K562 cells were treated with test compounds for 4 hours, washed, and exposed to T cells for 20 hours. Cytokine was measured by ELISA.

References

1. Foust, B. J.; Li, J.; Hsiao, C. C.; Wiemer, D. F.; Wiemer, A. J., Stability and Efficiency of Mixed Aryl Phosphonate Prodrugs. *Chemmedchem* **2019,** *14* (17), 1597-1603.

2. Lentini, N. A.; Foust, B. J.; Hsiao, C. H. C.; Wiemer, A. J.; Wiemer, D. F., Phosphonamidate Prodrugs of a Butyrophilin Ligand Display Plasma Stability and Potent V gamma 9 V delta 2 T Cell Stimulation. *J. Med. Chem.* **2018,** *61* (19), 8658-8669.

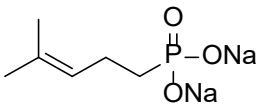
3. Tang, X. P.; Demiray, M.; Wirth, T.; Allemann, R. K., Concise synthesis of artemisinin from a farnesyl diphosphate analogue. *Bioorg. Med. Chem.* **2018,** *26* (7), 1314-1319.

4. Hsiao, C.-H. C.; Wiemer, A. J., A power law function describes the time-and dose-dependency of V gamma 9V delta 2 T cell activation by phosphoantigens. *Biochem. Pharmacol.* **2018,** *158*, 298-304.

5. Tong, H. X.; Kuder, C. H.; Wasko, B. M.; Hohl, R. J., Quantitative determination of isopentenyl diphosphate in cultured mammalian cells. *Anal. Biochem.* **2013,** *433* (1), 36-42.

6. Joachimiak, L.; Janczewski, L.; Ciekot, J.; Boratynski, J.; Blazewska, K., Applying the prodrug strategy to alpha-phosphonocarboxylate inhibitors of Rab GGTase - synthesis and stability studies. *Organic & Biomolecular Chemistry* **2015,** *13* (24), 6844-6856.

HPLC Traces



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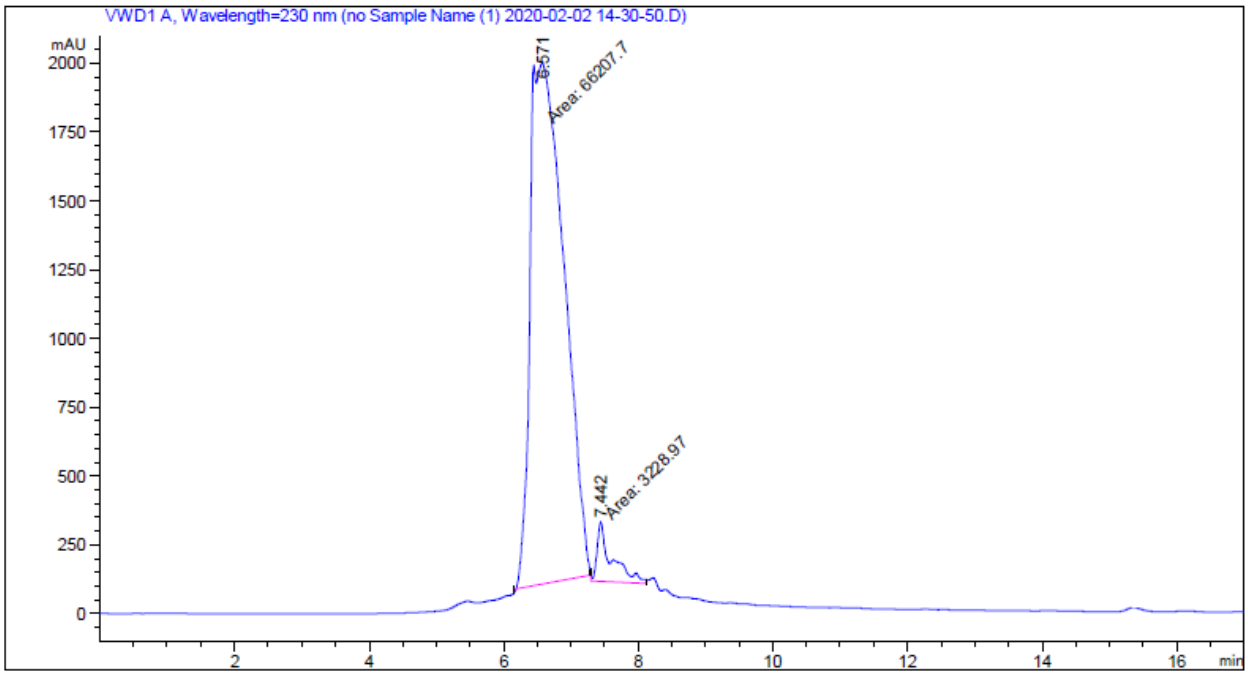


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Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


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   1   6.571 MM T  0.5834 6.62077e4  1891.51770  95.3498
   2   7.442 MM    0.2497 3228.96655  215.55539   4.6502

Totals :                  6.94366e4  2107.07309
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HPLC Trace of compound **8**

Conditions: wavelength: 230 nm, flow rate: 2 mL/min, column: Agilent Polaris C18A; column and sample temperature: ambient; Injection Volume: 70 µL; Isocratic 20% ACN in H_2O.

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Sample Operator : SYSTEM
Acq. Instrument : LC                                Location :   1
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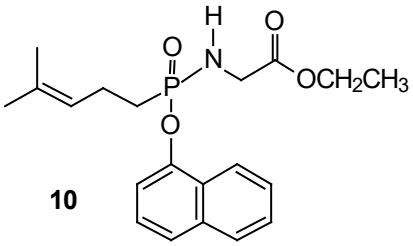

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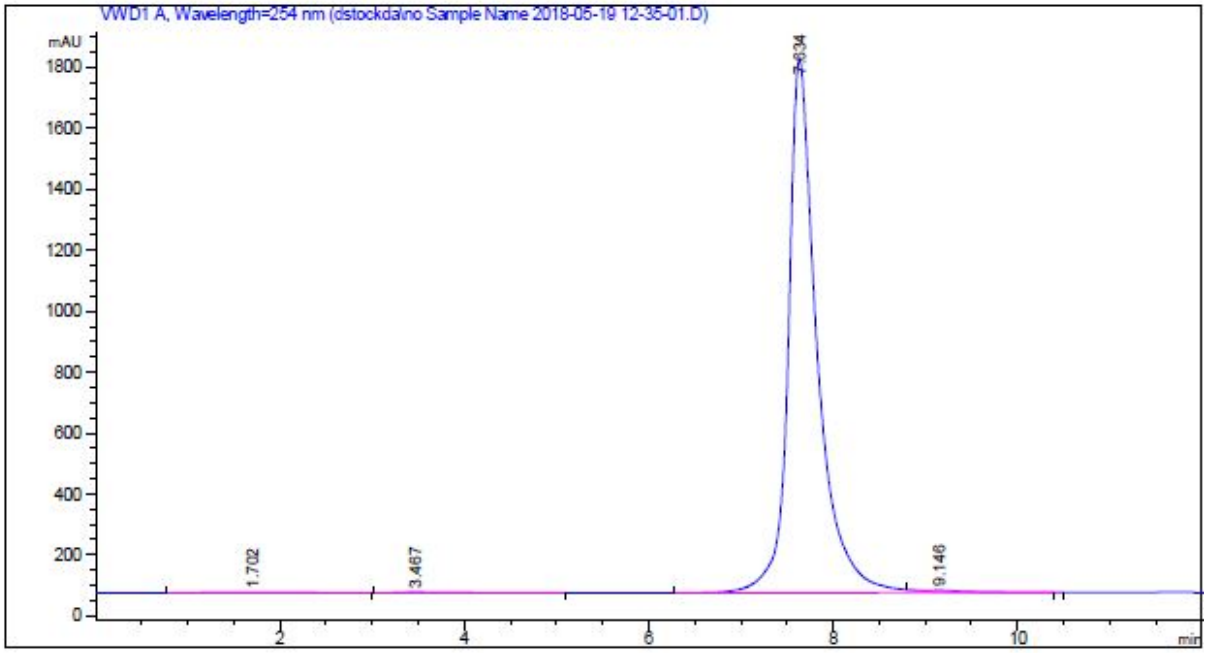


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Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=254 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   1.702 BB    0.6165   79.61287    1.81960   0.2056
   2   3.467 BB    0.3660   66.76842    2.56280   0.1724
   3   7.634 BV R  0.3166 3.84471e4  1748.30884  99.2917
   4   9.146 VB E  0.3857  127.86786    4.63712   0.3302

Totals :                  3.87214e4  1757.32835
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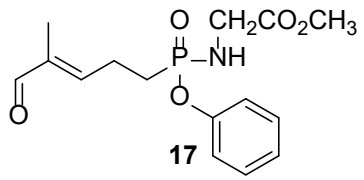
HPLC Trace of compound **10**



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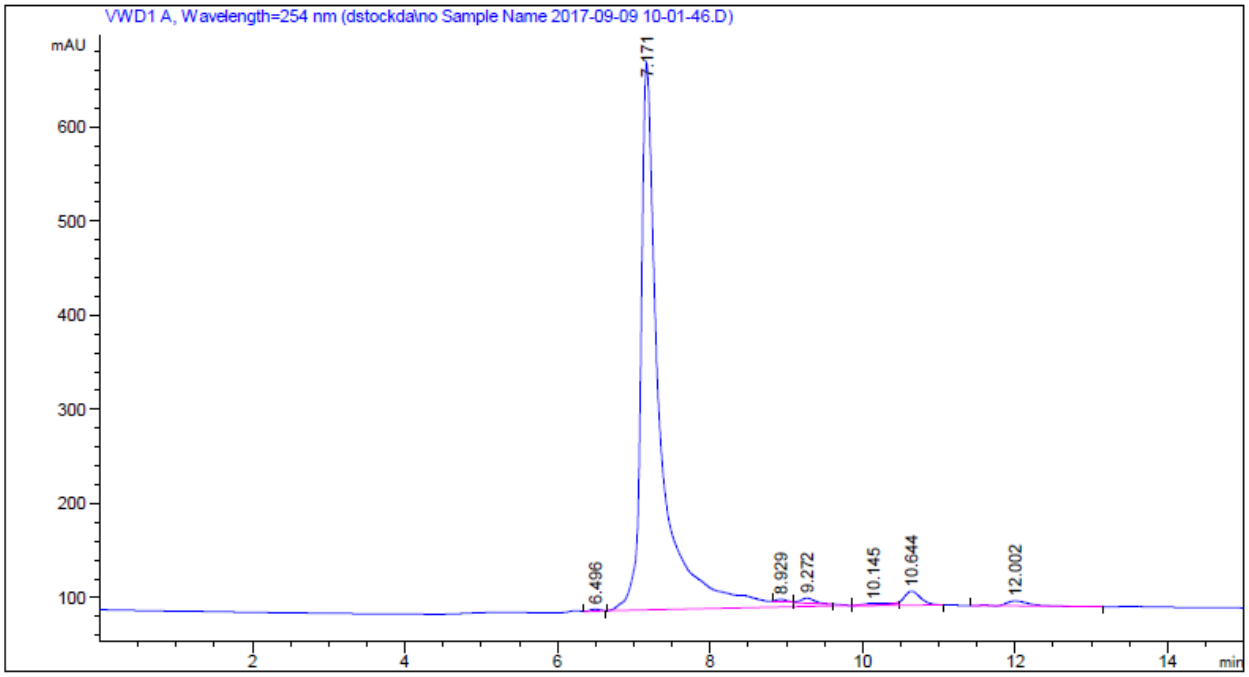


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Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=254 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   6.496 VB    0.1394   16.96753    1.89634   0.1580
   2   7.171 BV R  0.2456 1.02325e4   580.64319  95.2631
   3   8.929 VV E  0.1582   26.48608    2.63558   0.2466
   4   9.272 VB E  0.1935   67.60953    5.34888   0.6294
   5  10.145 BV E  0.2985   43.94265    2.08015   0.4091
   6  10.644 VB R  0.2319  226.48338   14.76711   2.1085
   7  12.002 VB R  0.3442  127.31445    5.49777   1.1853
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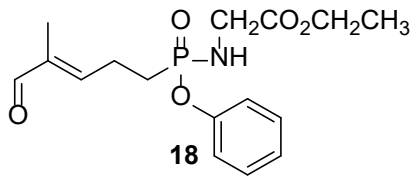
HPLC Trace of compound **17**



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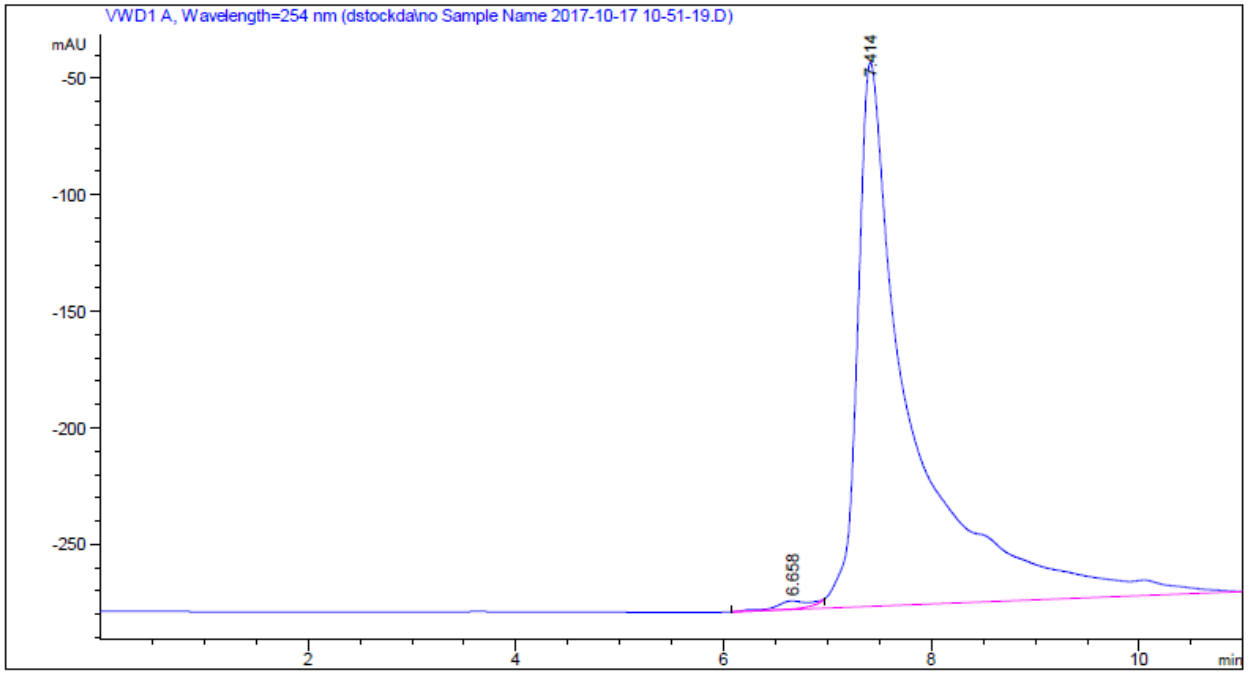


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Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=254 nm

Peak RetTime Type  Width     Area      Height     Area
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----|-------|----|-------|----------|----------|--------|
   1   6.658 BV E  0.3067   73.92787    3.42721   0.8502
   2   7.414 VV R  0.4897 8621.70801  233.52063  99.1498

Totals :                  8695.63588  236.94784
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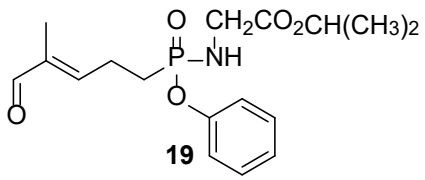
HPLC Trace of compound **18**



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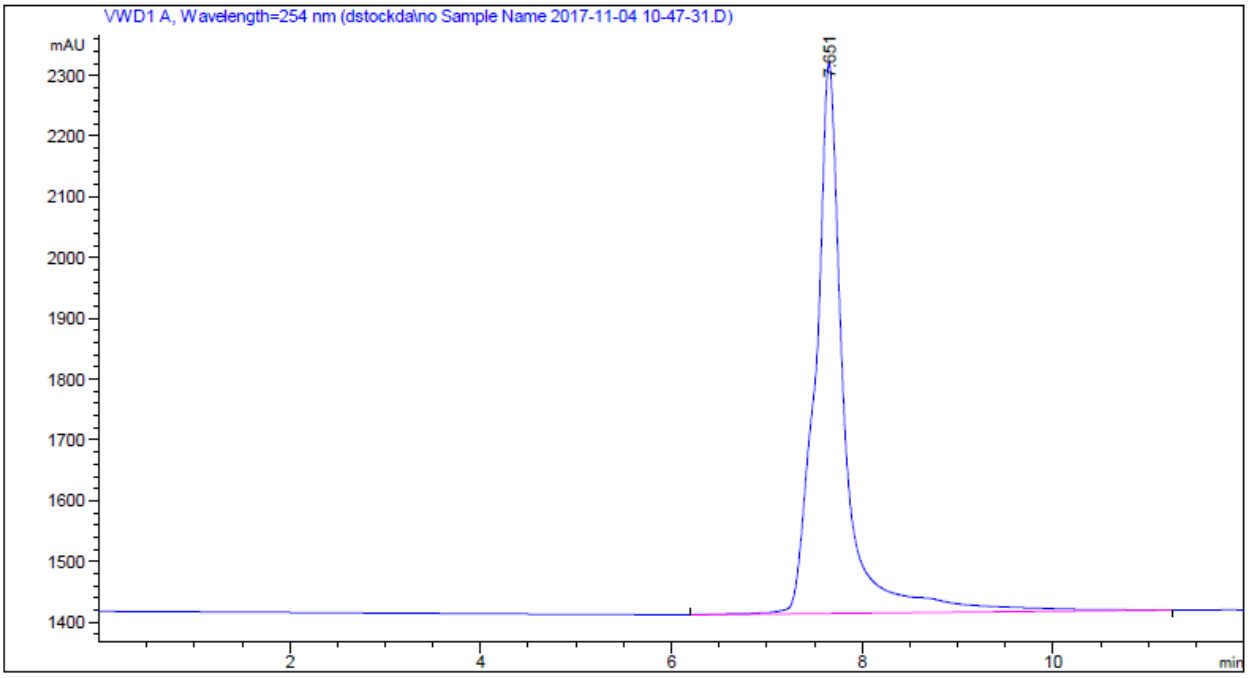


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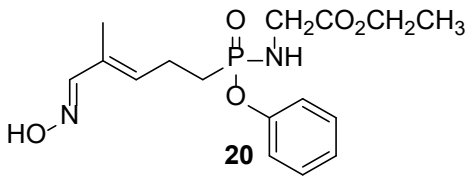
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=254 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]       [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   7.651 BB    0.2887 1.89189e4   905.86017 100.0000

Totals :                  1.89189e4   905.86017
```

HPLC Trace of compound **19**



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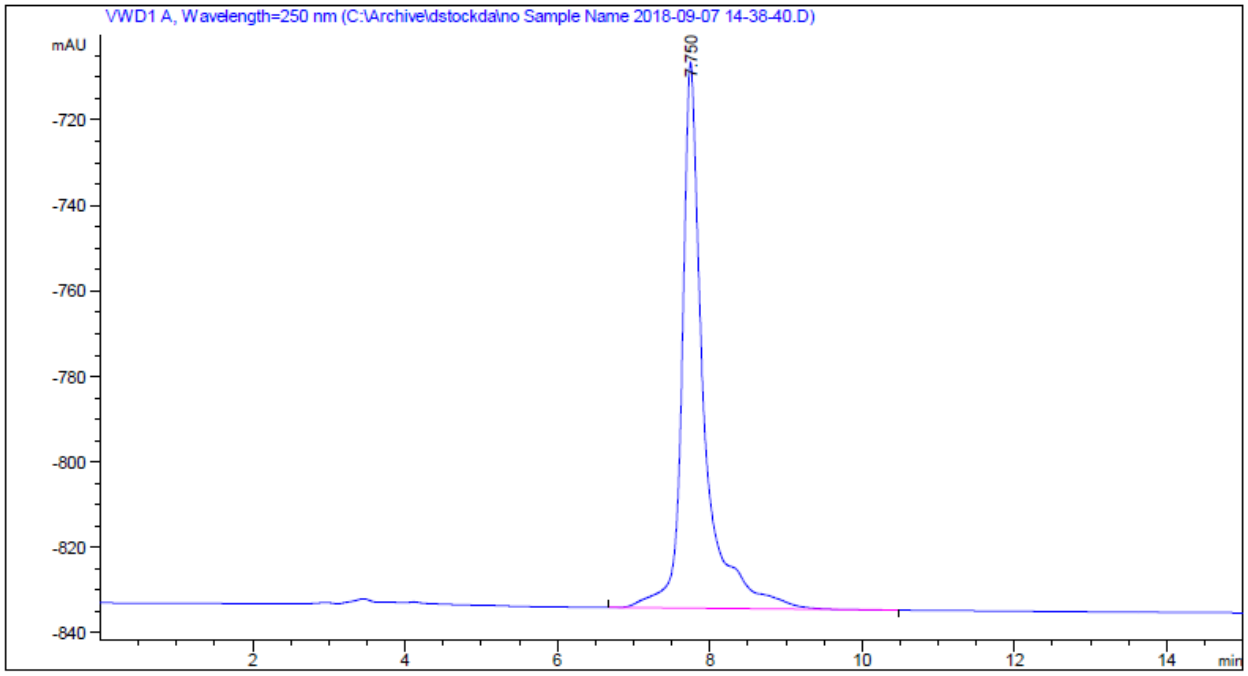


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Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs

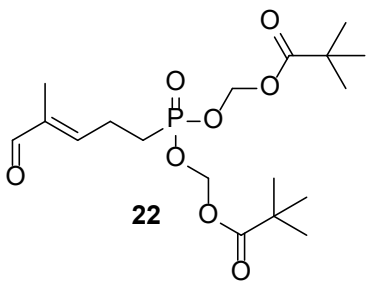

Signal 1: VWD1 A, Wavelength=250 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   7.750 BBA   0.2752 2529.31982  127.64165 100.0000

Totals :                  2529.31982  127.64165
```

HPLC Trace of compound **20**

Conditions: wavelength: 250 nm, flow rate: 2 mL/min, column: C18; column and sample temperature: ambient; Injection Volume: 100 µL; Gradient 75% ACN in H_2O to 100% ACN over 15 minutes



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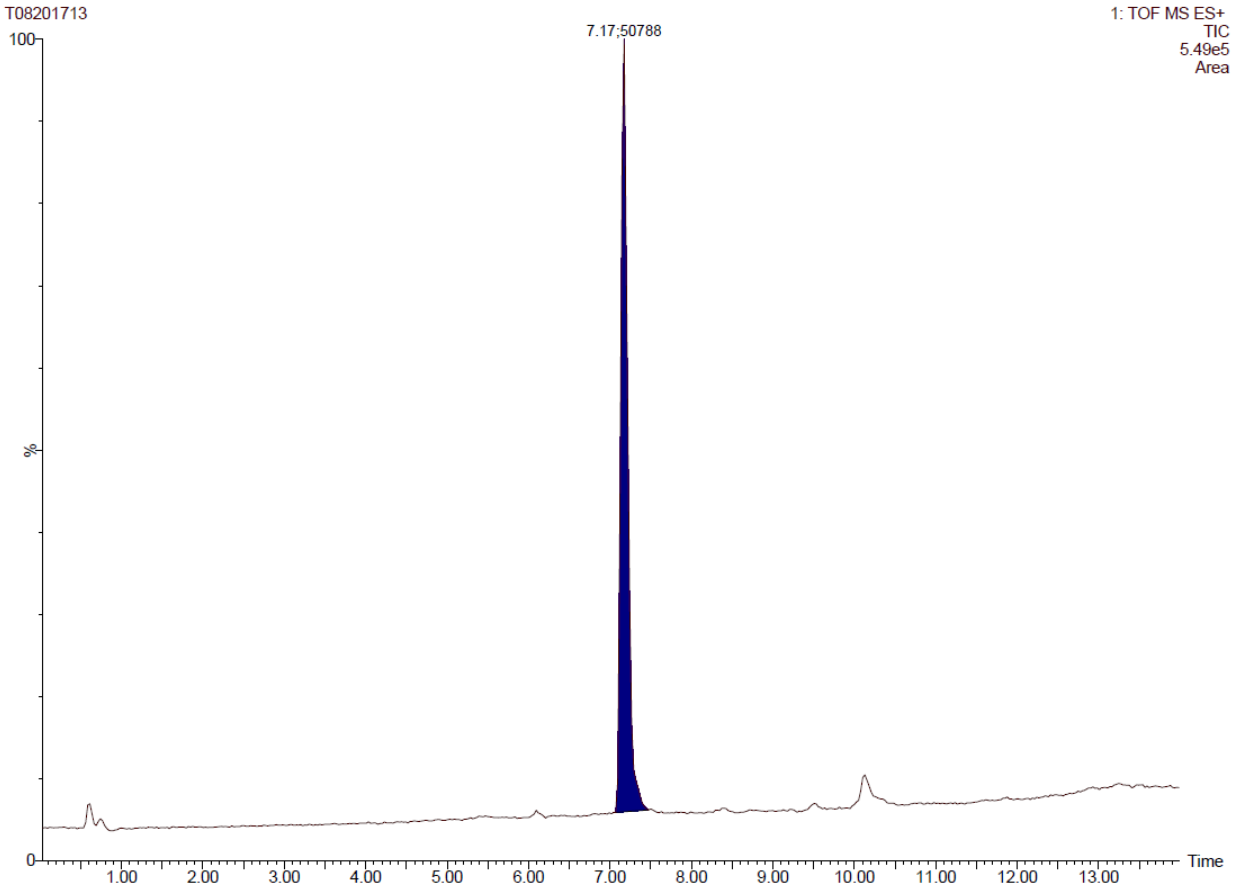
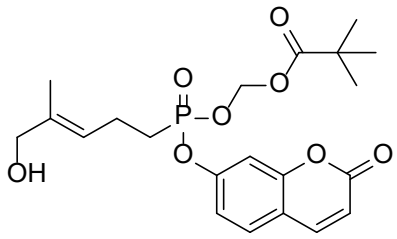


LCMS Trace of compound **22**



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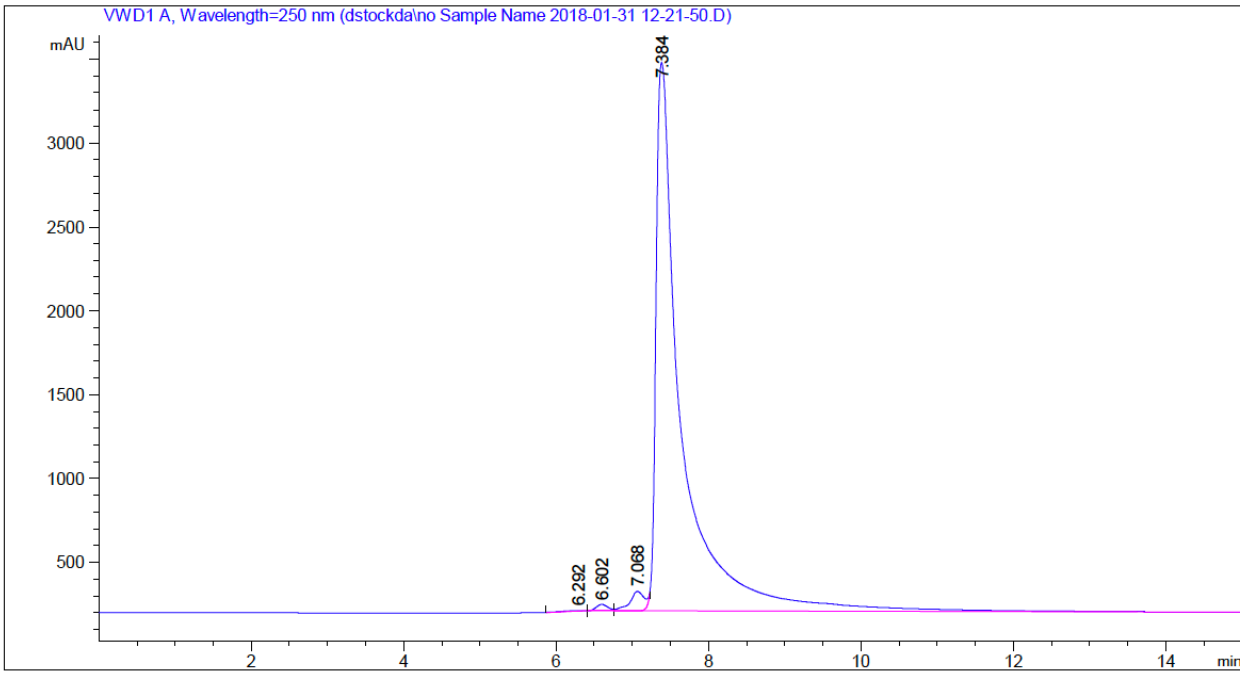


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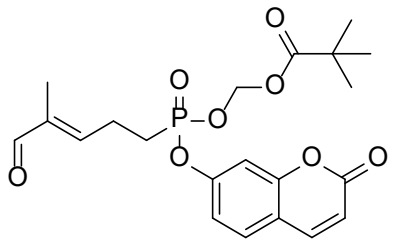
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=250 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   6.292 BB    0.2591   68.46365    4.09414   0.0866
   2   6.602 BV E  0.1655  403.47595   38.10117   0.5106
   3   7.068 VV E  0.1916 1508.82935  115.34275   1.9094
   4   7.384 VBAR  0.3229 7.70403e4  3270.03516  97.4934
```

LCMS Trace of compound **27**



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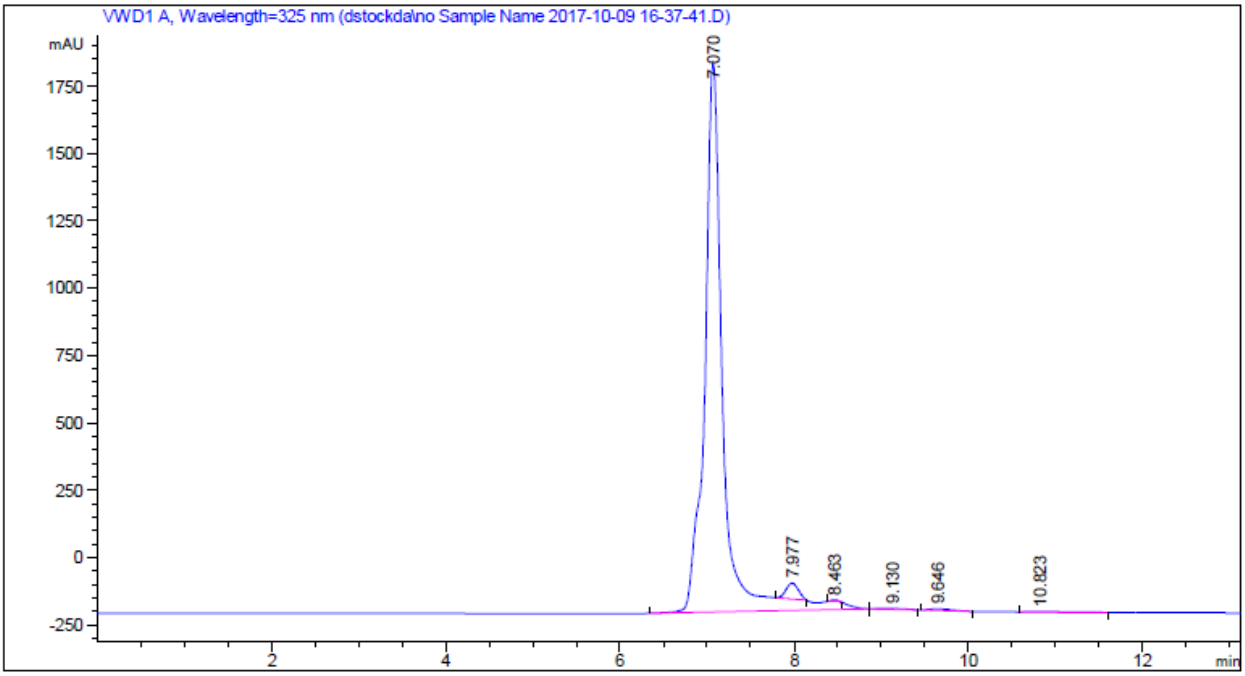


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Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=325 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   7.070 BV R  0.2098 2.96535e4  2037.88367  97.3431
   2   7.977 VV E  0.1563  583.06189   59.01612   1.9140
   3   8.463 VB E  0.1147   37.90584    6.43895   0.1244
   4   9.130 BB    0.2900   51.33621    2.65041   0.1685
   5   9.646 BB    0.2335   91.29733    5.65109   0.2997
   6  10.823 BBA   0.3345   45.75524    1.98826   0.1502
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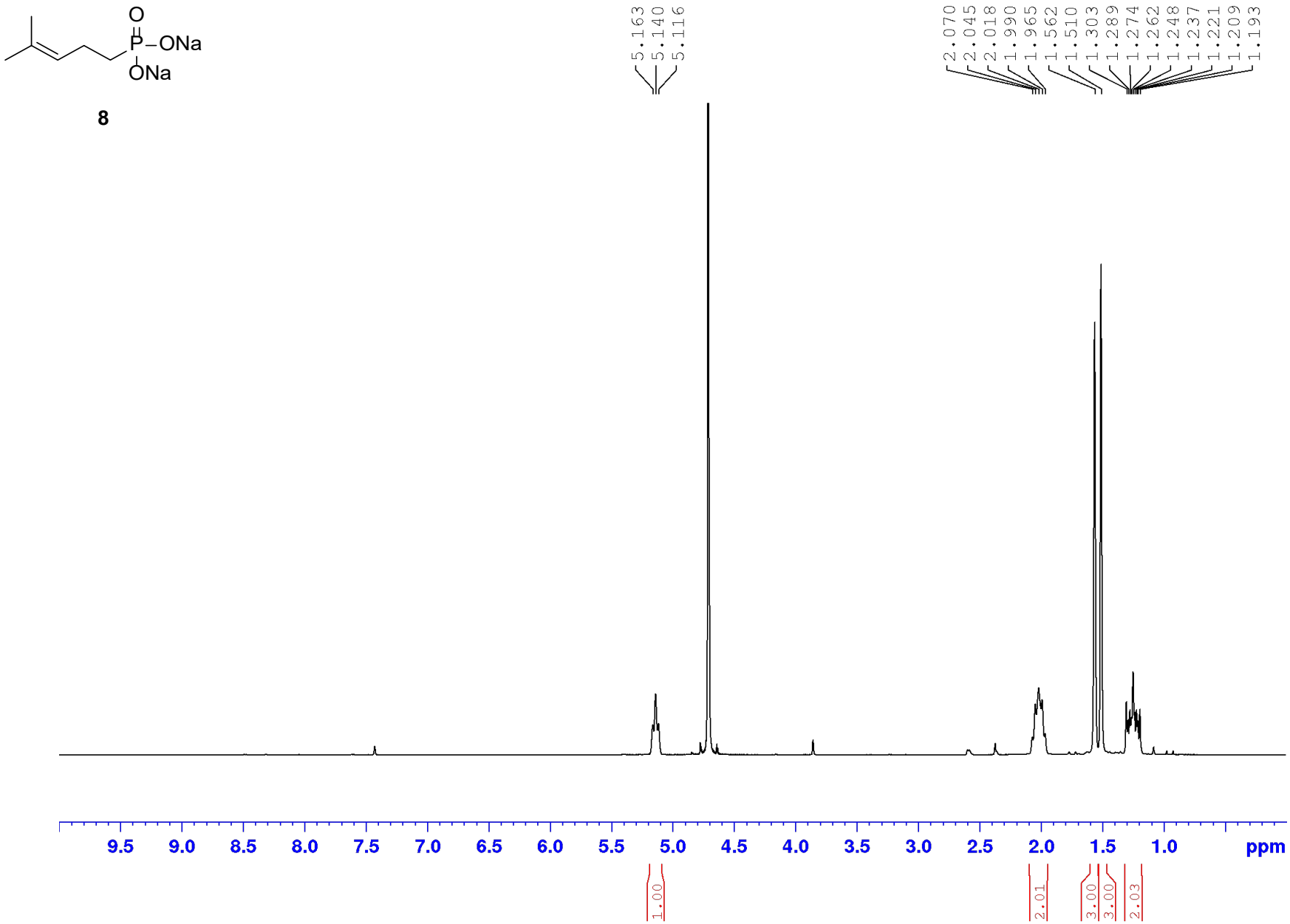
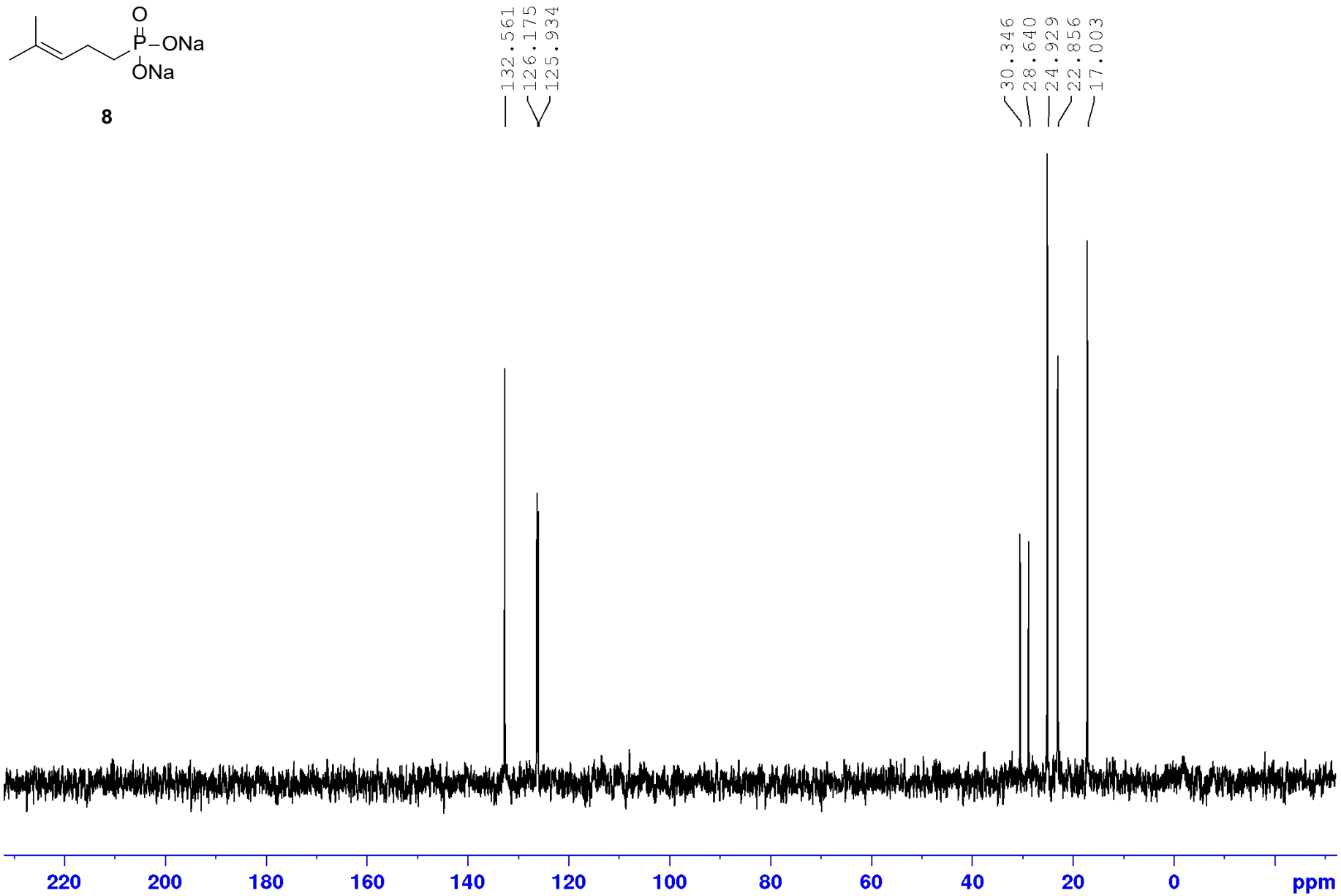
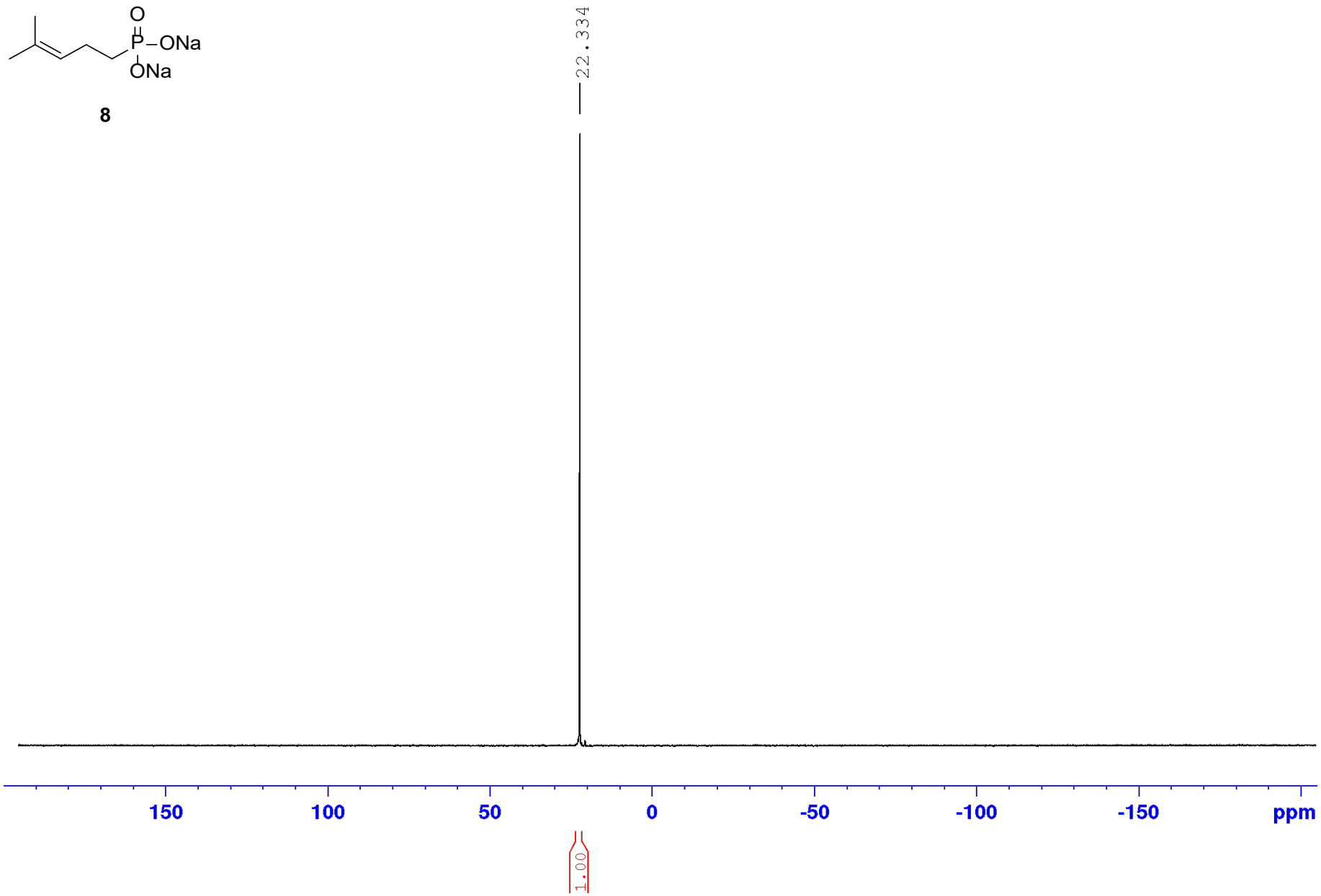
HPLC Trace of compound **28**



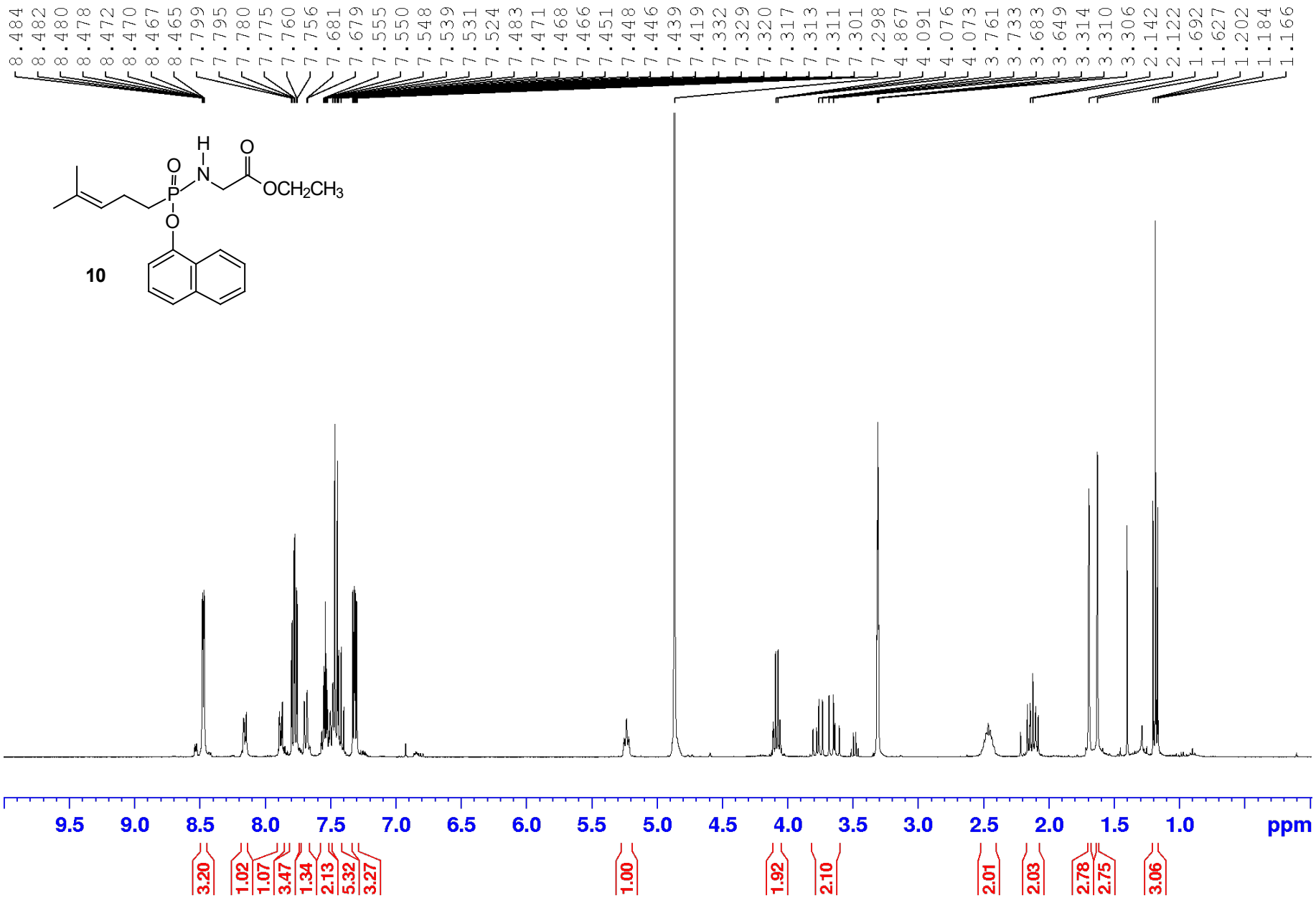
300 MHz ^{1}H NMR spectrum of compound **8** (D_2O)



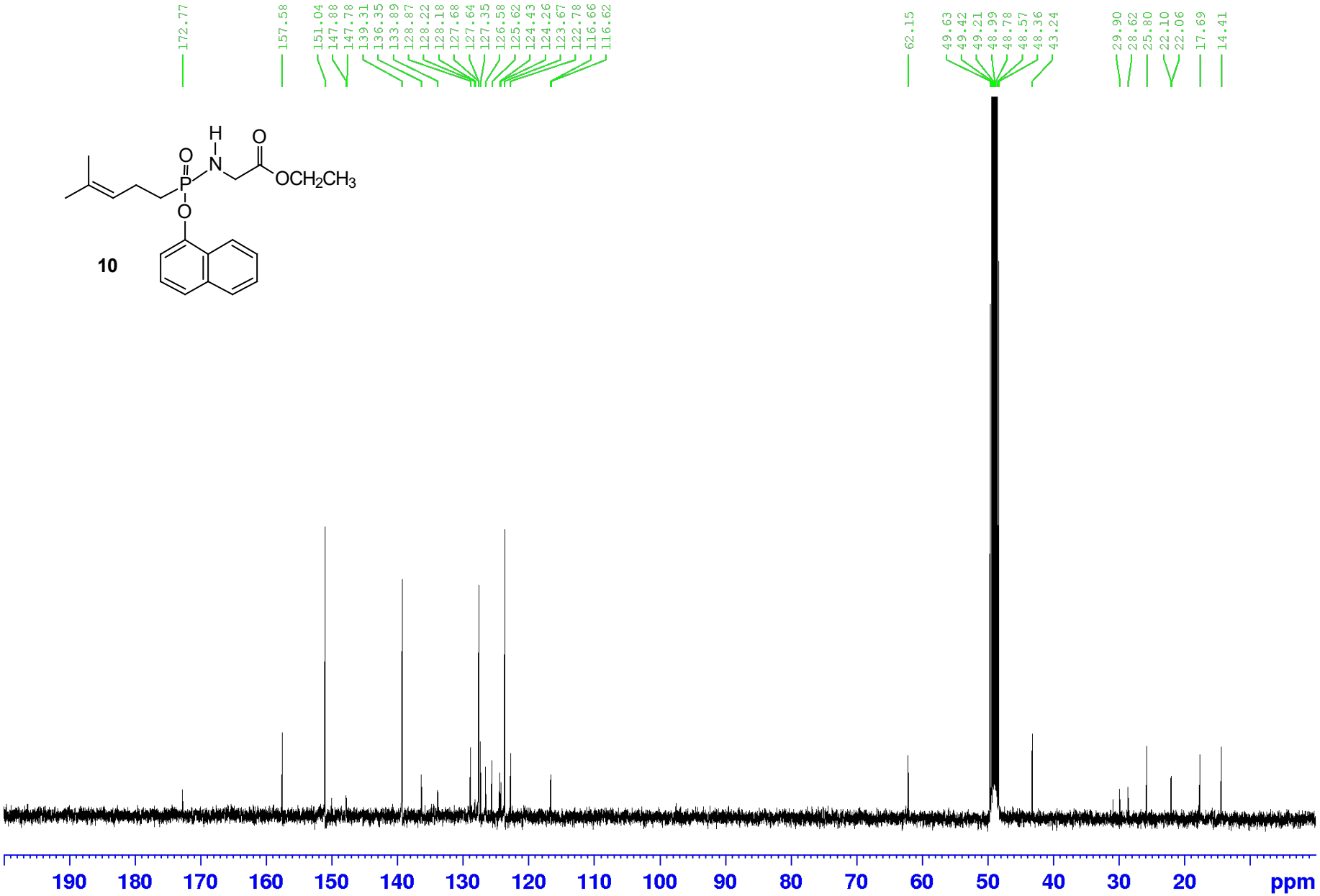
75 MHz ^{13}C NMR spectrum of compound **8** (D_2O)



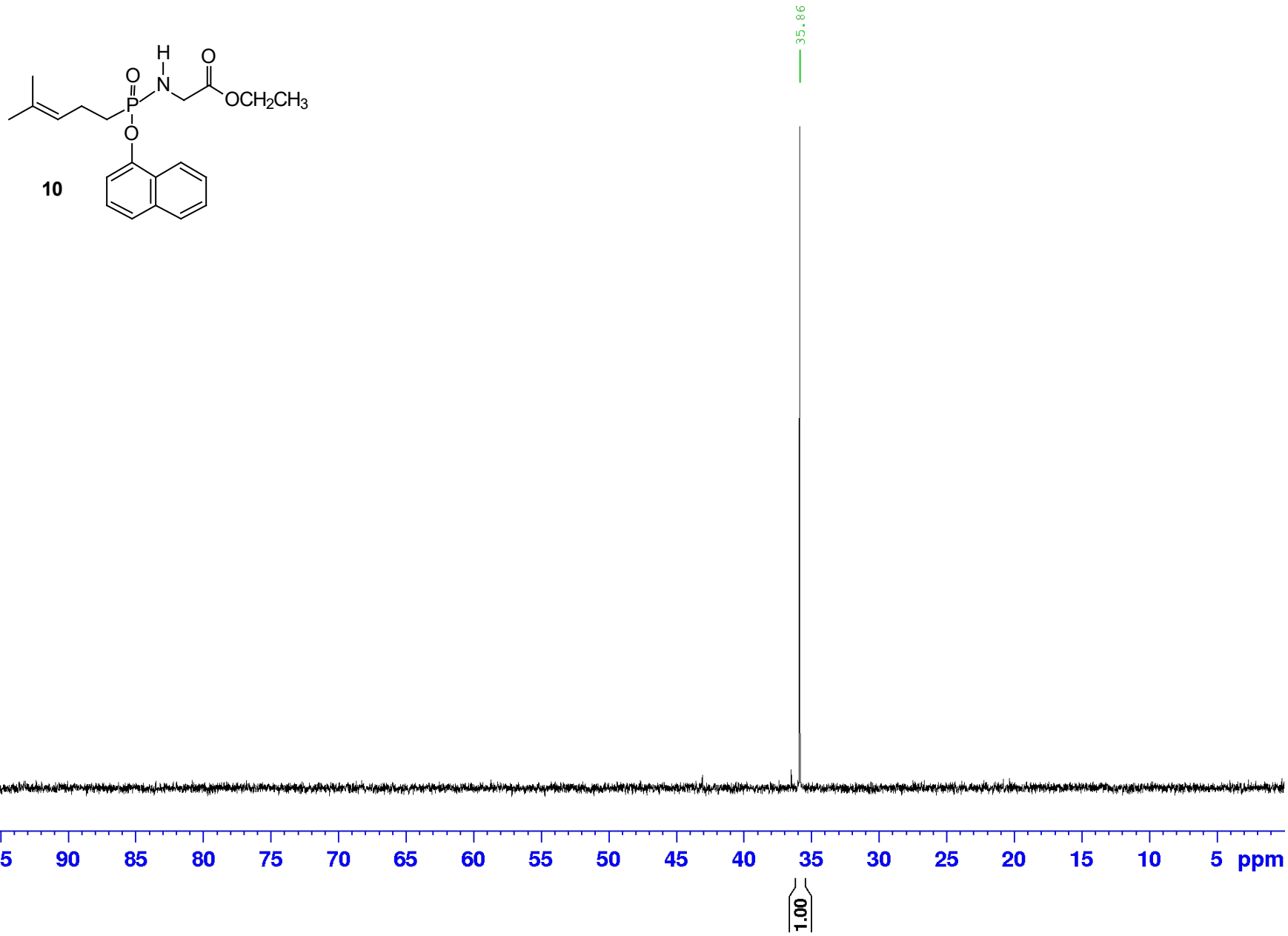
121 MHz ^{31}P NMR spectrum of compound **8** (D_2O)



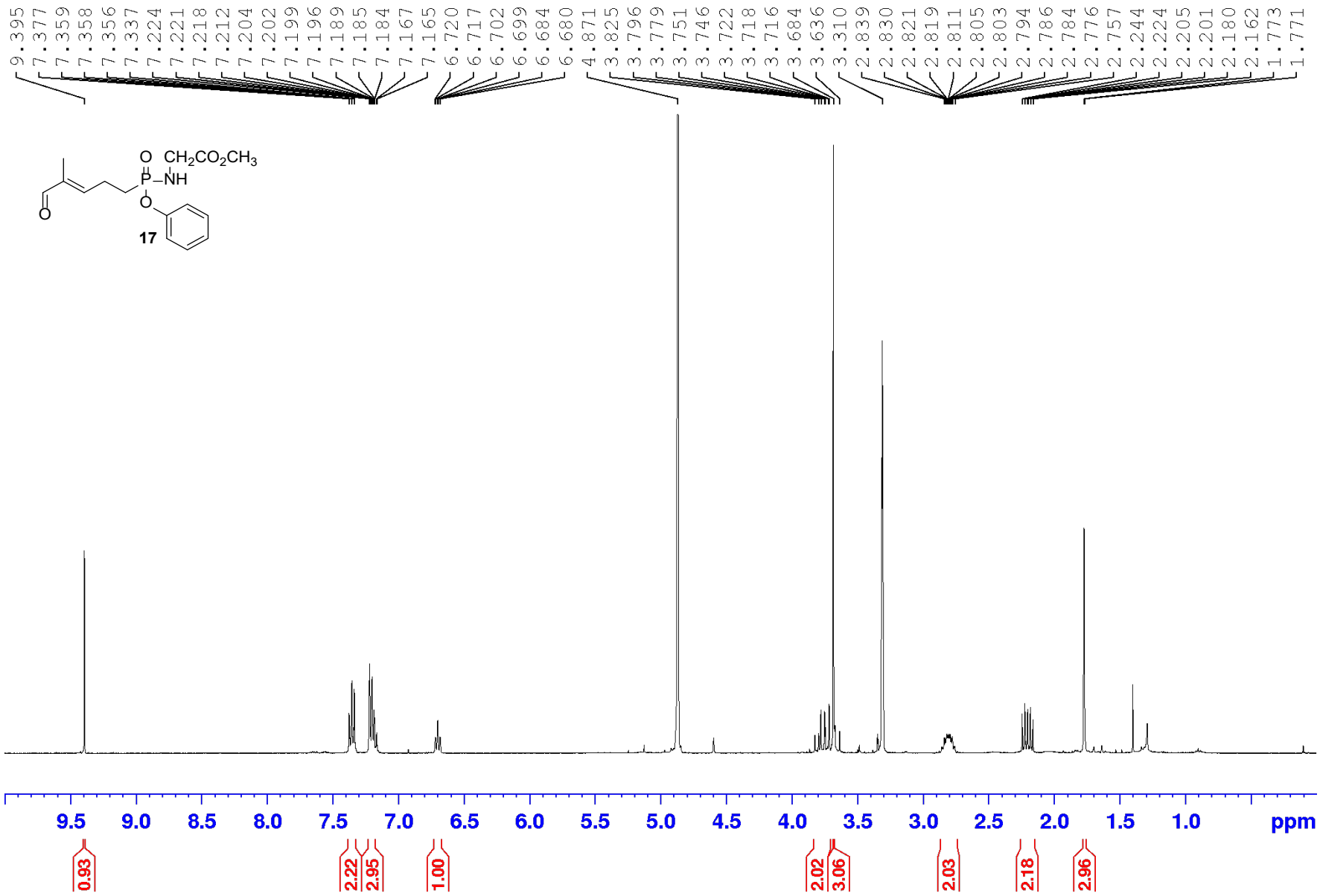
400 MHz ^{1}H NMR spectrum of compound **10** (CD_3OD)



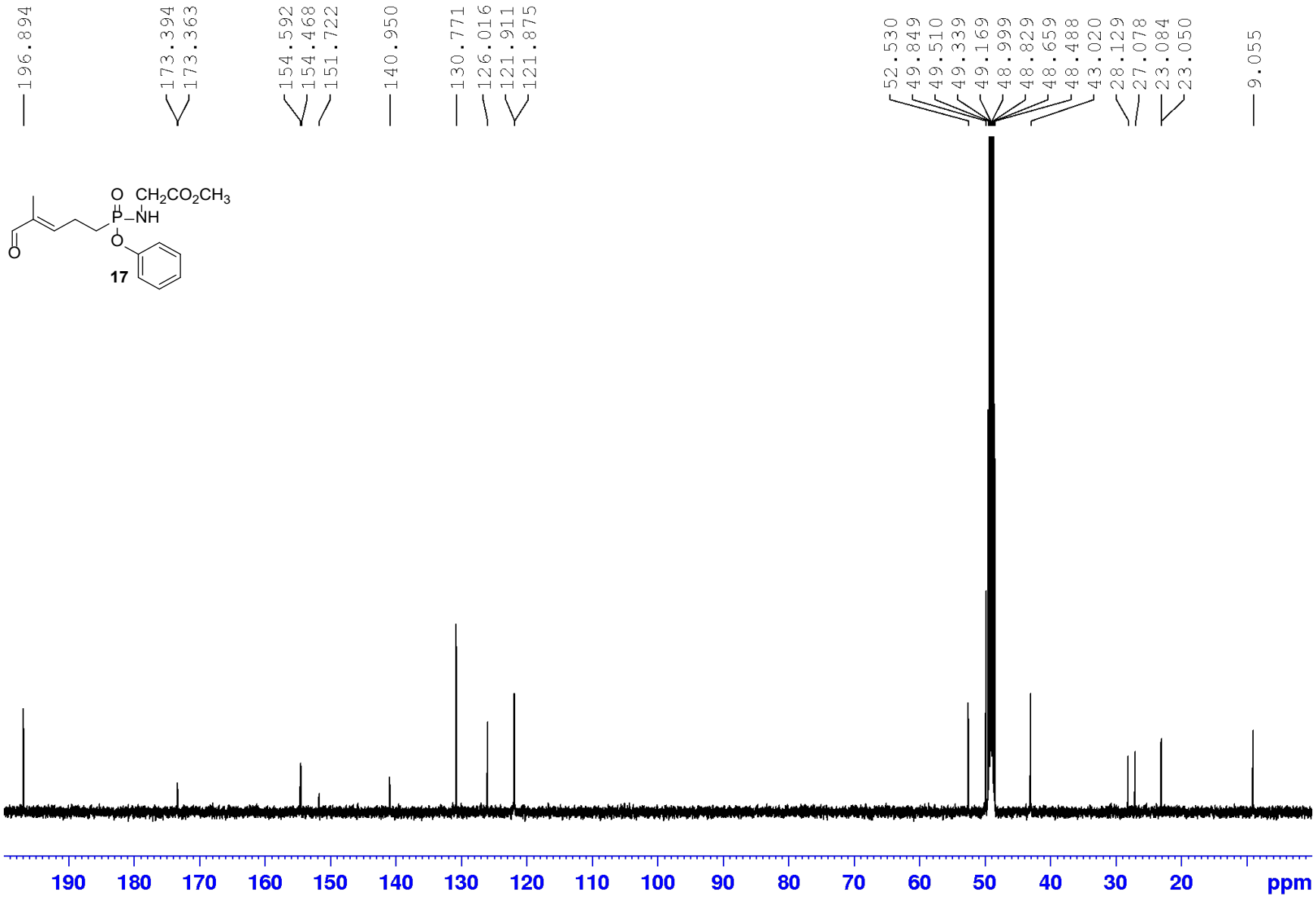
100 MHz ^{13}C NMR spectrum of compound **10** (CD_3OD)



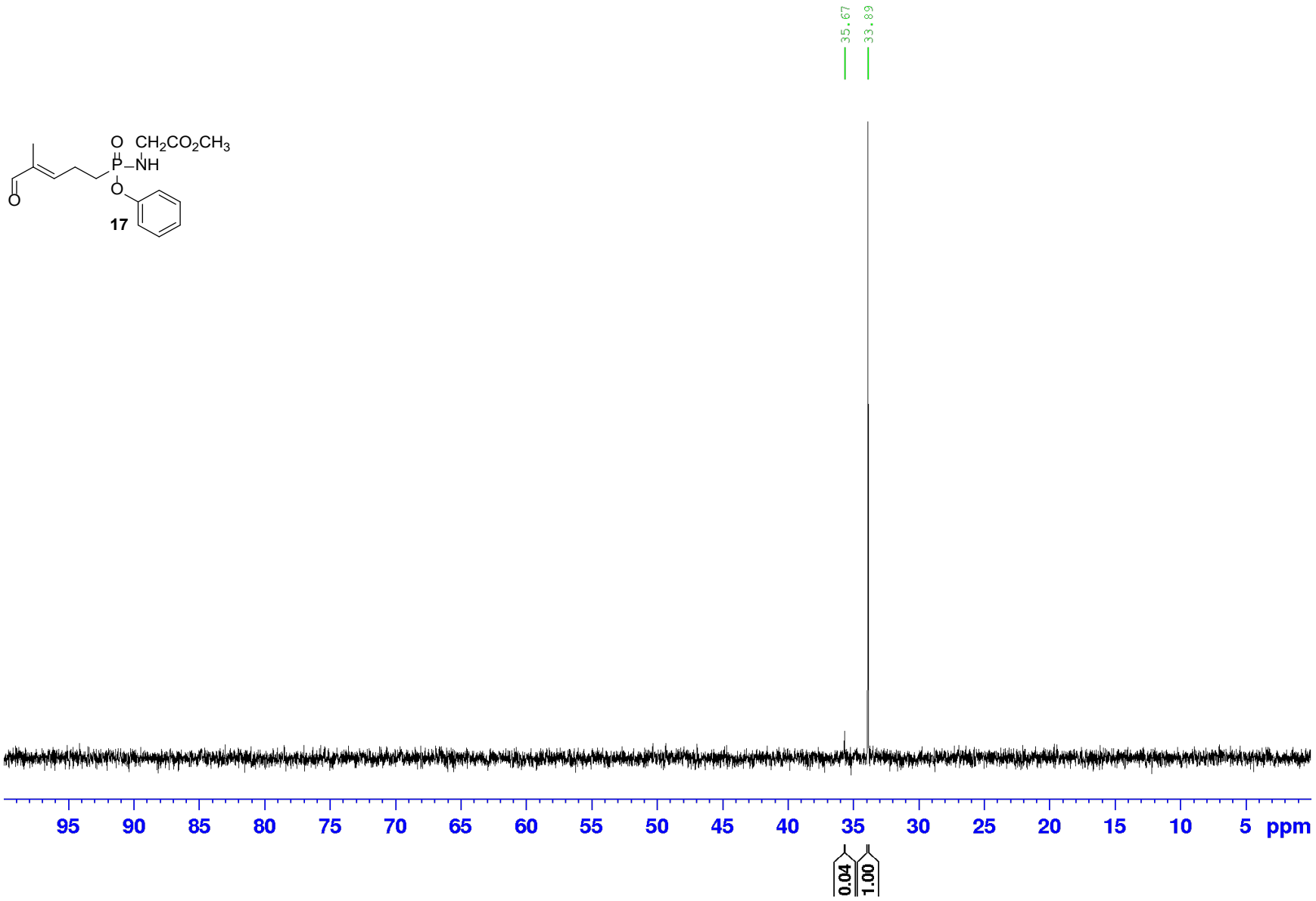
161 MHz ^{31}P NMR spectrum of compound **10** (CD_3OD)



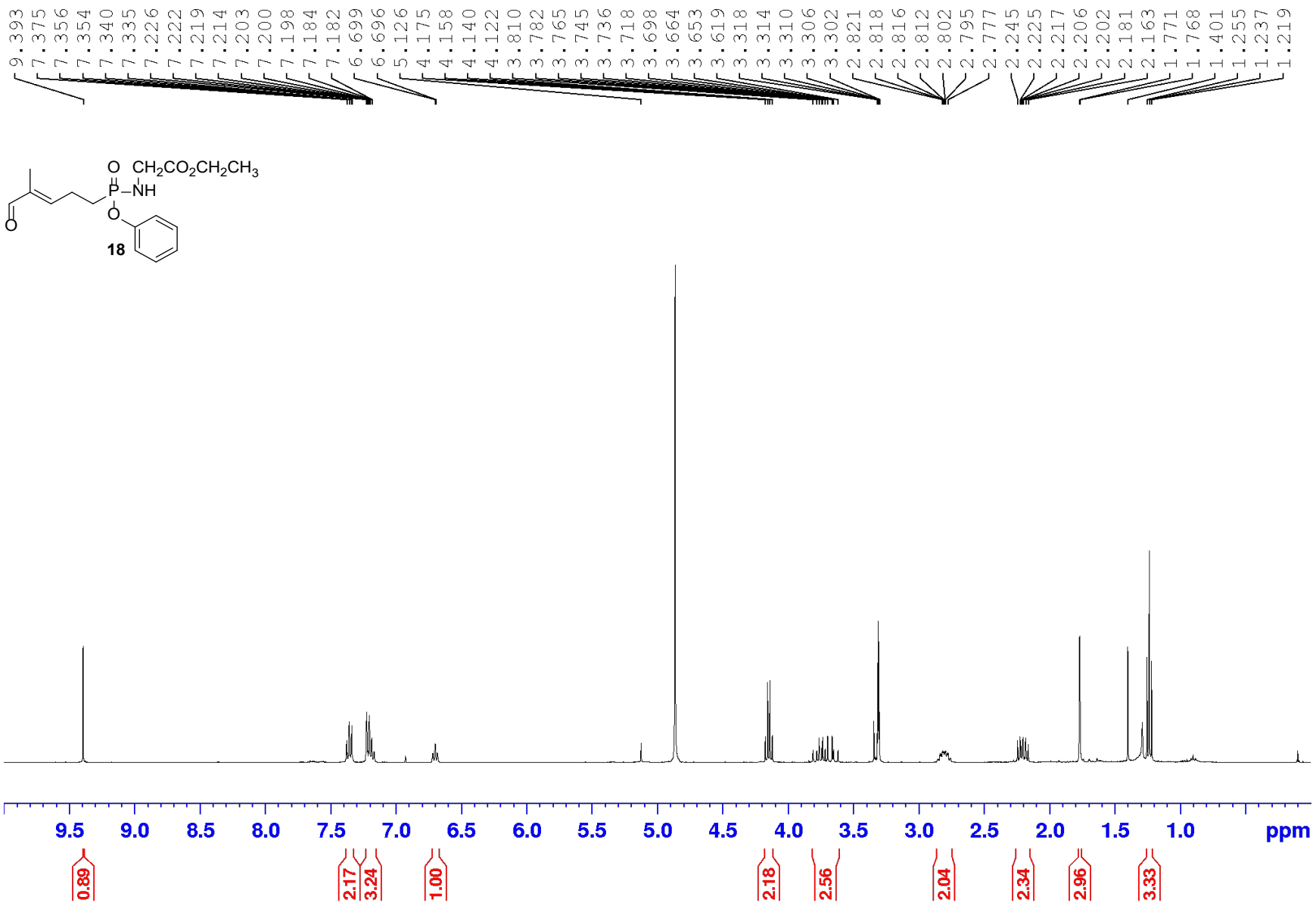
400 MHz ^{1}H NMR spectrum of compound **17** (CD_3OD)



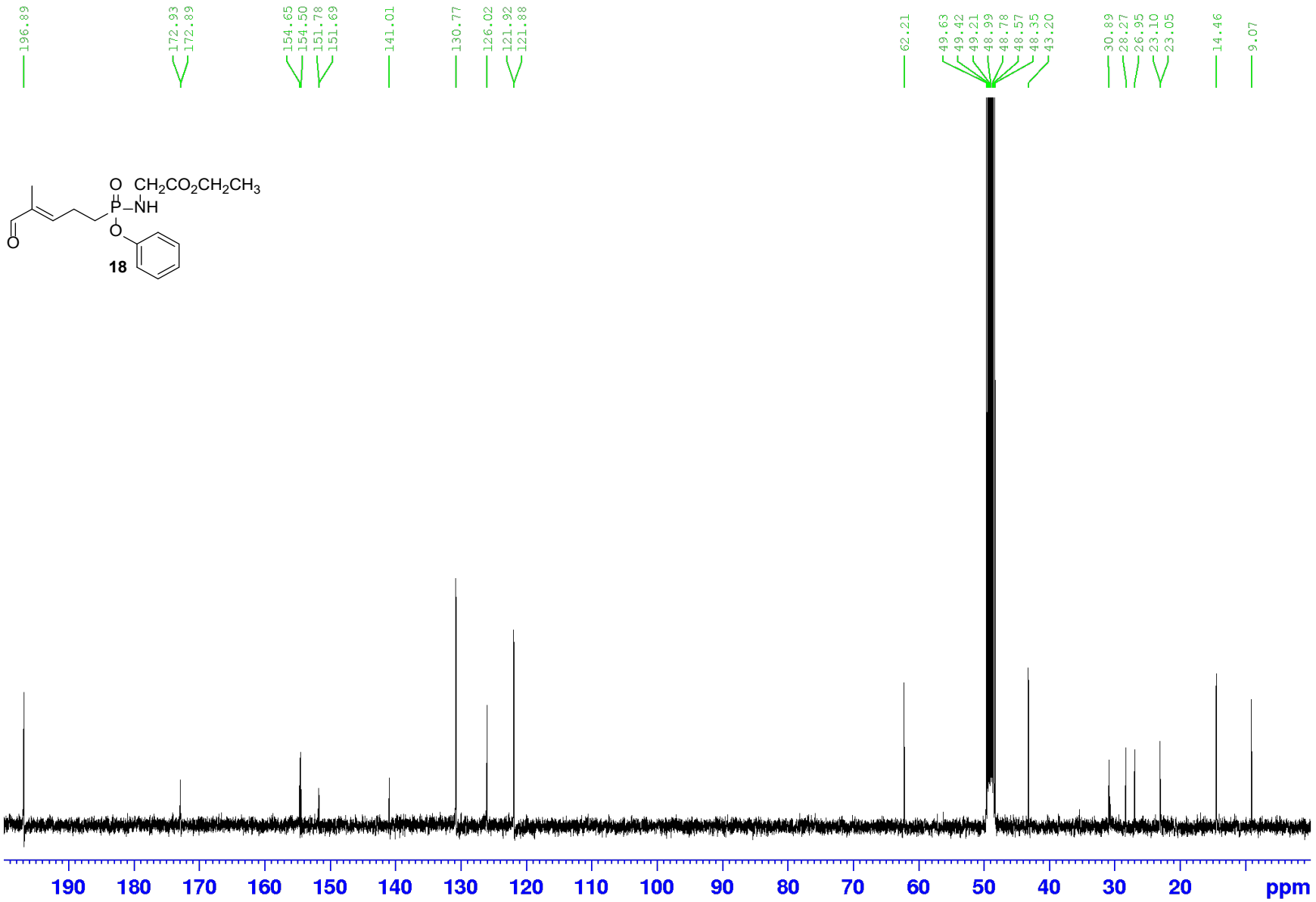
125 MHz ^{13}C NMR spectrum of compound **17** (CD_3OD)



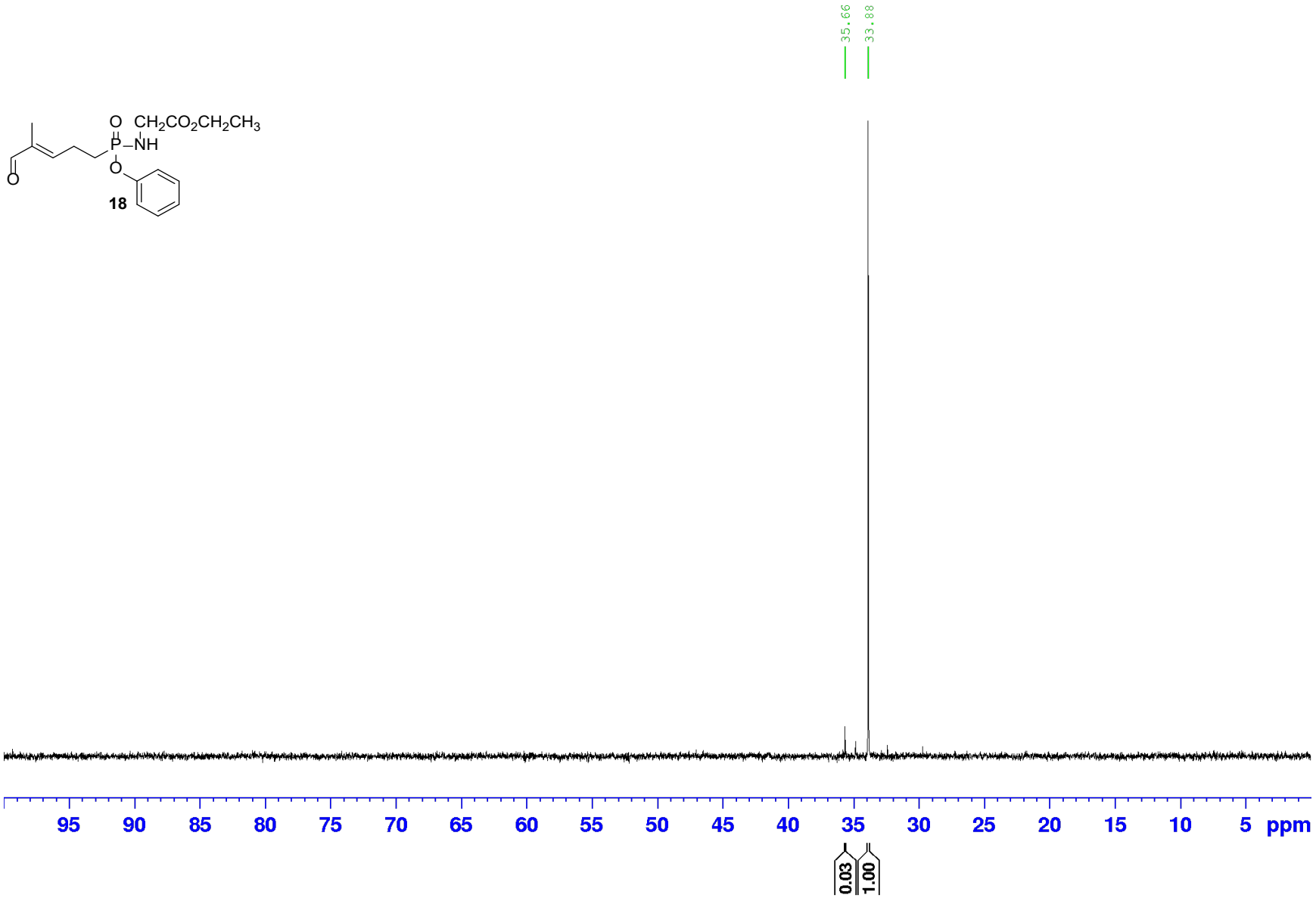
161 MHz ^{31}P NMR spectrum of compound **17** (CD_3OD)



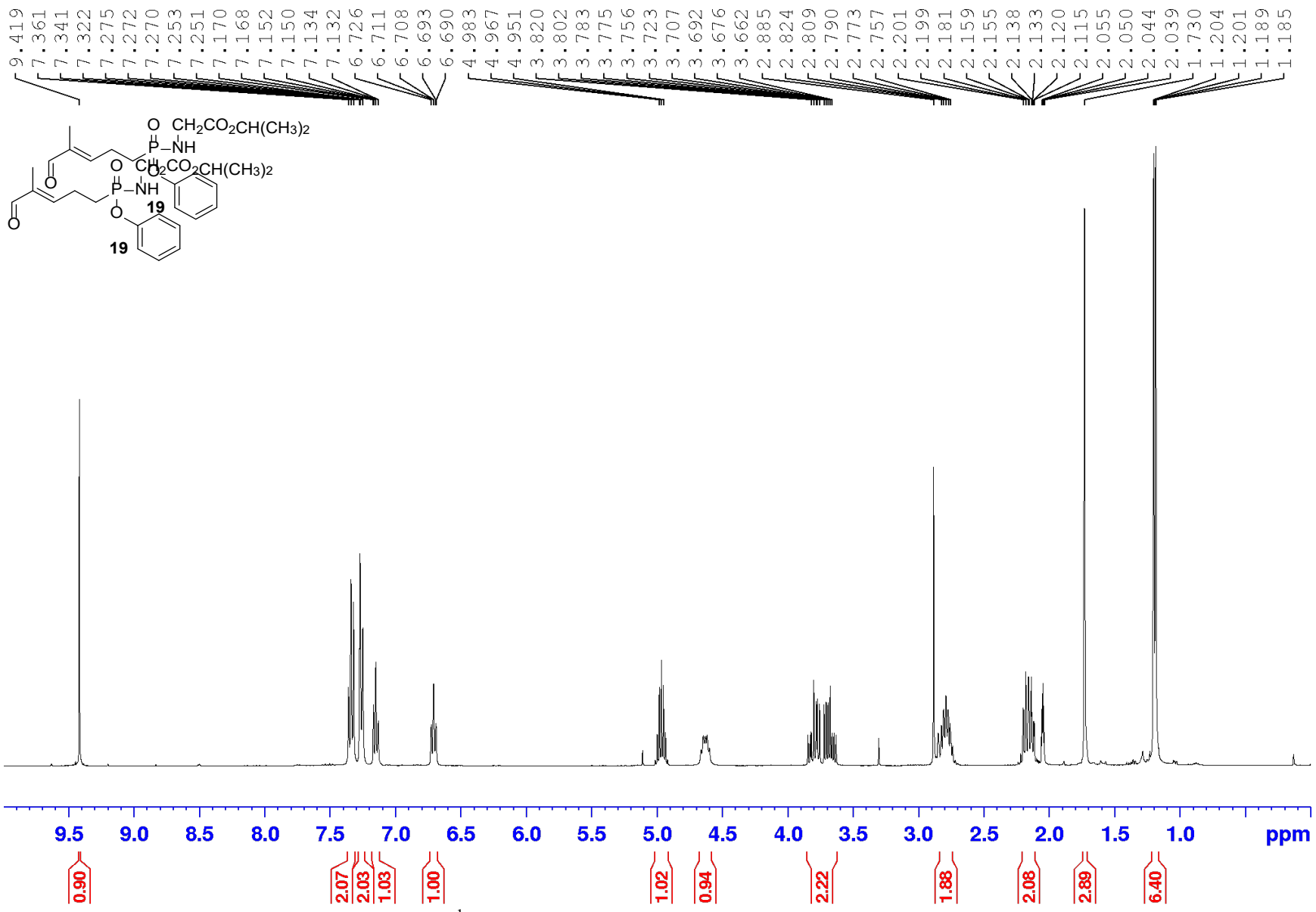
400 MHz ^{1}H NMR spectrum of compound **18** (CD_3OD)



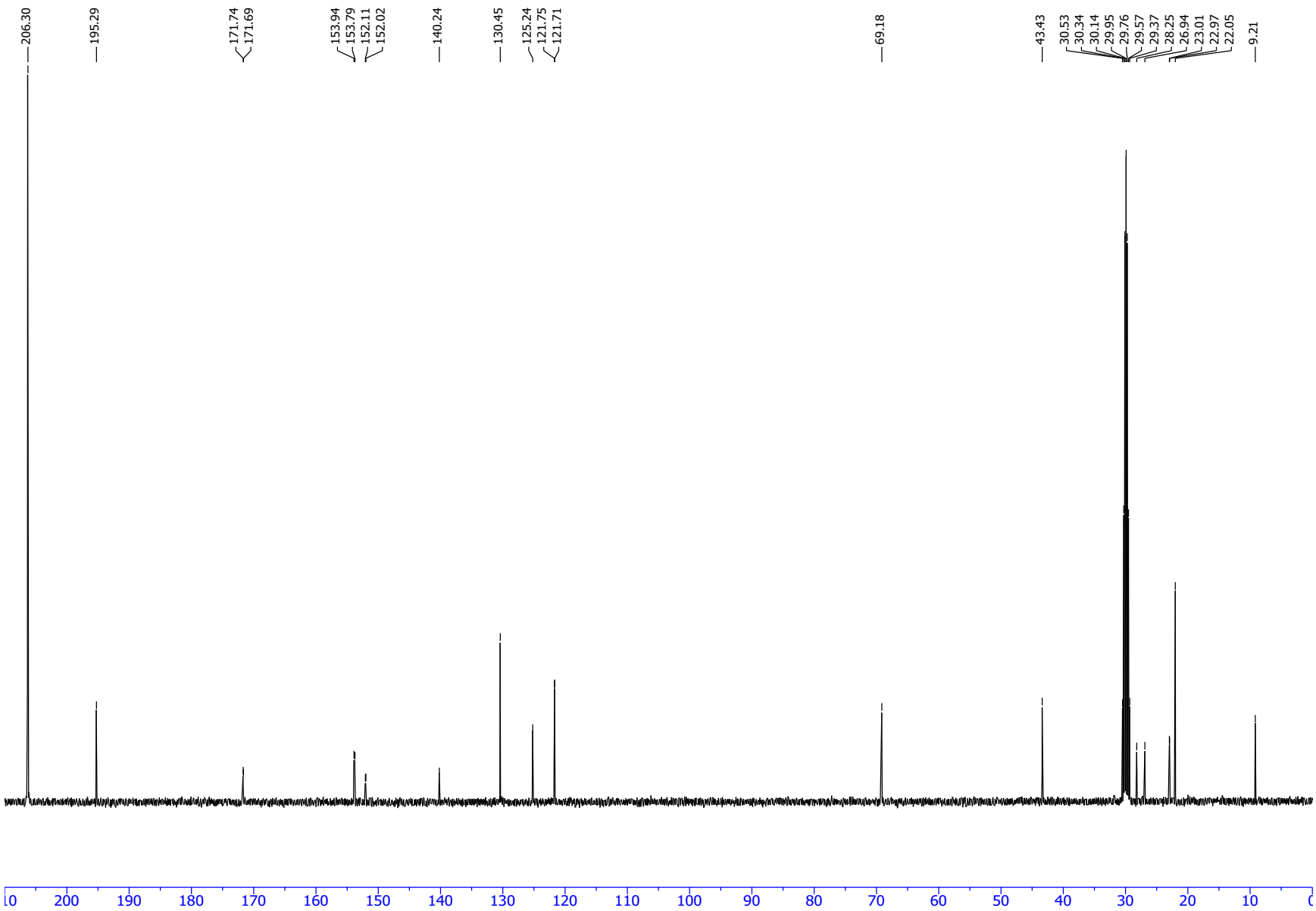
100 MHz ^{13}C NMR spectrum of compound **18** (CD_3OD)



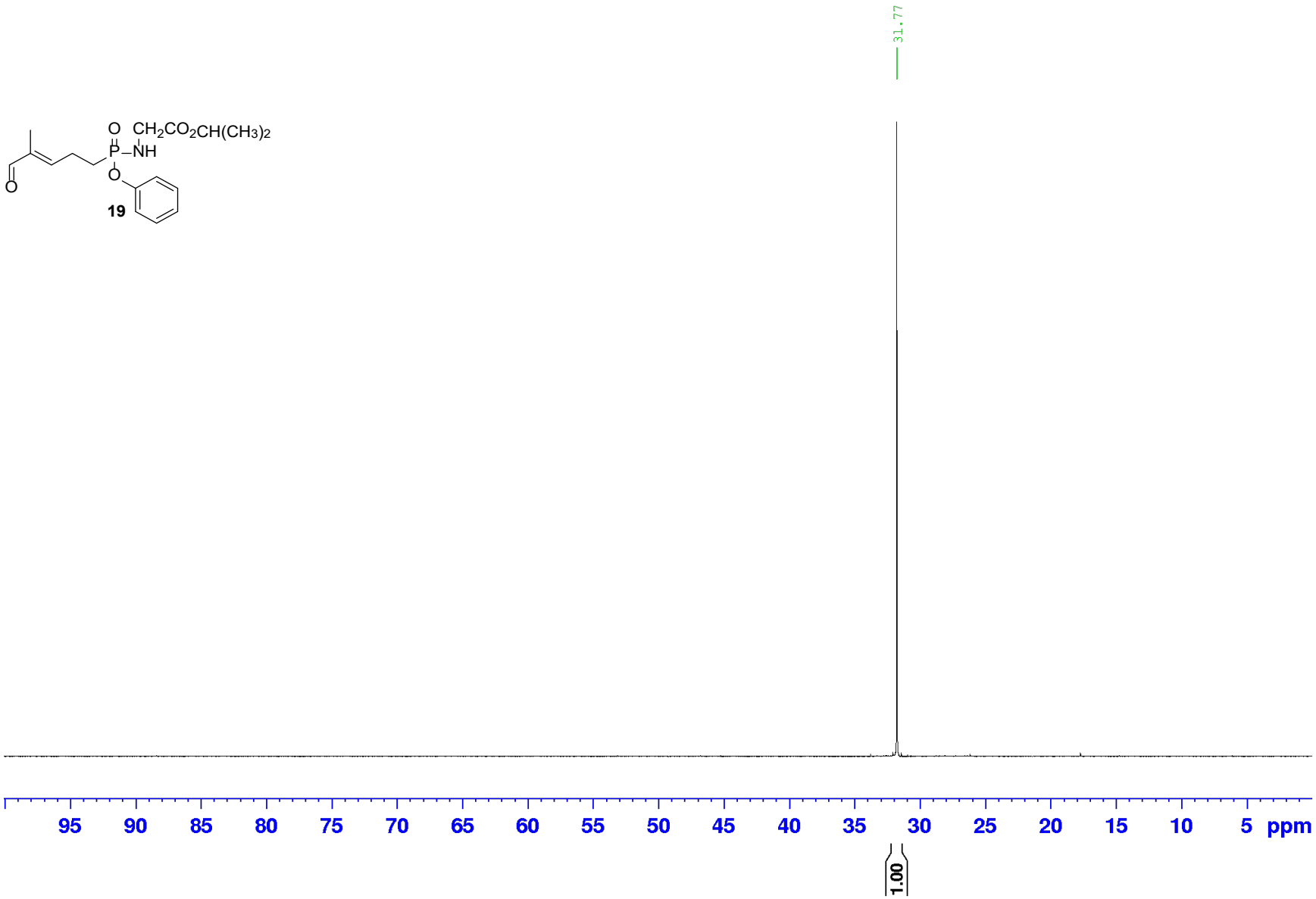
161 MHz ^{31}P NMR spectrum of compound **18** (CD_3OD)



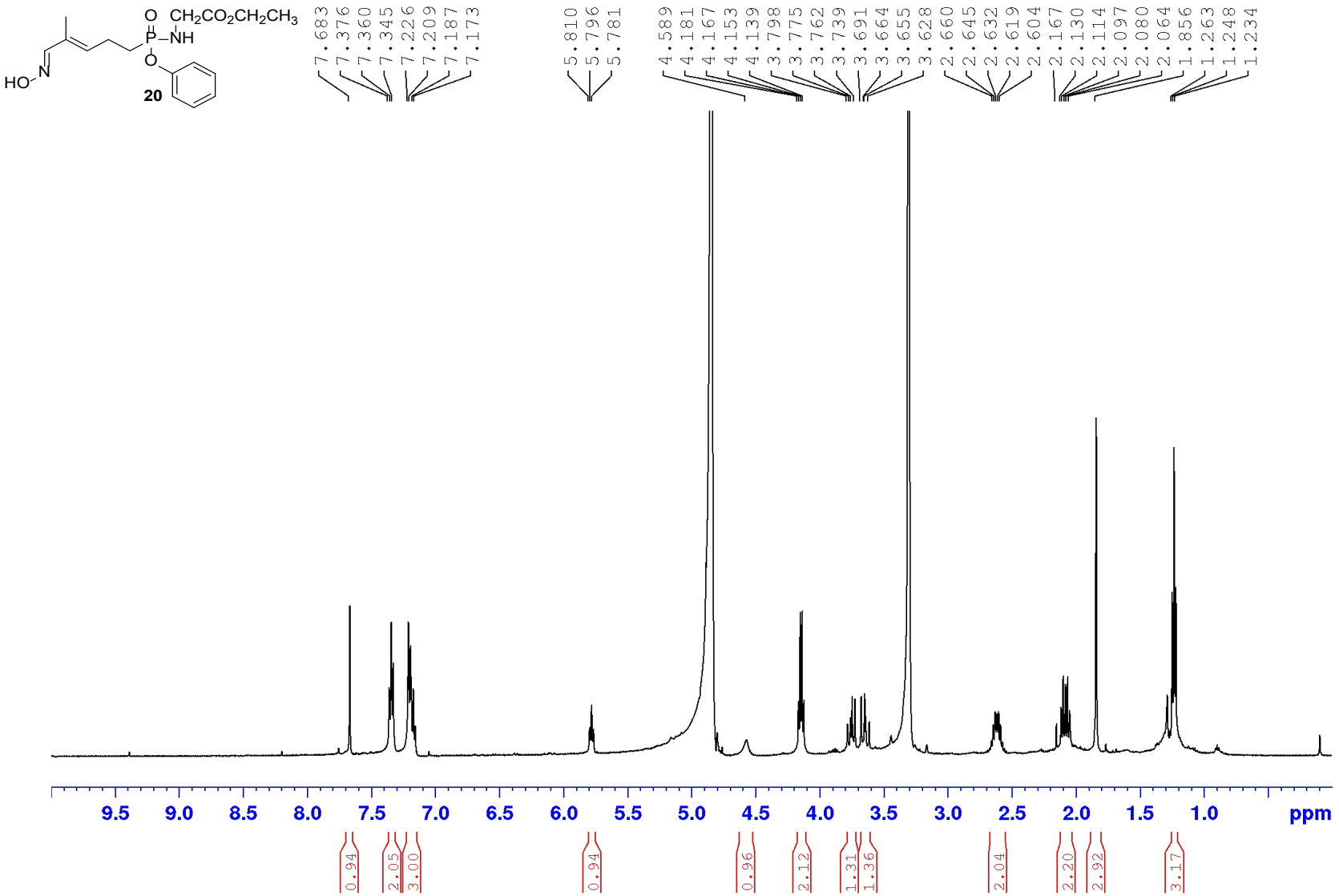
400 MHz ^{1}H NMR spectrum of compound **19** ($CD_3C(O)CD_3$)



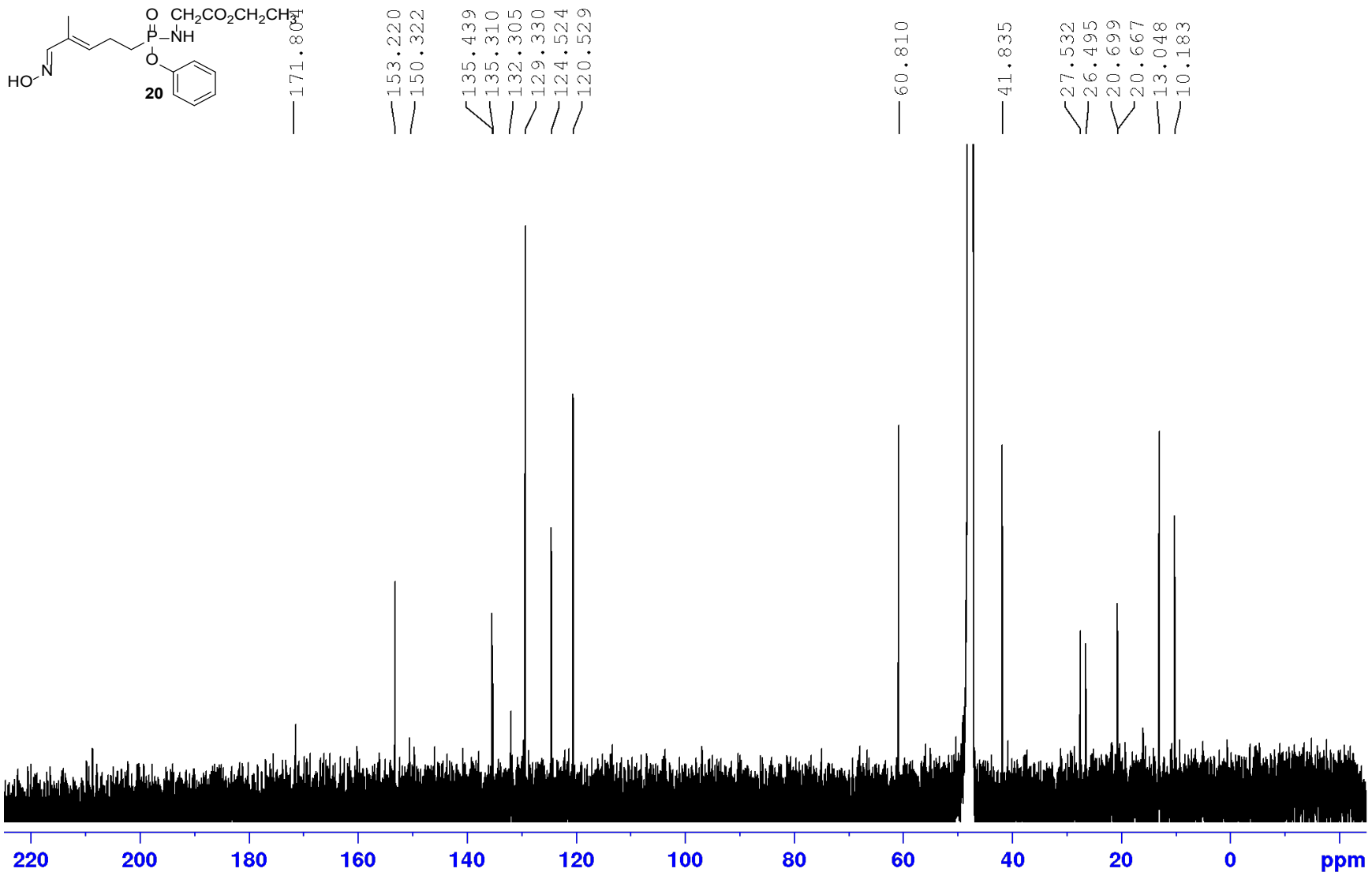
100 MHz ^{13}C NMR spectrum of compound **19** ($CD_3C(O)CD_3$)



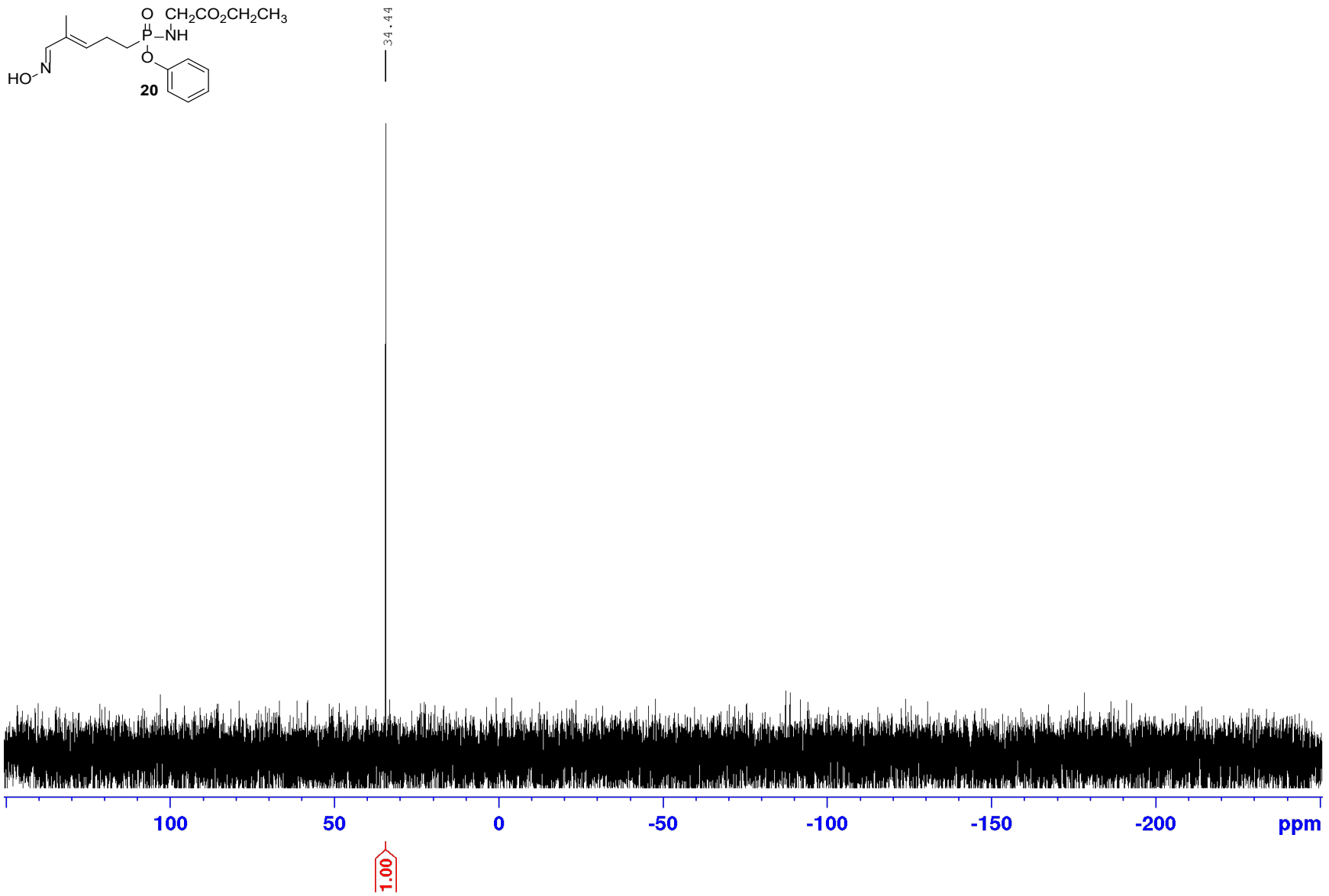
202 MHz ^{31}P NMR spectrum of compound **19** (CD_3CN)



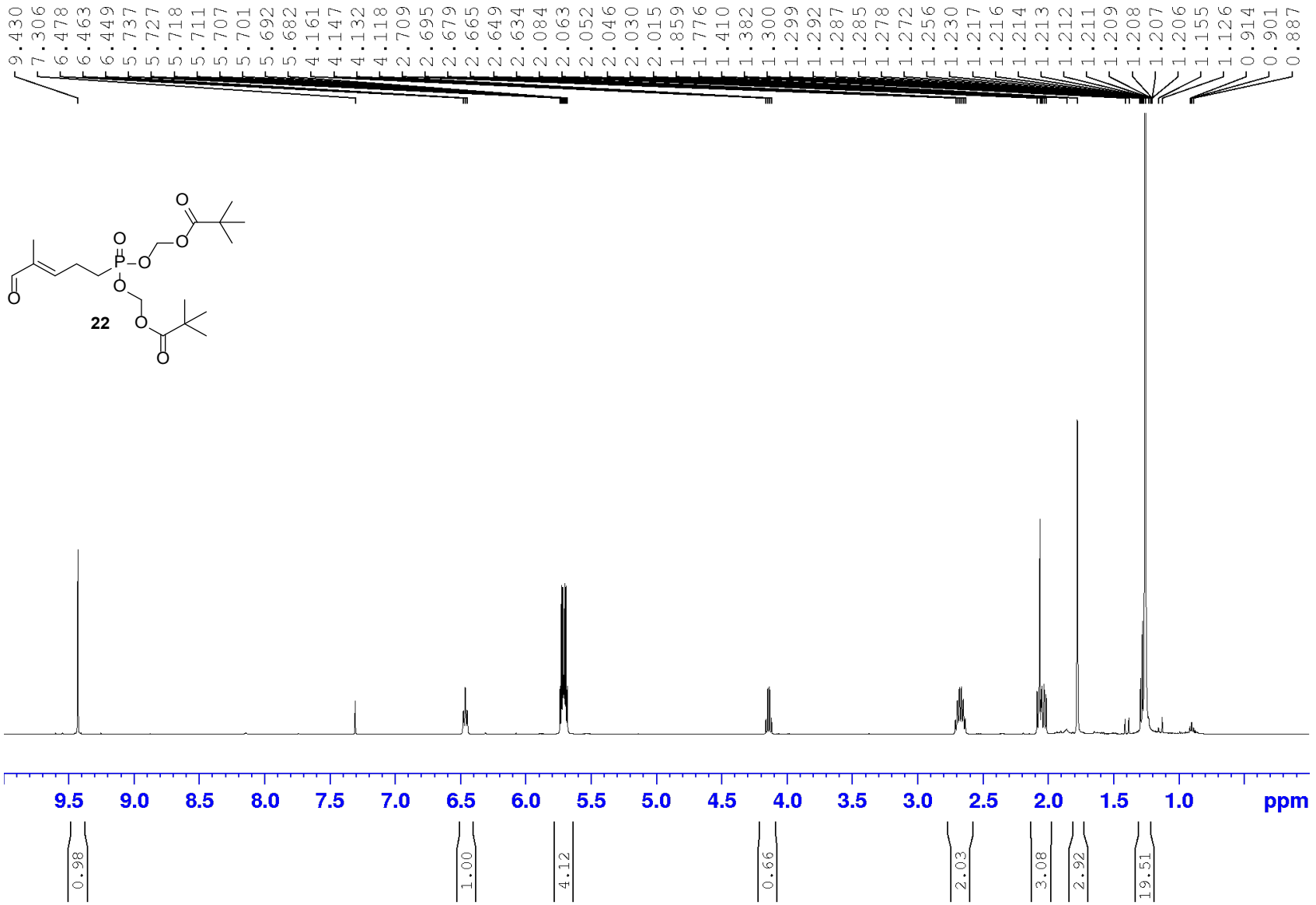
400 MHz ^{1}H NMR spectrum of compound **20** (CD_3OD)



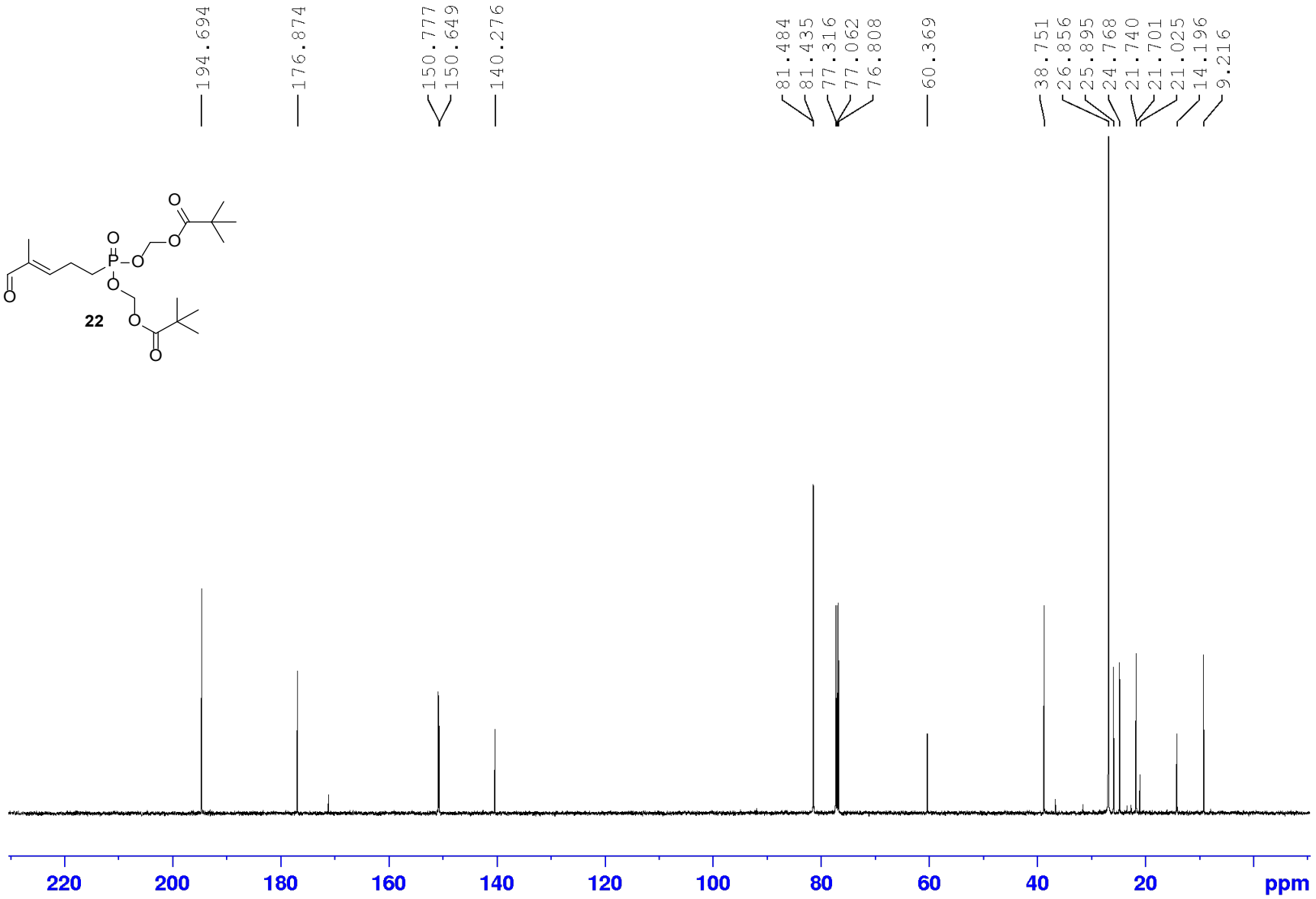
125 MHz ^{13}C NMR spectrum of compound **20** (CD_3OD)



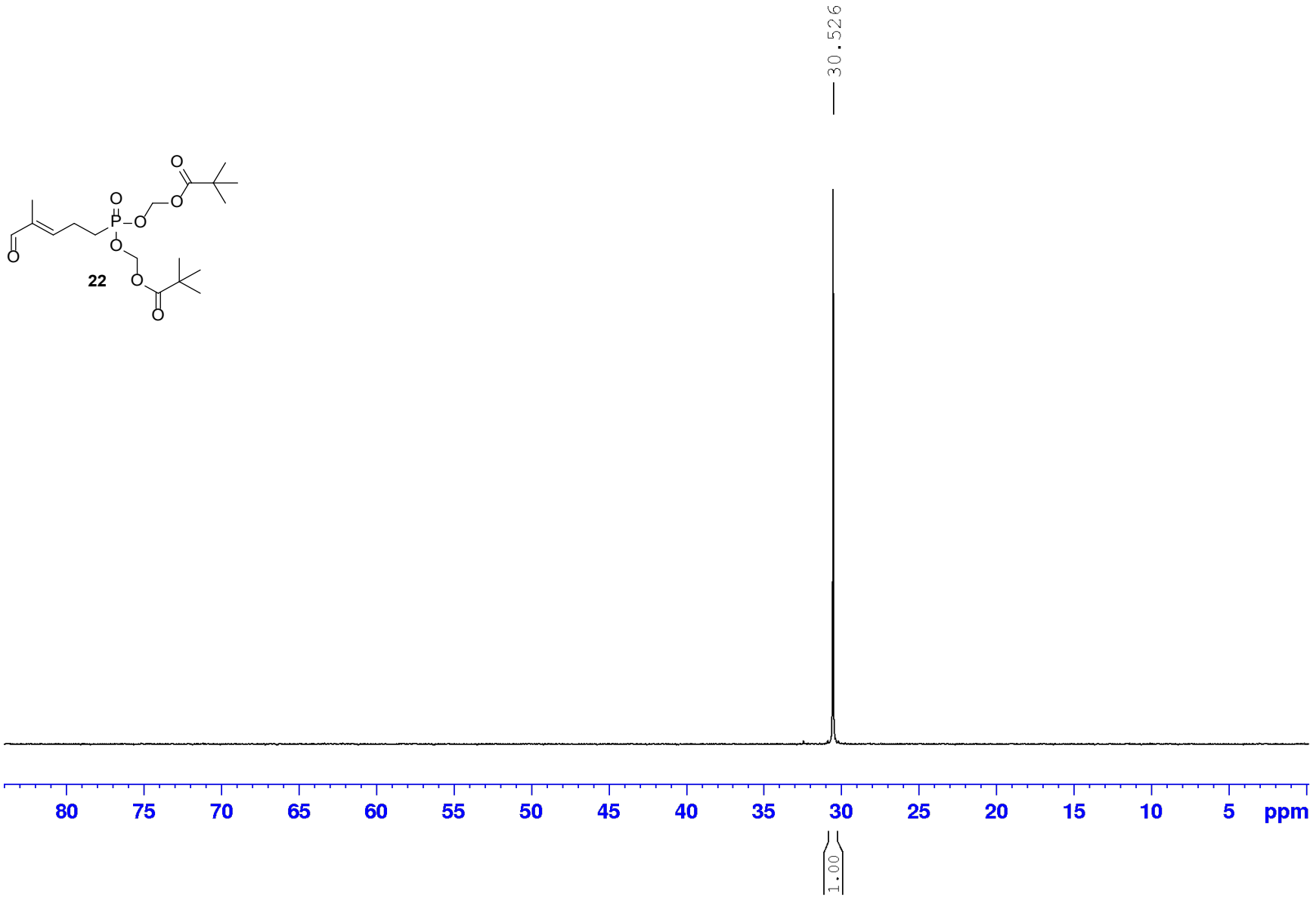
202 MHz ^{31}P NMR spectrum of compound **20** (CD_3OD)



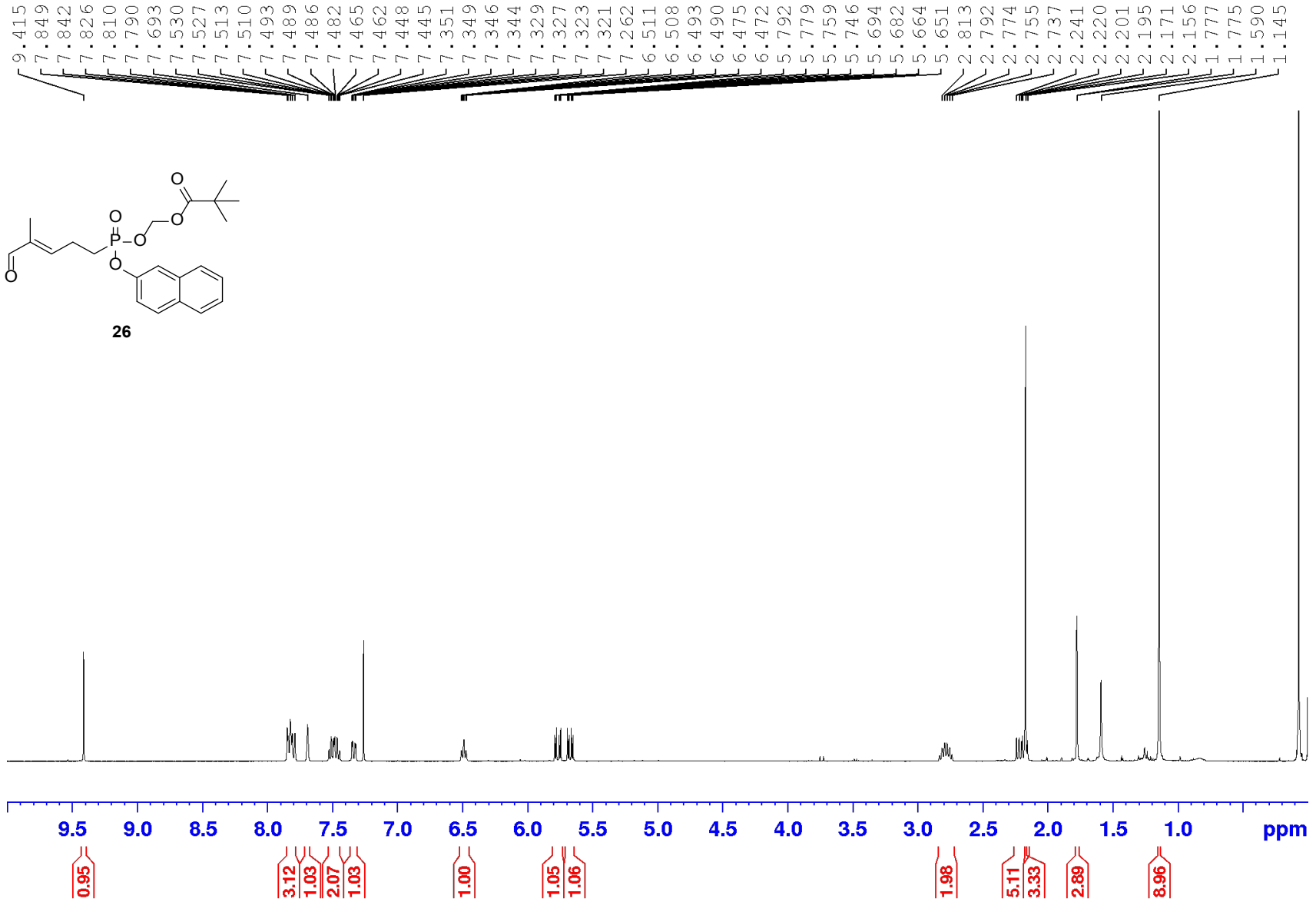
500 MHz ^{1}H NMR spectrum of compound **22** ($CDCl_3$)



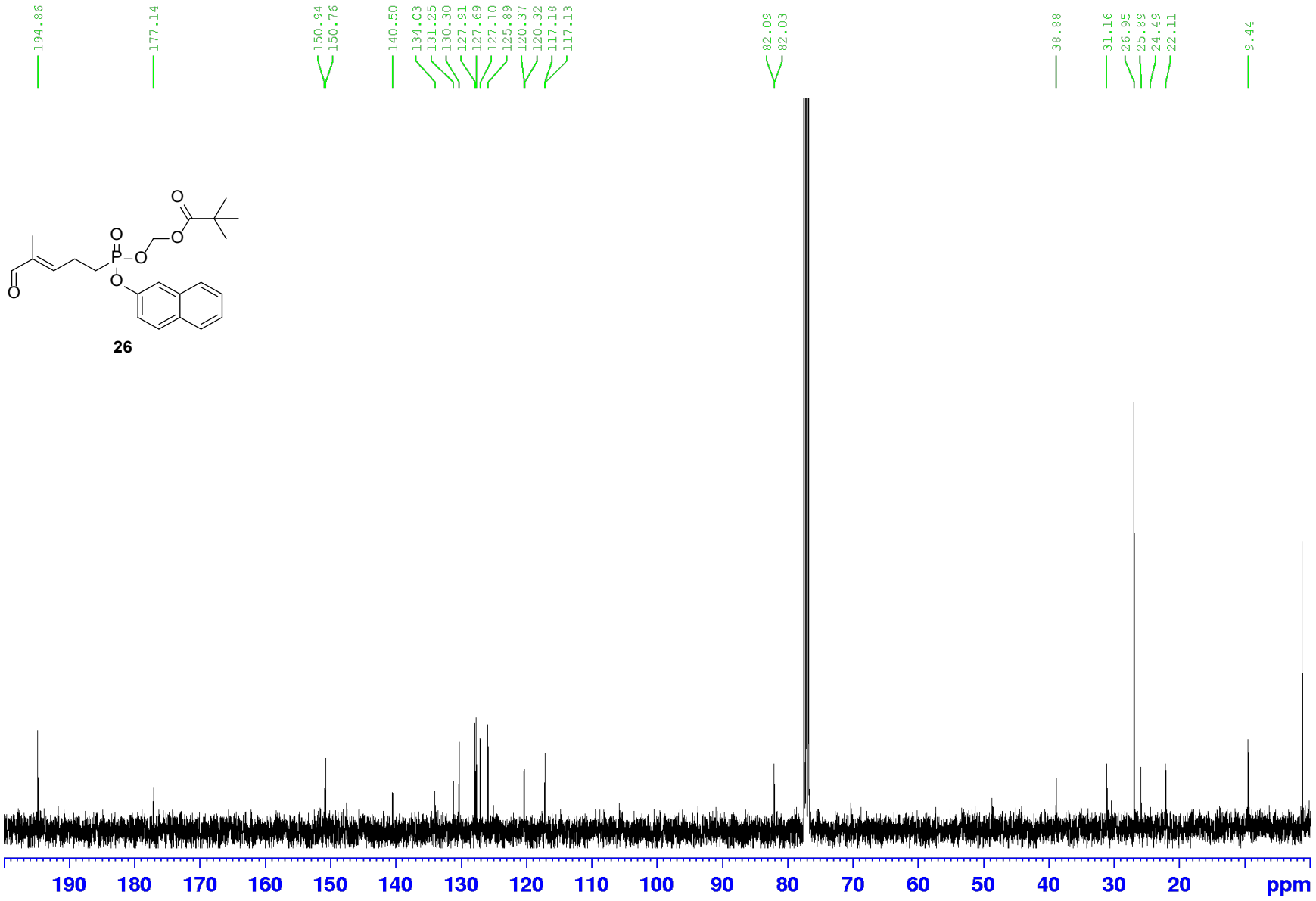
125 MHz ^{13}C NMR spectrum of compound **22** ($CDCl_3$)



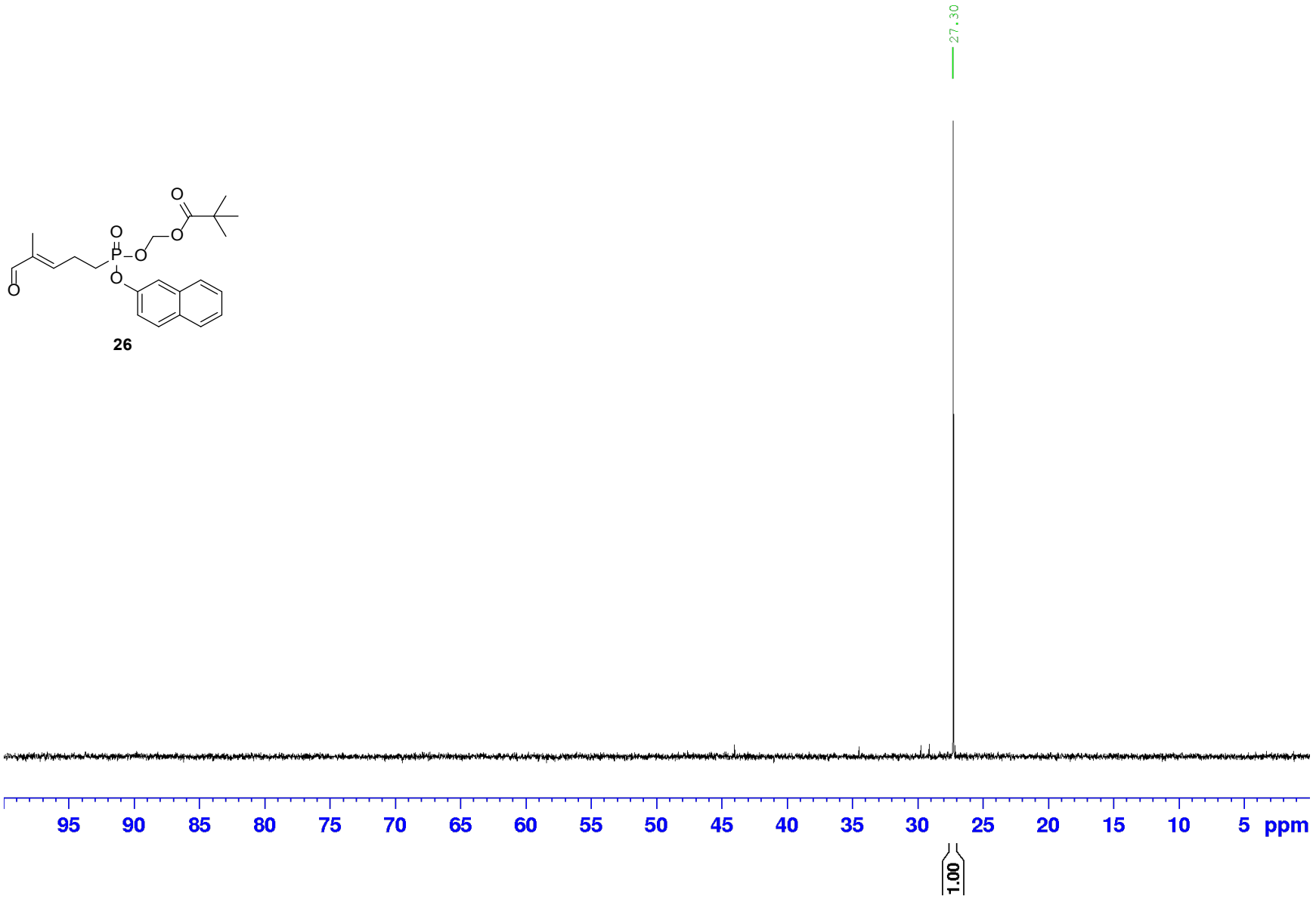
202 MHz ^{31}P NMR spectrum of compound **22** ($CDCl_3$)



400 MHz ^{1}H NMR spectrum of compound **26** ($CDCl_3$)

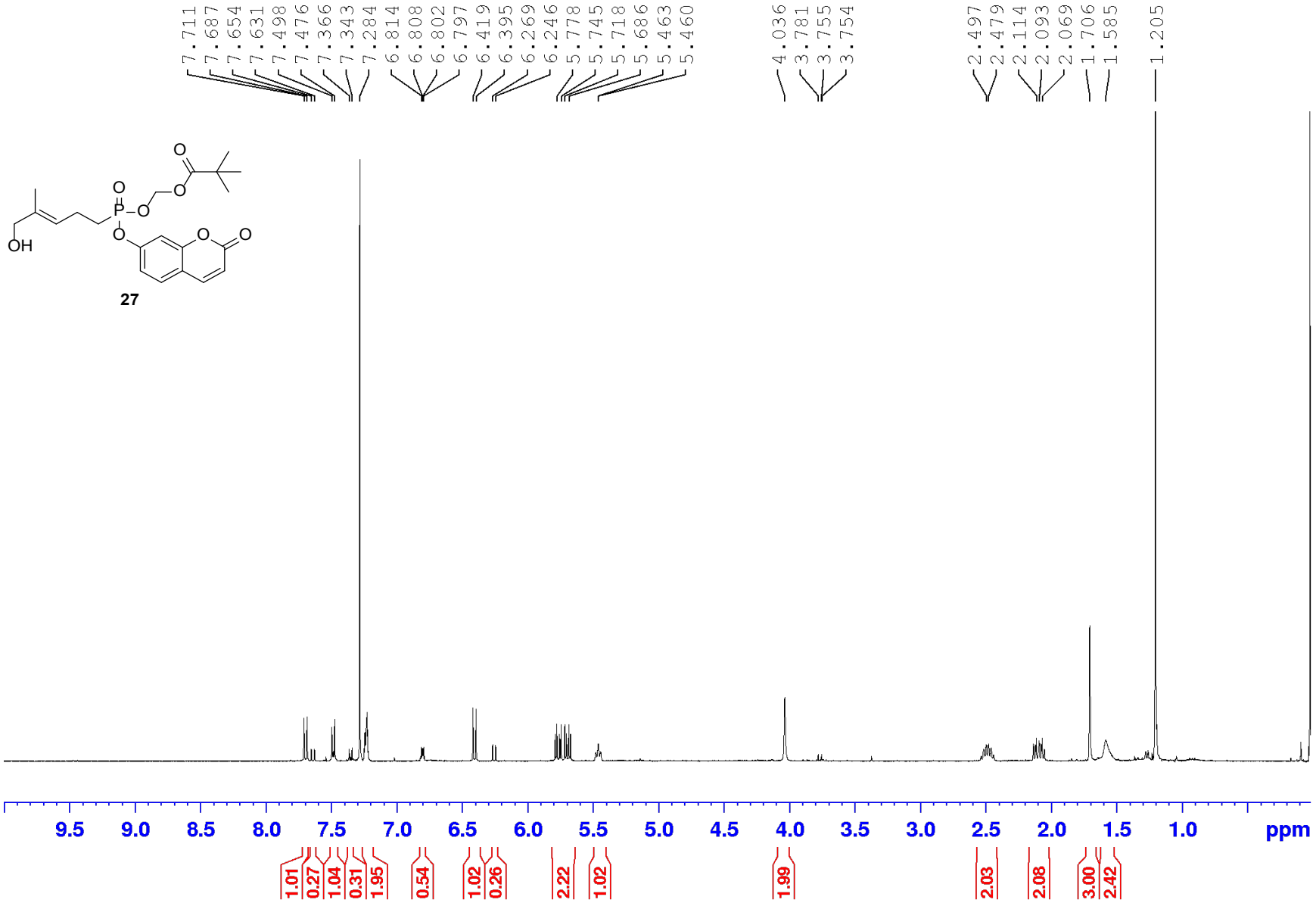


100 MHz ^{13}C NMR spectrum of compound **26** ($CDCl_3$)

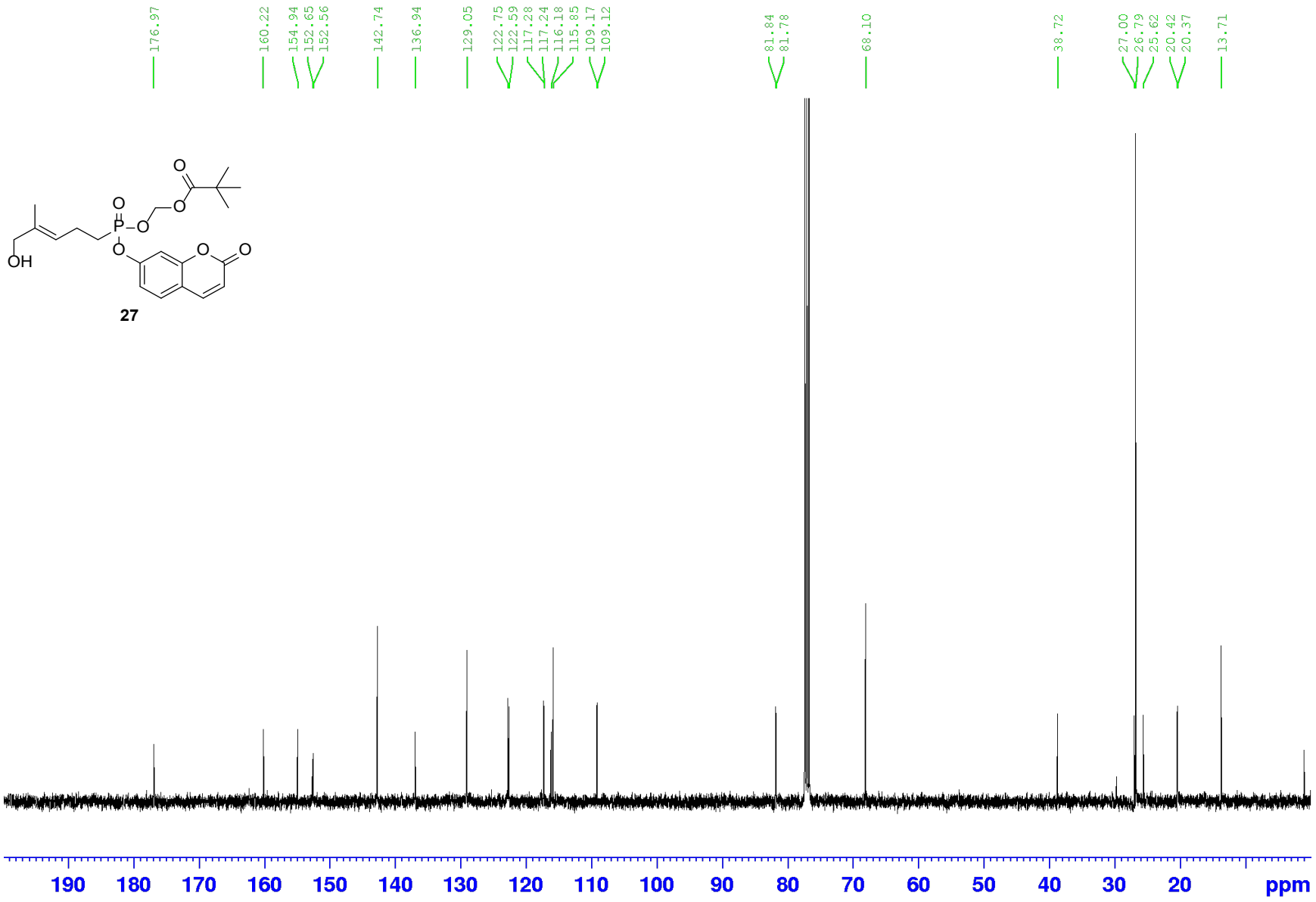


26

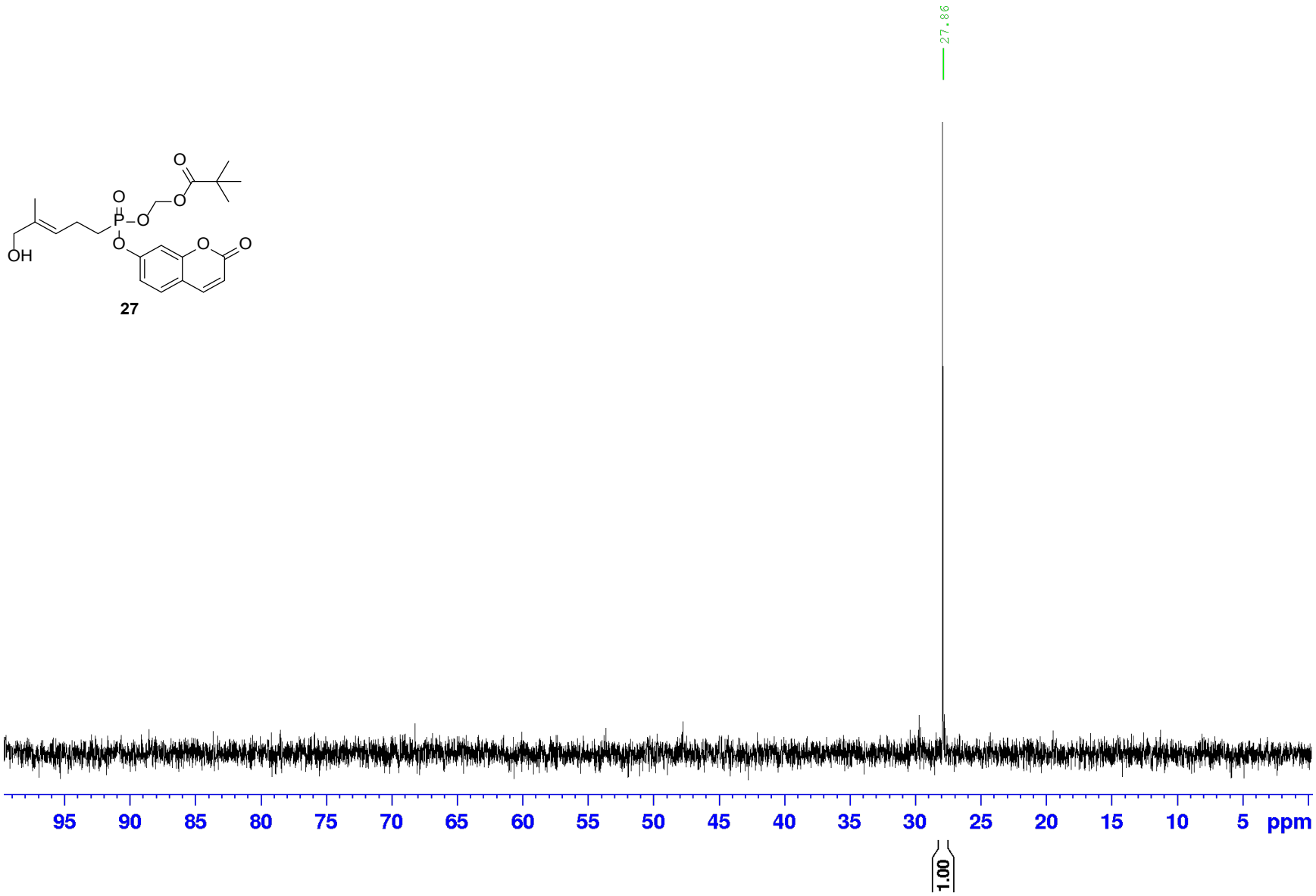
161 MHz ^{31}P NMR spectrum of compound **26** ($CDCl_3$)



400 MHz ^{1}H NMR spectrum of compound **27** ($CDCl_3$)

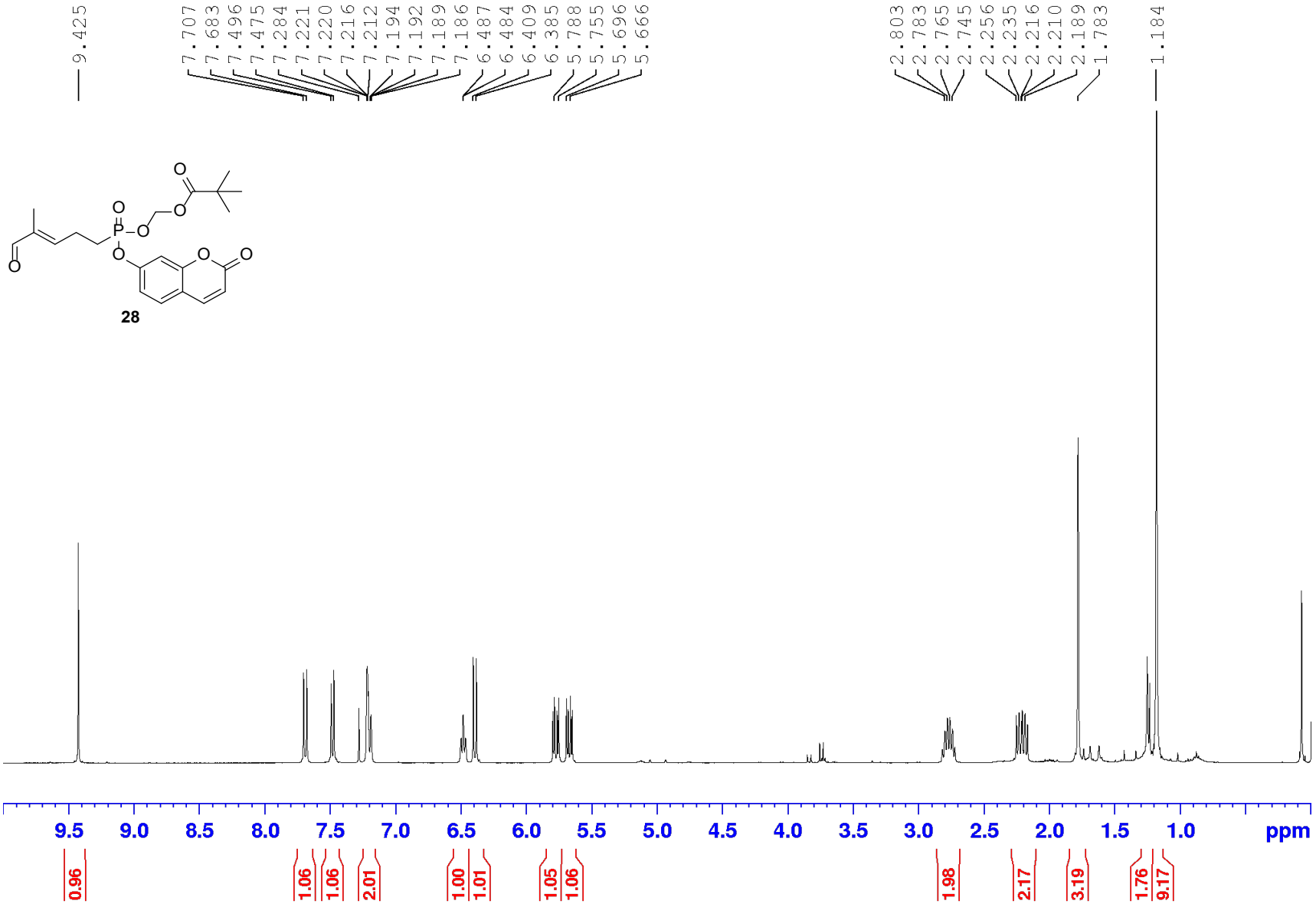


100 MHz ^{13}C NMR spectrum of compound **27** ($CDCl_3$)

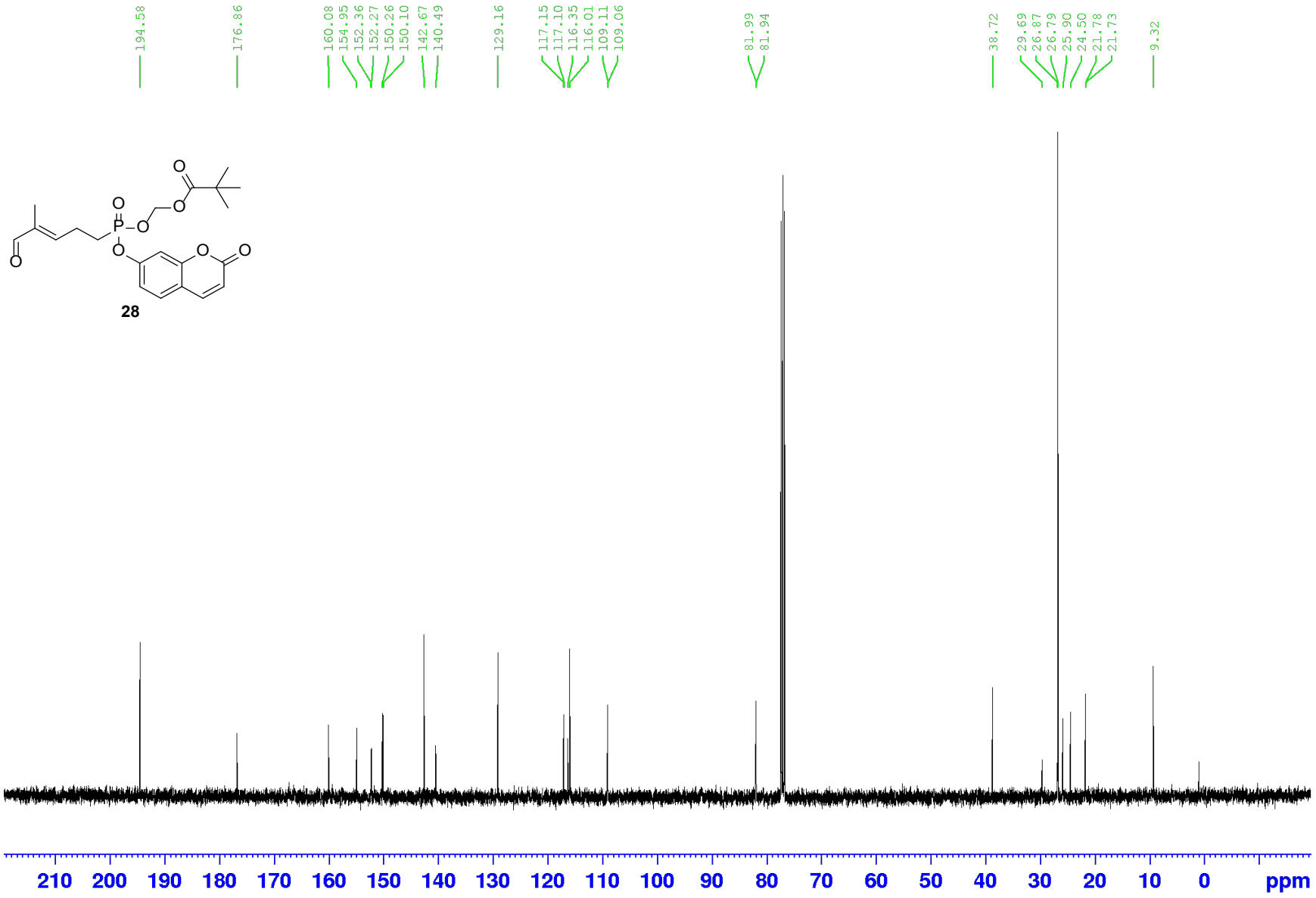


27

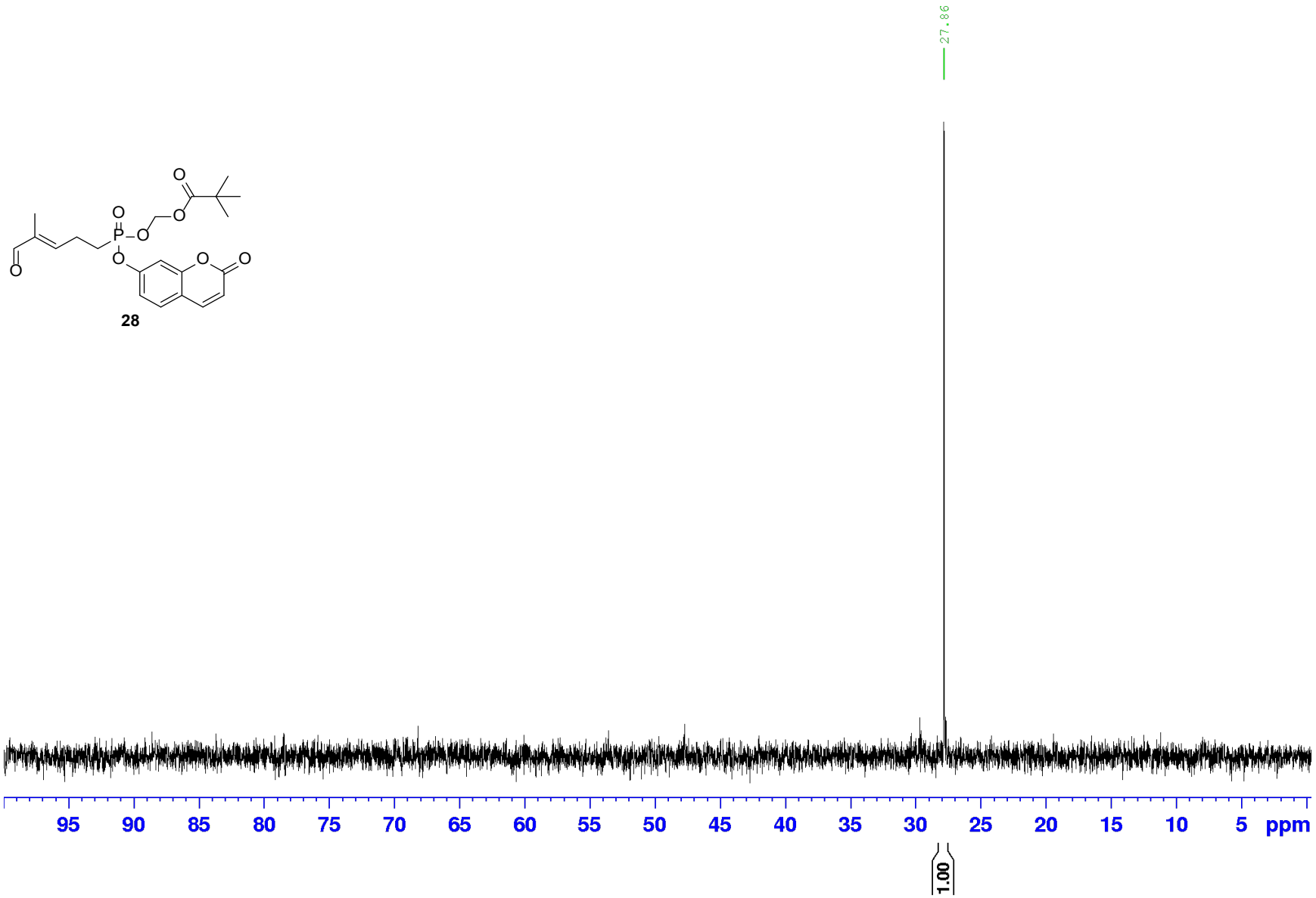
161 MHz ^{31}P NMR spectrum of compound **27** ($CDCl_3$)



400 MHz ^{1}H NMR spectrum of compounds **28** ($CDCl_3$)

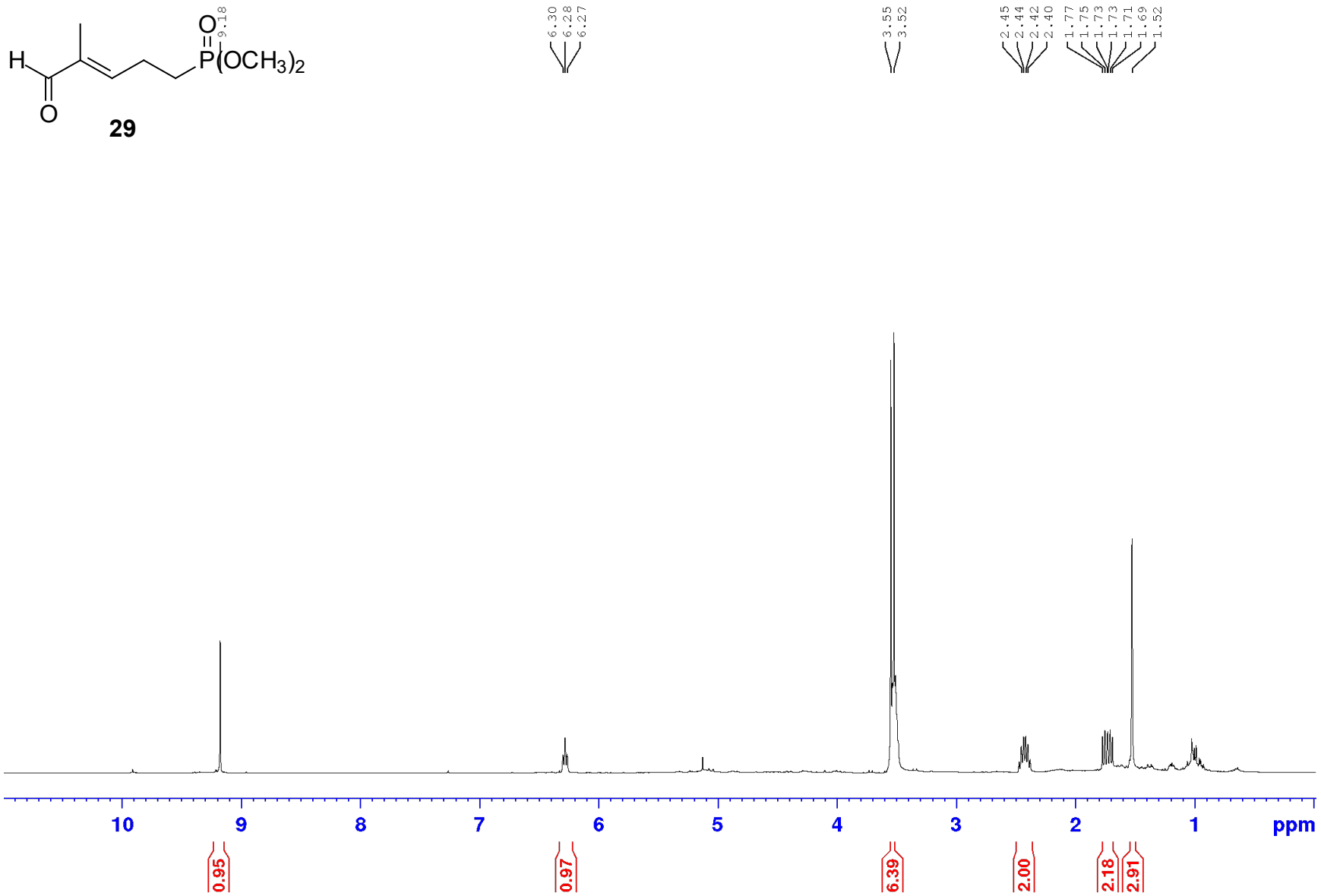


100 MHz ^{13}C NMR spectrum of compound **28** ($CDCl_3$)

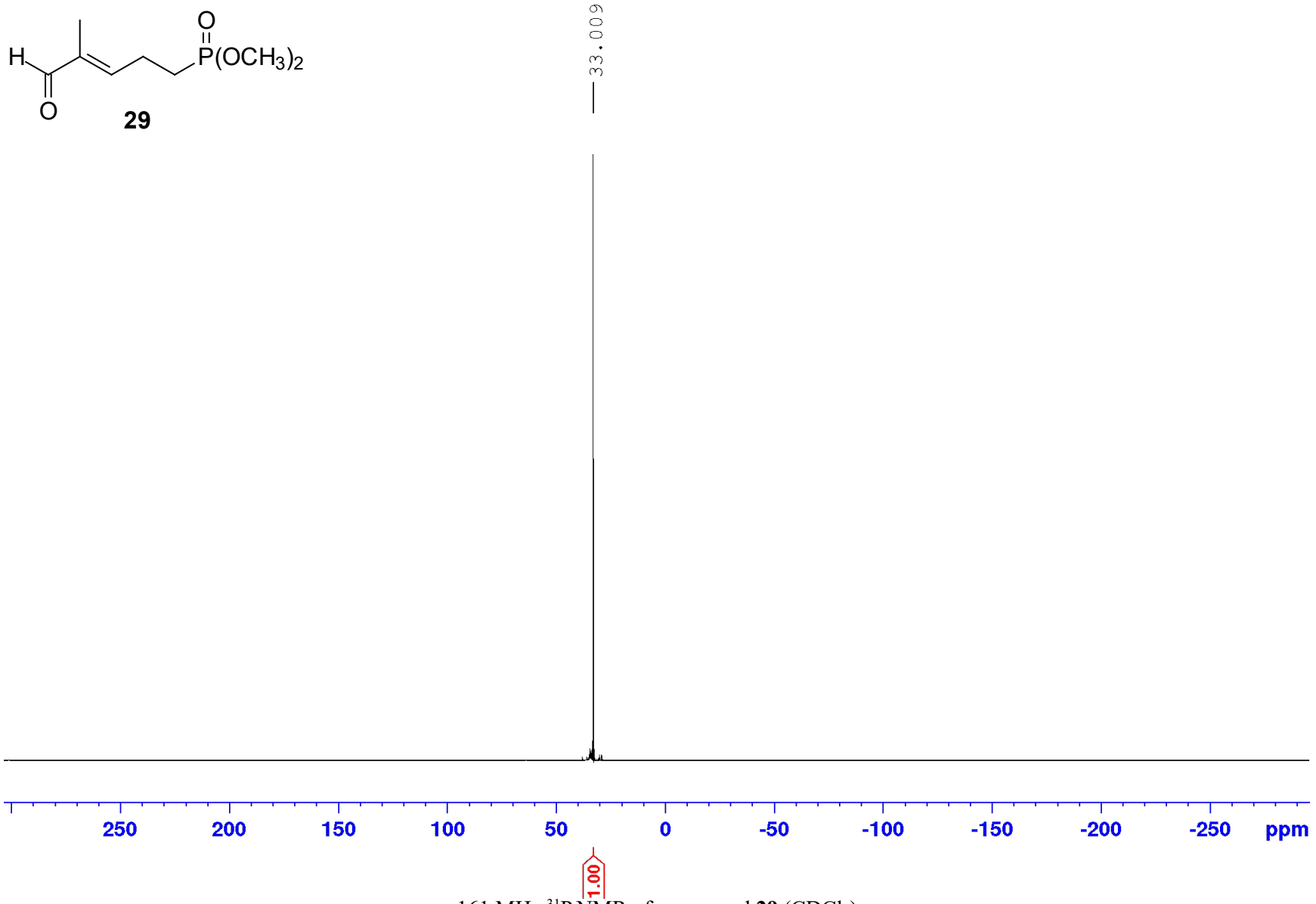


28

161 MHz ^{31}P NMR spectrum of compound **28** ($CDCl_3$)



400 MHz ^{1}H NMR of compound **29** ($CDCl_3$)



161 MHz ^{31}P NMR of compound **29** ($CDCl_3$)