Supplemental Table of Contents

Biological assays

Materials. Human peripheral blood mononuclear cell (PBMCs) were isolated from buffy coat obtained from Research Blood Components (Boston, MA). Pooled human plasma was from Fisher Scientific (Hampton, NH). HMBPP was purchased from Echelon (Salt Lake City, UT) and Cayman Chemical (Ann Arbor, MI). K562 cells were from Sigma Aldrich (Saint Louis, MO). Cell Quantiblue viability reagent was purchased from Bioassay Systems (Hayward, CA). The interferon-γ enzyme-linked immunosorbent assay kit was purchased from Biolegend (San Diego, CA). Interleukin 2 and the TCR γ/δ+ T Cell Isolation Kit were purchased from Miltenyi (Bergisch Gladbach, Germany).

Solubility. The prodrugs were dissolved in ethanol at 50 mM. For stability studies, compounds were diluted with water to a stock concentration of 1 mM. For cellular studies, compounds were diluted with tissue culture media to a stock concentration of 1 mM. Some minor cloudiness in 1 mM aqueous solutions was observed only with compounds **9** and **12**, which was no longer apparent upon dilution to 100 µM or lower concentrations.

Stability studies. Stability studies were performed as described previously with some modifications for the specific compounds.[1] Pooled human plasma was diluted to 50% with phosphate buffered saline at pH 7.5. Test compounds were added at a final concentration of 100 µM in a volume of 100 µL. Compounds were incubated as indicated in the text, then extracted from the plasma with 300 µL of LCMS grade acetonitrile and vigorous mixing. 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 positive mode using a gradient of water and acetonitrile and a C18 column. The gradient started at 25% acetonitrile then increased to 80% acetonitrile over 8 minutes and held there for 1 minute before re-equilibration. Masses corresponding to the molecular ion $[M+H]^+$, the sodium adduct $[M + Na]^+$, and the dehydration product $[M - OH]^+$ were generally observed, though varied by compound. The integrated peak values were compared to those of t = 0 minutes for each test compound and expressed as a fraction of the initial compound.

Expansion of Vγ9Vδ2 T cells. All compounds were evaluated for their ability to promote growth of human Vγ9Vδ2 T cells from peripheral blood as previously described.[2] In each replicate, 100 nM of HMBPP **1** and 100 nM of POM_2-C-HMBP **2** were used as positive controls. Negative controls contained cells without test compounds. EC_{50} values were determined as the concentration that induced 50% of the maximum proliferative effect.

ELISA for interferon-γ. Interferon-γ was measured by ELISA as previously described.[2b, 3] K562 cells were treated with test compounds for indicated times at indicated concentrations. They were washed twice with media, then diluted and mixed with Vγ9Vδ2 T cells that had been expanded by treatment with HMBPP (3 days on 7-11 days off) then purified by negative selection. Each well contained a 3:1 effector: target ratio in 200 µL. After 20 hours of co-incubation, the concentration of interferon-γ in the supernatant was determined relative to a standard curve.

Cell viability. Viability assays were performed using K562 cells with various concentrations of test compounds. K562 cells (0.5×10^4 cells in 100 µL of RPMI media) were distributed into each well of a 96-well plate. The aryl phosphonates were added for 72 hours, during the last 2 hours the cell-QB reagent was added. Signals were quantified with a

fluorescence plate reader. Viable cells are expressed as a percentage of untreated control cells after subtraction of a media-only blank.

Statistical analysis. All experiments involving primary human cells were performed at least three times (n=3) using cells from a minimum of two different donors. Graphs were constructed and data analyzed using GraphPad Prism 6. The proliferation dose response curves were analyzed using a log (agonist) versus response -- variable slope (four parameters) model in which the bottom was constrained at the level of the negative control. The ELISA dose response curves were analyzed using a log (agonist) versus response -- variable slope (four parameters) model in which the top, bottom, and Hill slope parameters were assigned as shared values within each compound. EC_{50} values for each assay and time point were calculated, and the EC_{50} values with 95% confidence intervals are reported. Log EC_{50} values for each compound and time point from the ELISA assay were plotted on the X-axis versus log *t* on the Y-axis (log time for 50% interferon-γ response). Power constants (k_1) were calculated according to the formula $k_1 = \log t + \alpha \log C$ where *C* is the concentration (μM) and *t* the incubation time (minutes), and both α and *k* were variables. The fit (R^2) was assessed for each compound.[3b]

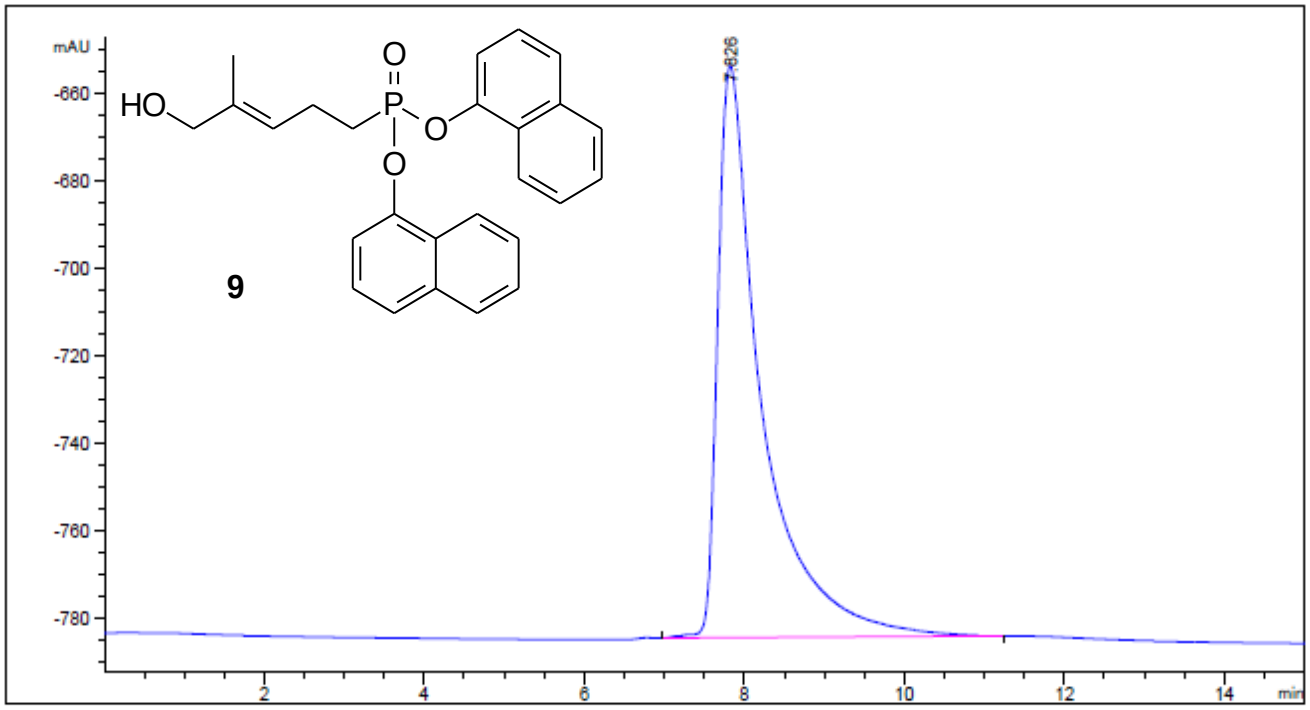
SI References.

1. Lentini, N. A.; Foust, B. J.; Hsiao, C. C.; Wiemer, A. J.; Wiemer, D. F., Phosphonamidate Prodrugs of a Butyrophilin Ligand Display Plasma Stability and Potent Vgamma9 Vdelta2 T Cell Stimulation. *J. Med. Chem.* **2018,** *61* (19), 8658-8669.
2. (a) Hsiao, C. H.; Lin, X.; Barney, R. J.; Shippy, R. R.; Li, J.; Vinogradova, O.; Wiemer, D. F.; Wiemer, A. J., Synthesis of a phosphoantigen prodrug that potently activates Vgamma9Vdelta2 T-lymphocytes. *Chem. Biol.* **2014,** *21* (8), 945-54; (b) Shippy, R. R.; Lin, X.; Agabiti, S. S.; Li, J.; Zangari, B. M.; Foust, B. J.; Poe, M. M.; Hsiao, C. C.; Vinogradova, O.; Wiemer, D. F.; Wiemer, A. J., Phosphinophosphonates and Their Tris-pivaloyloxymethyl Prodrugs Reveal a Negatively Cooperative Butyrophilin Activation Mechanism. *J. Med. Chem.* **2017,** *60* (6), 2373-2382.
3. (a) Kilcollins, A. M.; Li, J.; Hsiao, C. H.; Wiemer, A. J., HMBPP Analog Prodrugs Bypass Energy-Dependent Uptake To Promote Efficient BTN3A1-Mediated Malignant Cell Lysis by Vgamma9Vdelta2 T Lymphocyte Effectors. *J. Immunol.* **2016,** *197* (2), 419-28; (b) Hsiao, C. C.; Wiemer, A. J., A power law function describes the time- and dose-dependency of Vgamma9Vdelta2 T cell activation by phosphoantigens. *Biochem. Pharmacol.* **2018,** *158*, 298-304.

Table S1. Stability of compounds in 50% human plasma in PBS.[a]

Compound	2 hour fraction remaining (SD)	20 hour fraction remaining (SD)
2[1-2]	0.025 (0.007)	0.050 (0.057)
4	0.085 (0.021)	0.070 (0.014)
5	0.60 (0.25)	0.10 (0.04)
12	1.0 (0.2)	0.60 (0.14)
14	0.59 (0.00)	0.040 (0.028)
17	0.20 (0.04)	0.00 (0.00)
9	1.0 (0.1)	0.98 (0.08)

[a] Data represent the mean +/- standard deviation (SD) of 2 independent experiments

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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]       %
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   1   7.826 BB    0.5471 5038.22021  130.88841 100.0000

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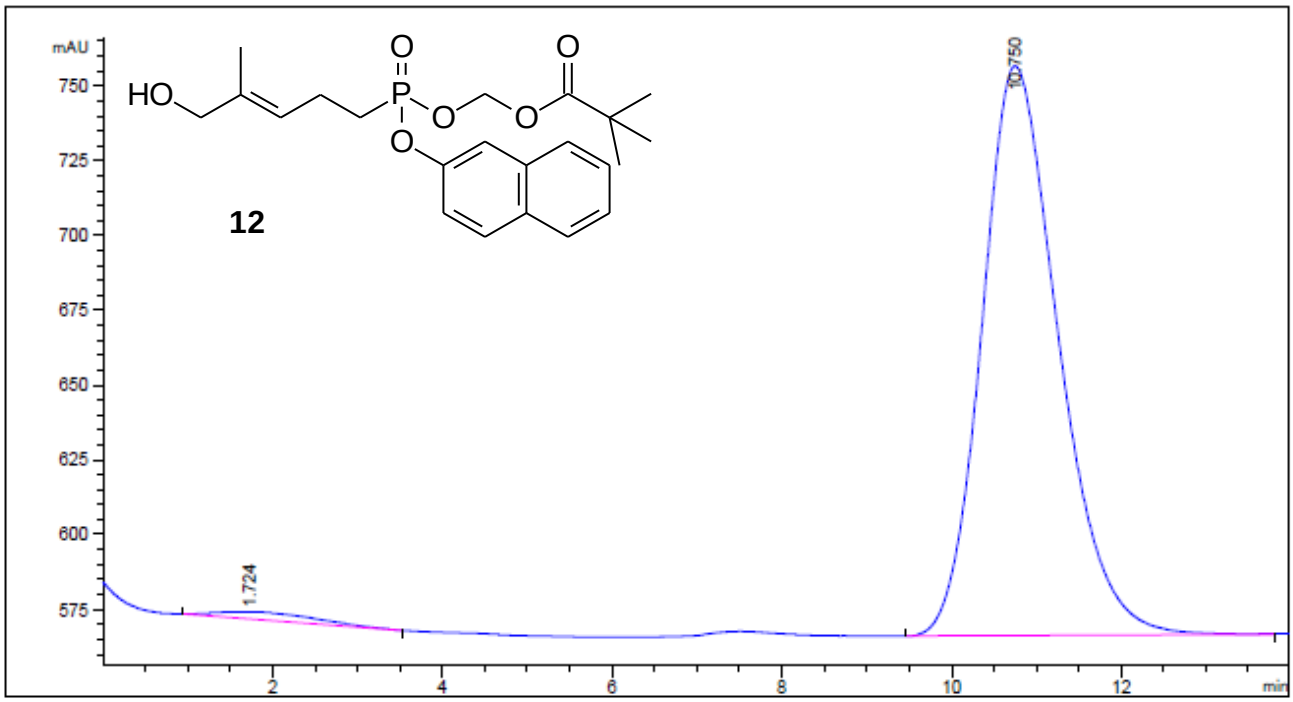


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Peak RetTime Type  Width     Area      Height     Area
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   1   1.724 BB    1.0233  202.45877    2.33112   1.6714
   2  10.750 BB    0.9377 1.19108e4   190.42981  98.3286

Totals :                  1.21132e4   192.76093
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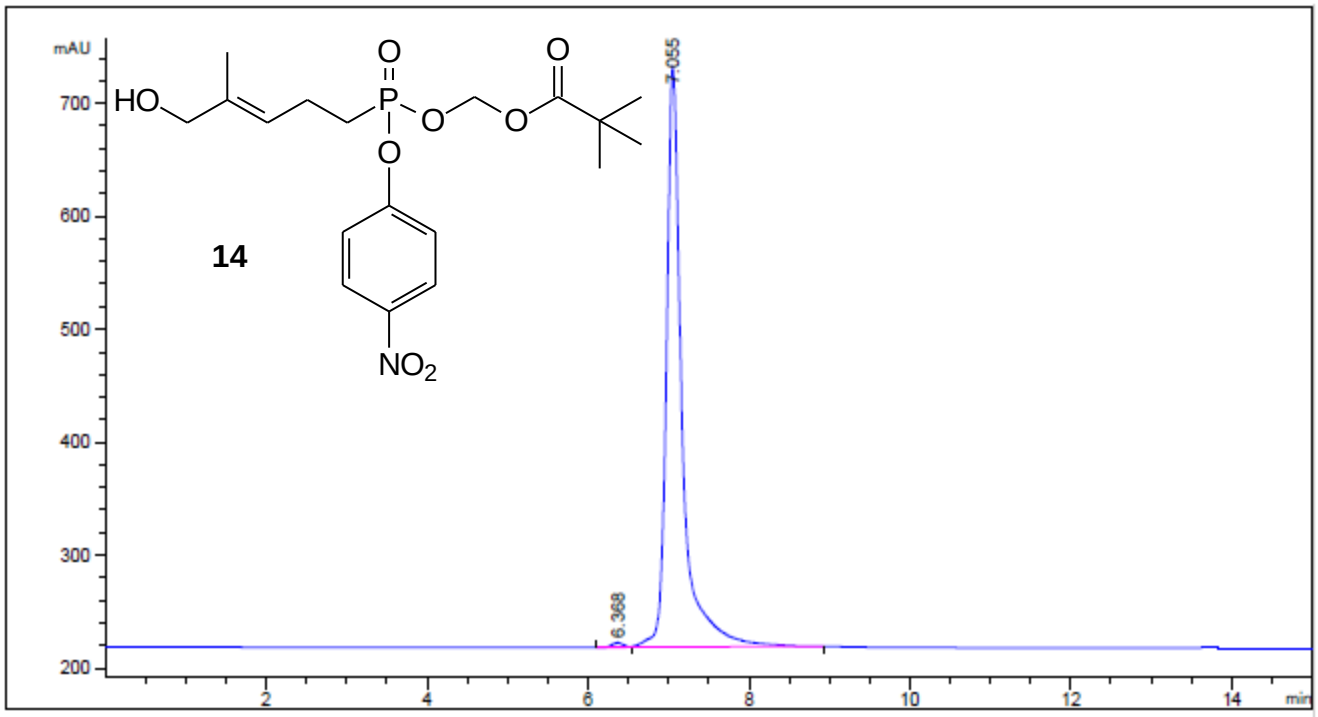


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Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=250 nm

Peak RetTime Type  Width     Area      Height     Area
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   1   6.368 BV E  0.1423   39.38525    4.20225   0.5582
   2   7.055 VB R  0.1984 7016.47070  513.45758  99.4418

Totals :                  7055.85596  517.65983
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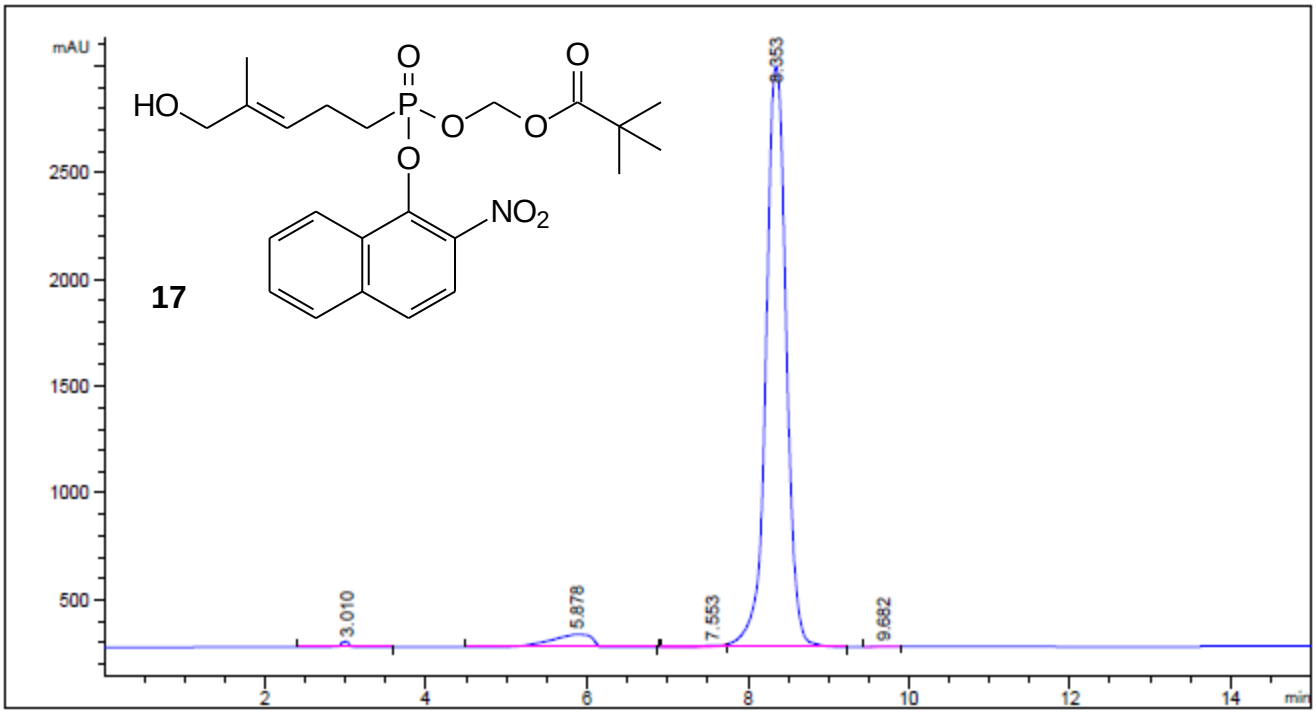


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Peak RetTime Type  Width     Area      Height     Area
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   1   3.010 VV R  0.0988  195.06570   26.52928   0.3889
   2   5.878 BB    0.4490 2146.83105   60.79145   4.2796
   3   7.553 BV E  0.2177   91.48296    6.51959   0.1824
   4   8.353 VB R  0.2728 4.77047e4  2717.02173  95.0970
   5   9.682 BB    0.1692   26.17470    1.96760   0.0522

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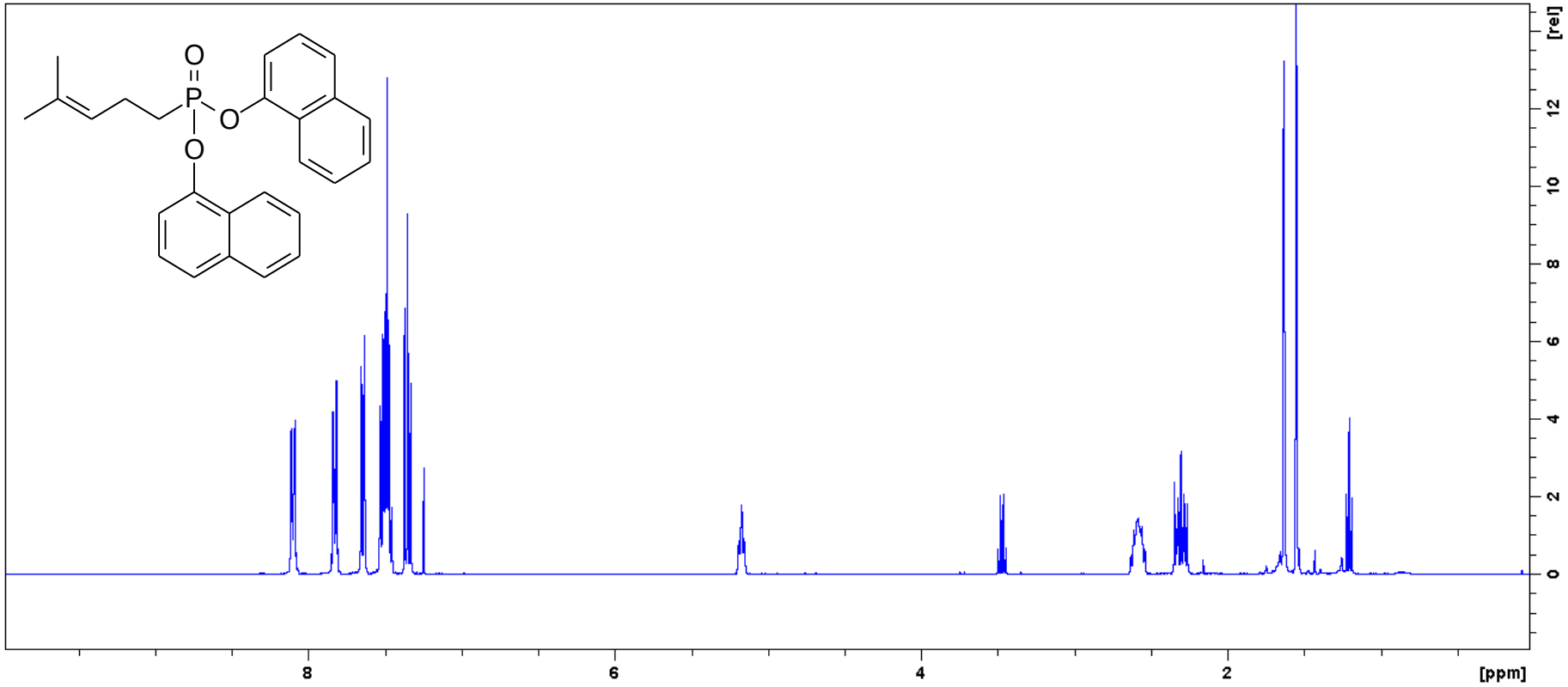
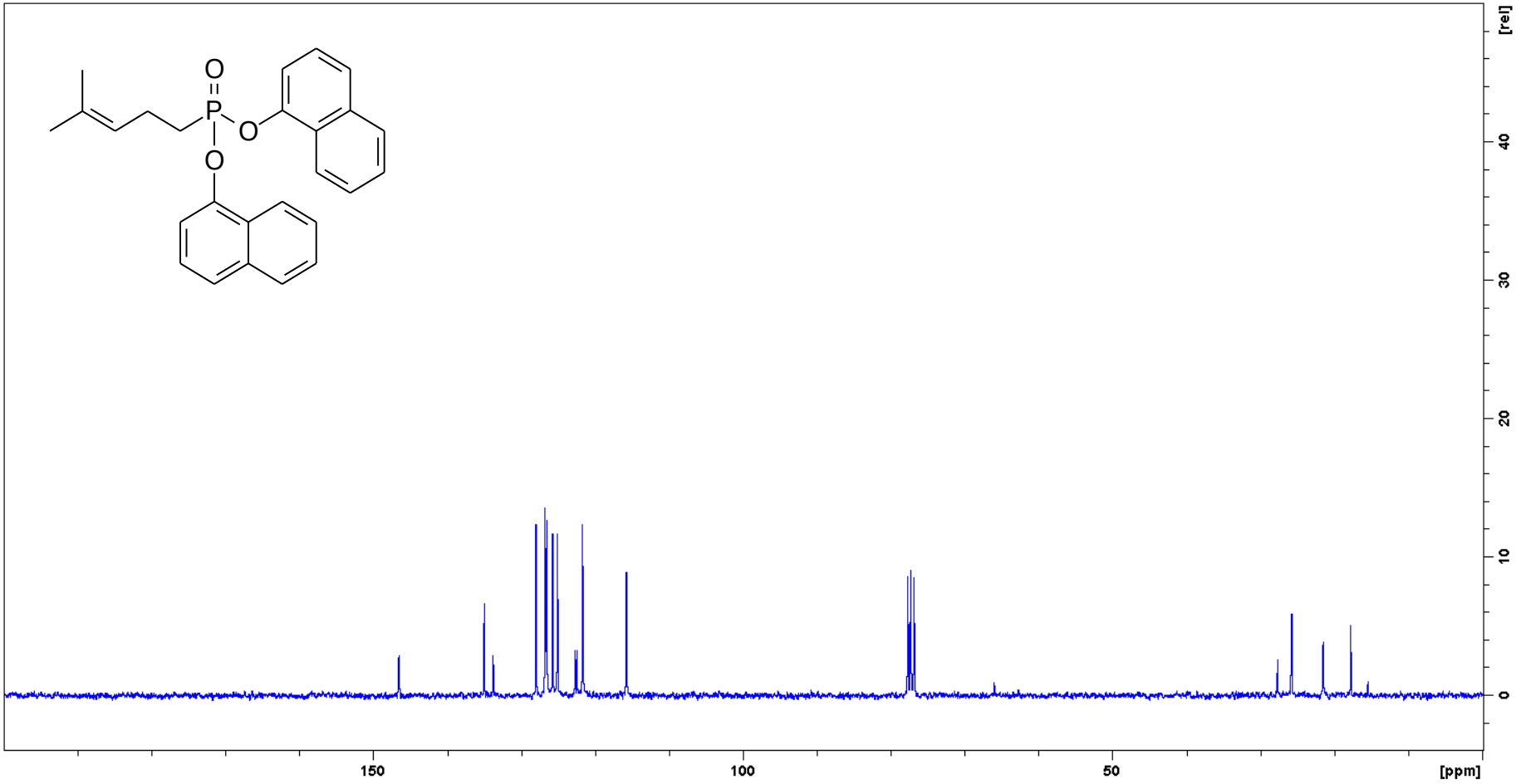
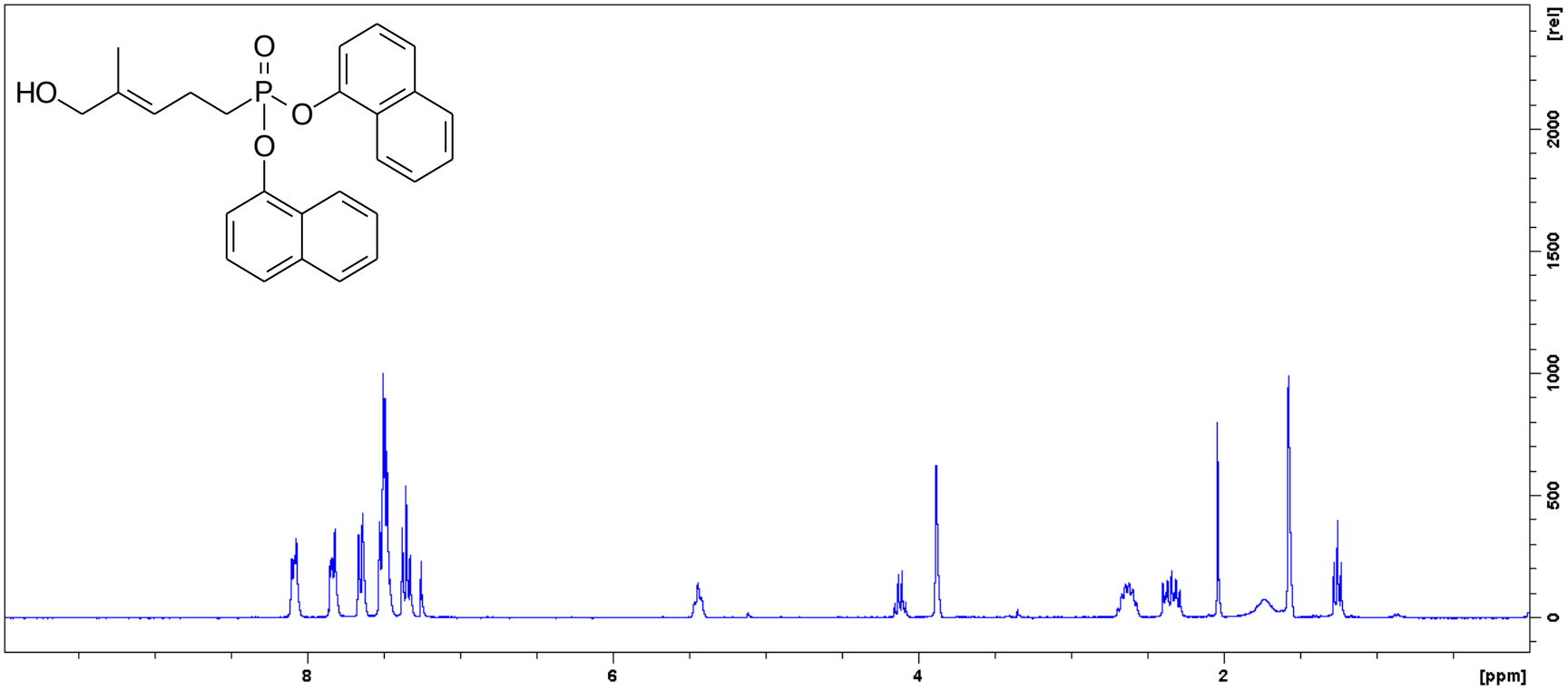


400 MHz ^{1}H NMR Spectrum of Compound **8**



75 MHz ^{13}C NMR Spectrum of Compound **8**



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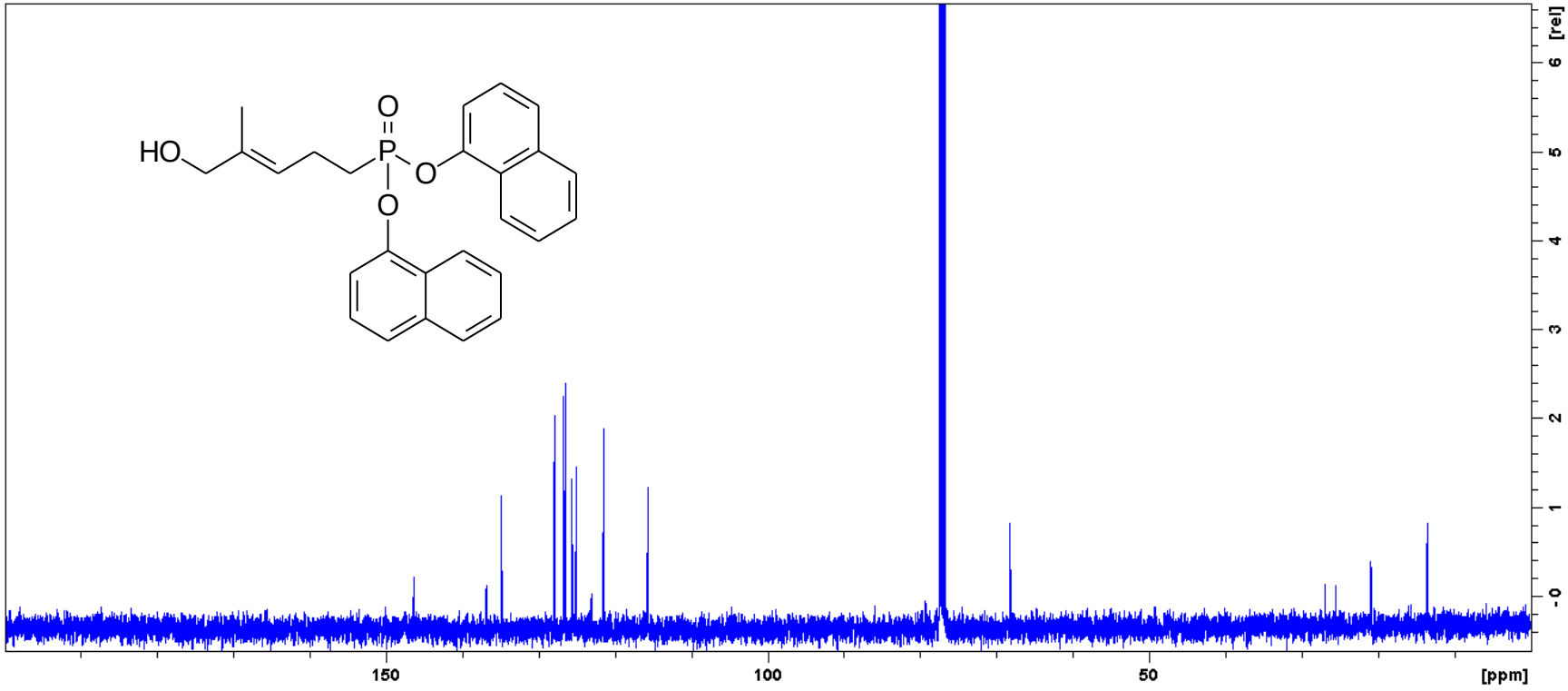

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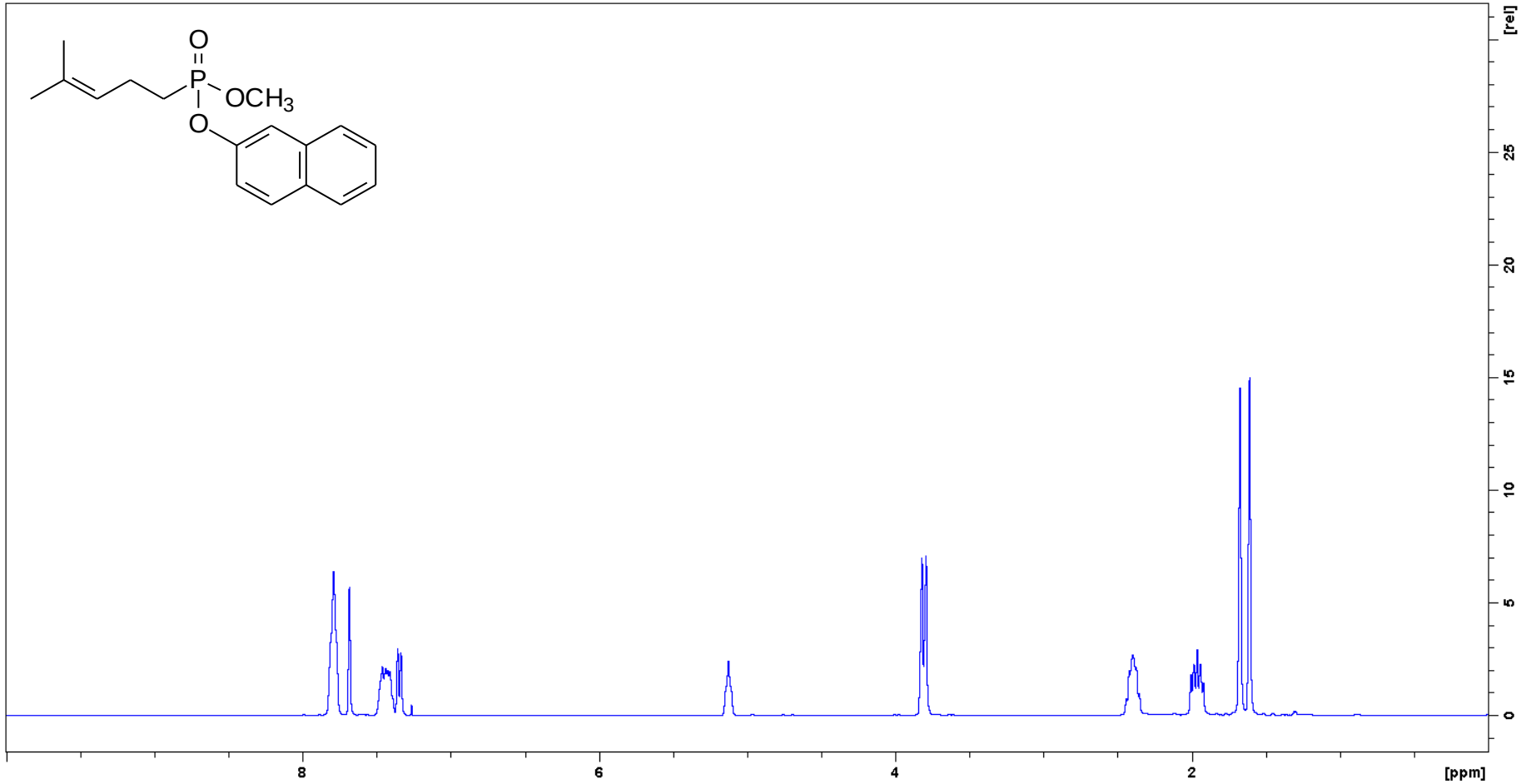

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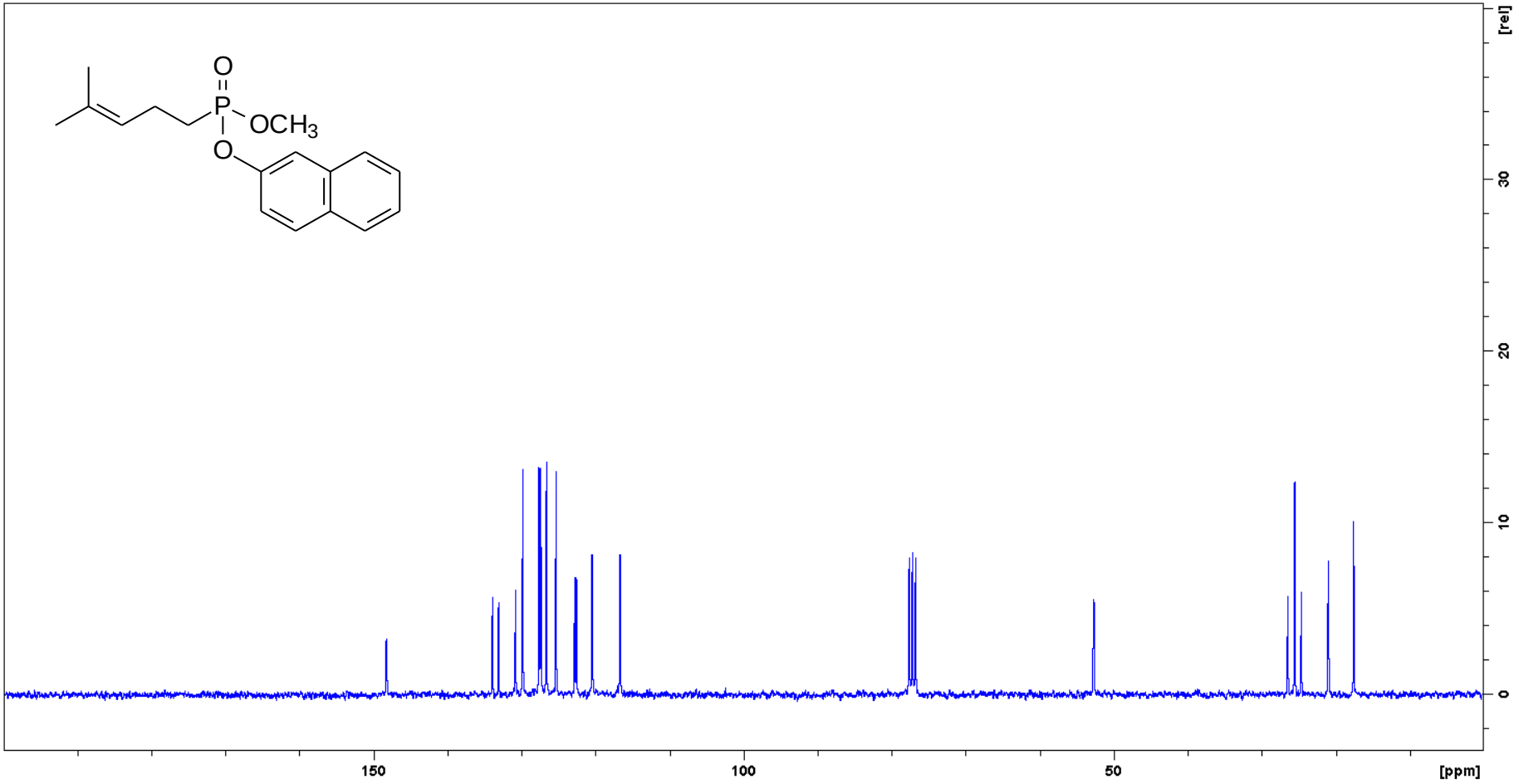
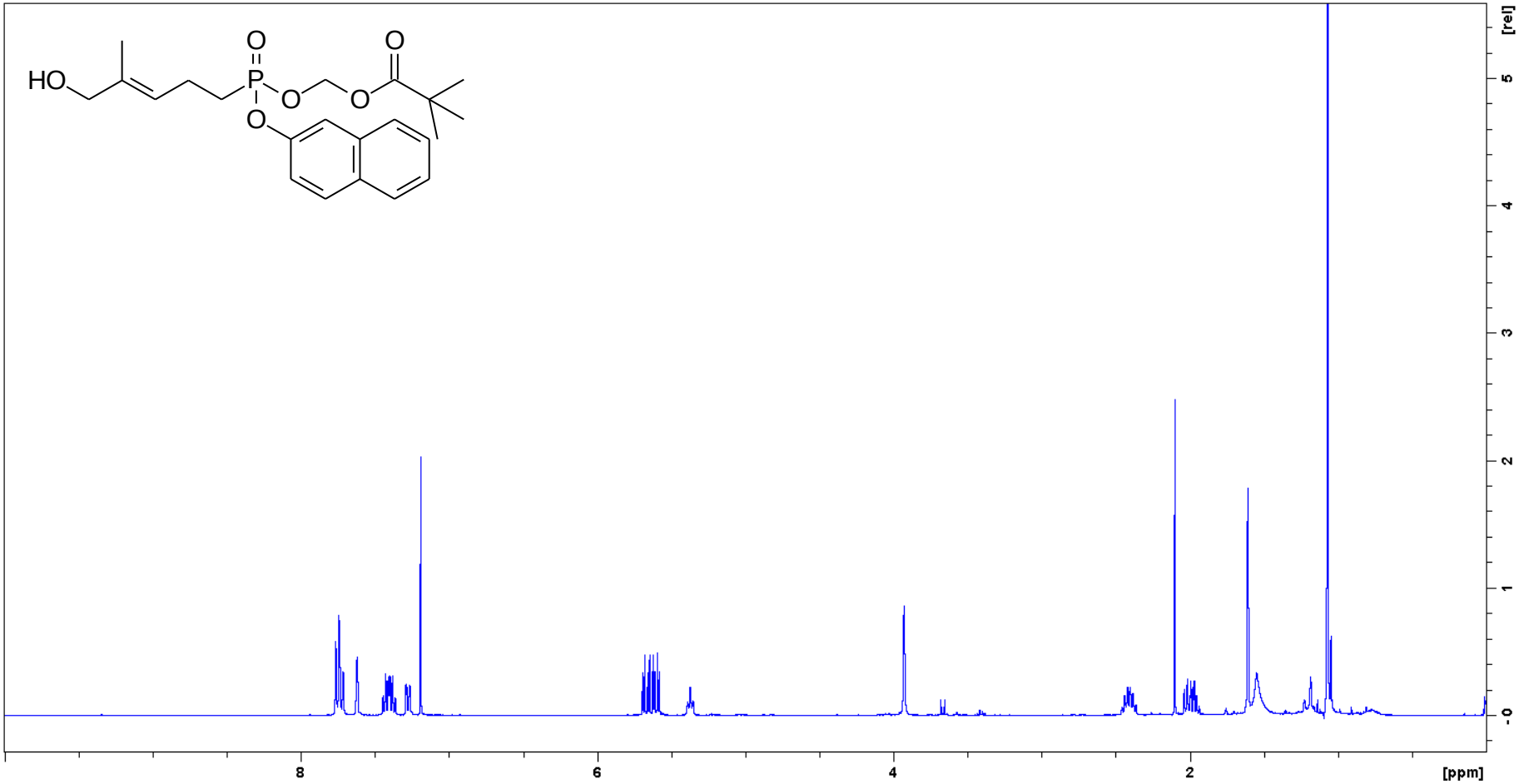


100 MHz ^{13}C NMR Spectrum of Compound **11**



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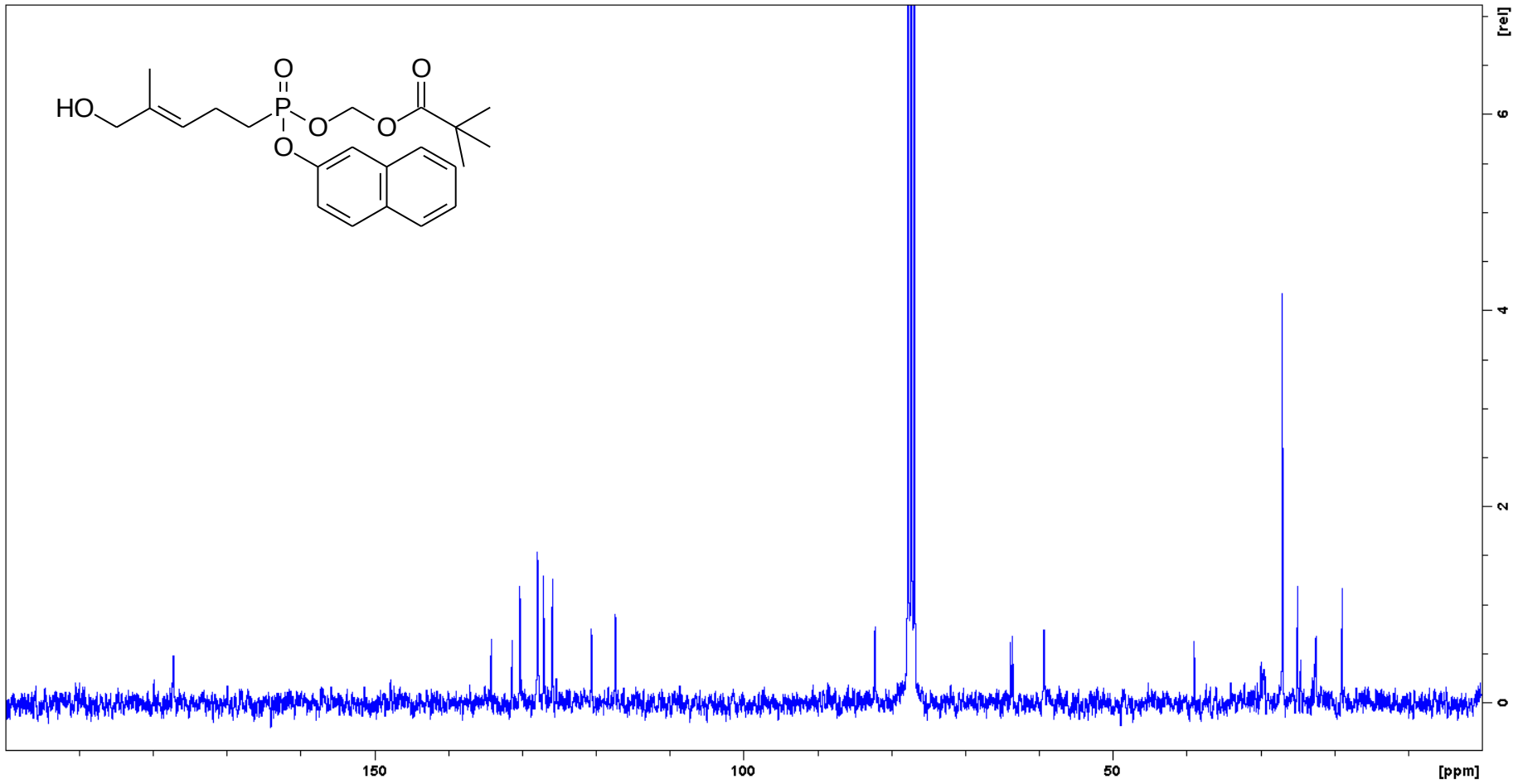

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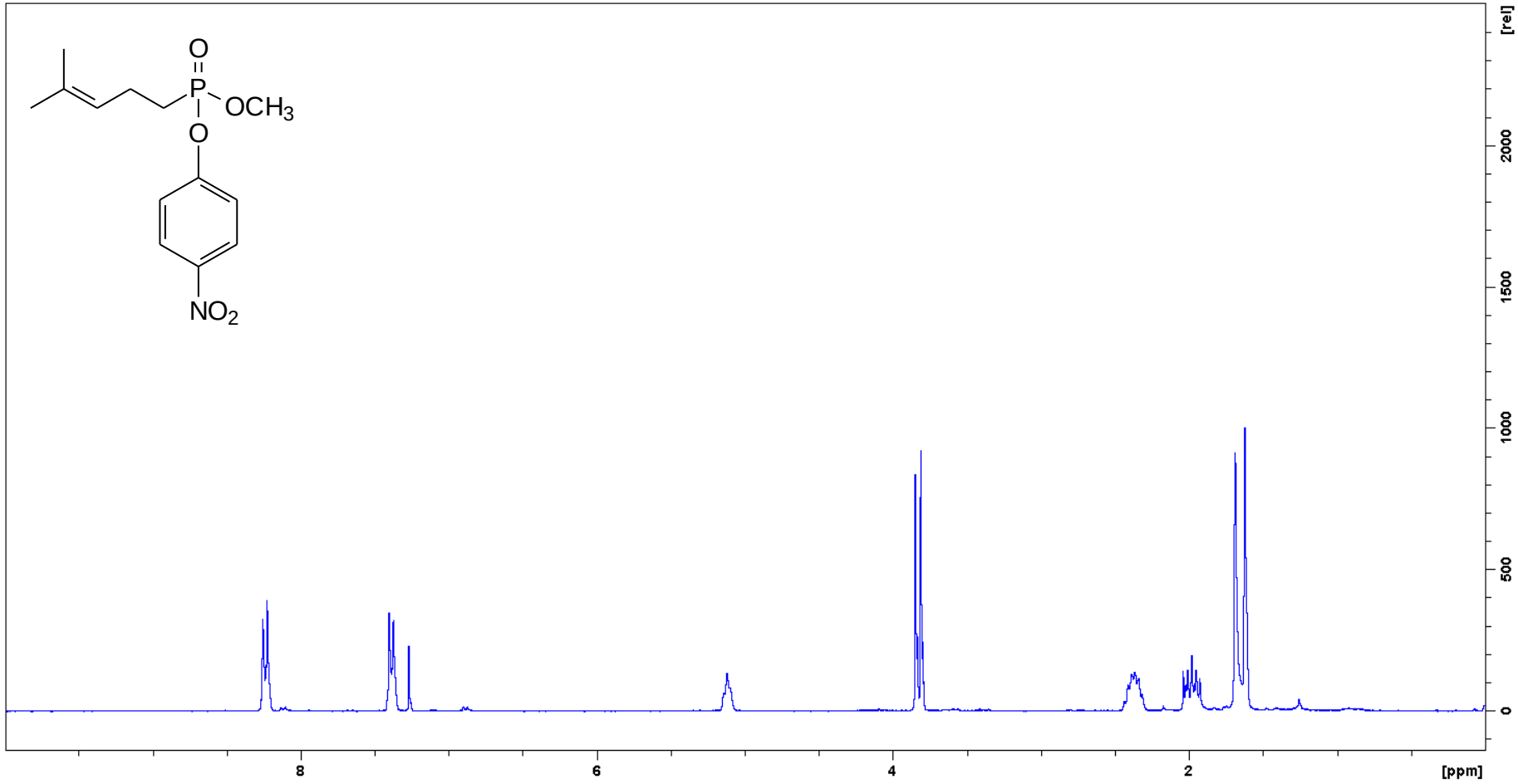

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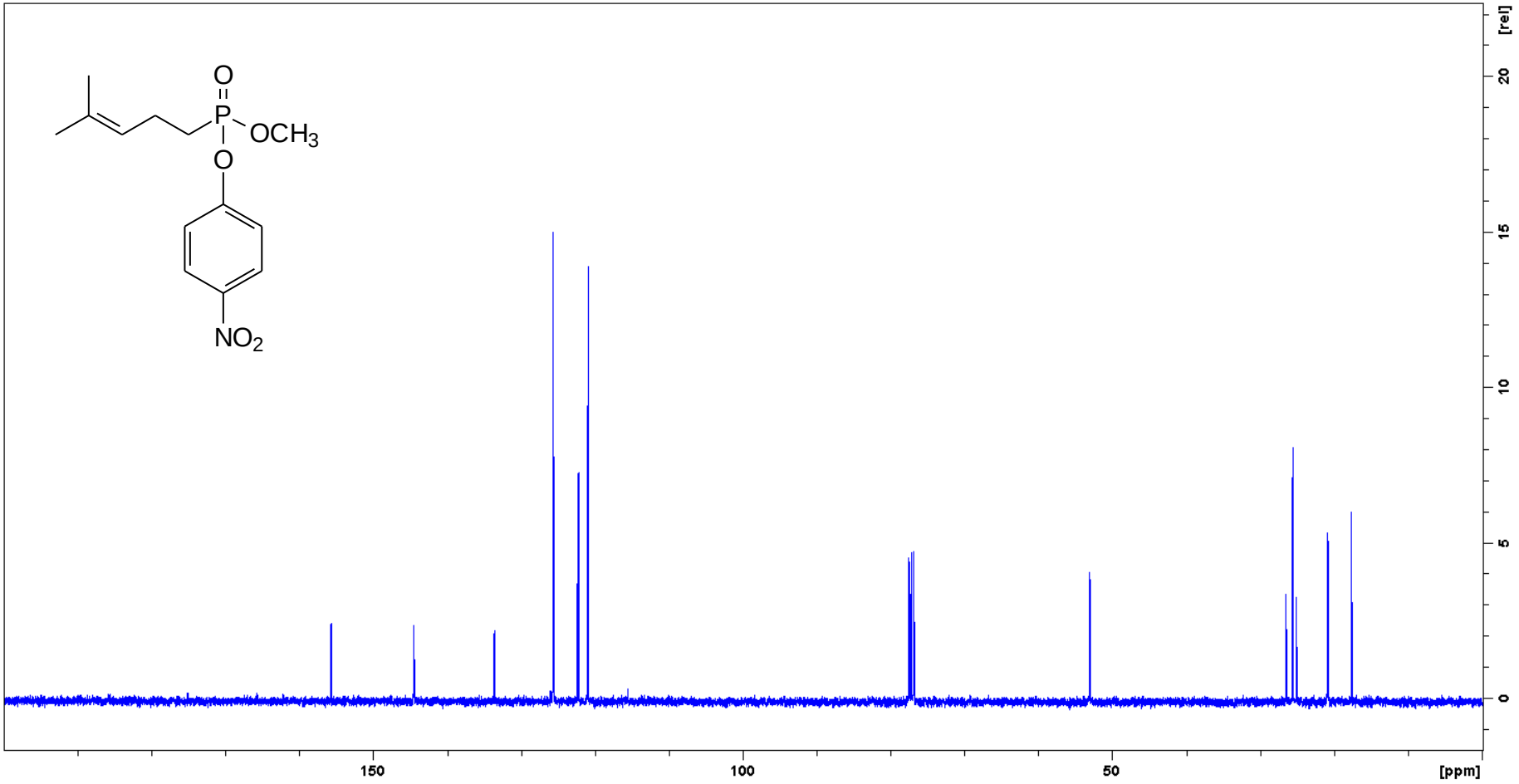

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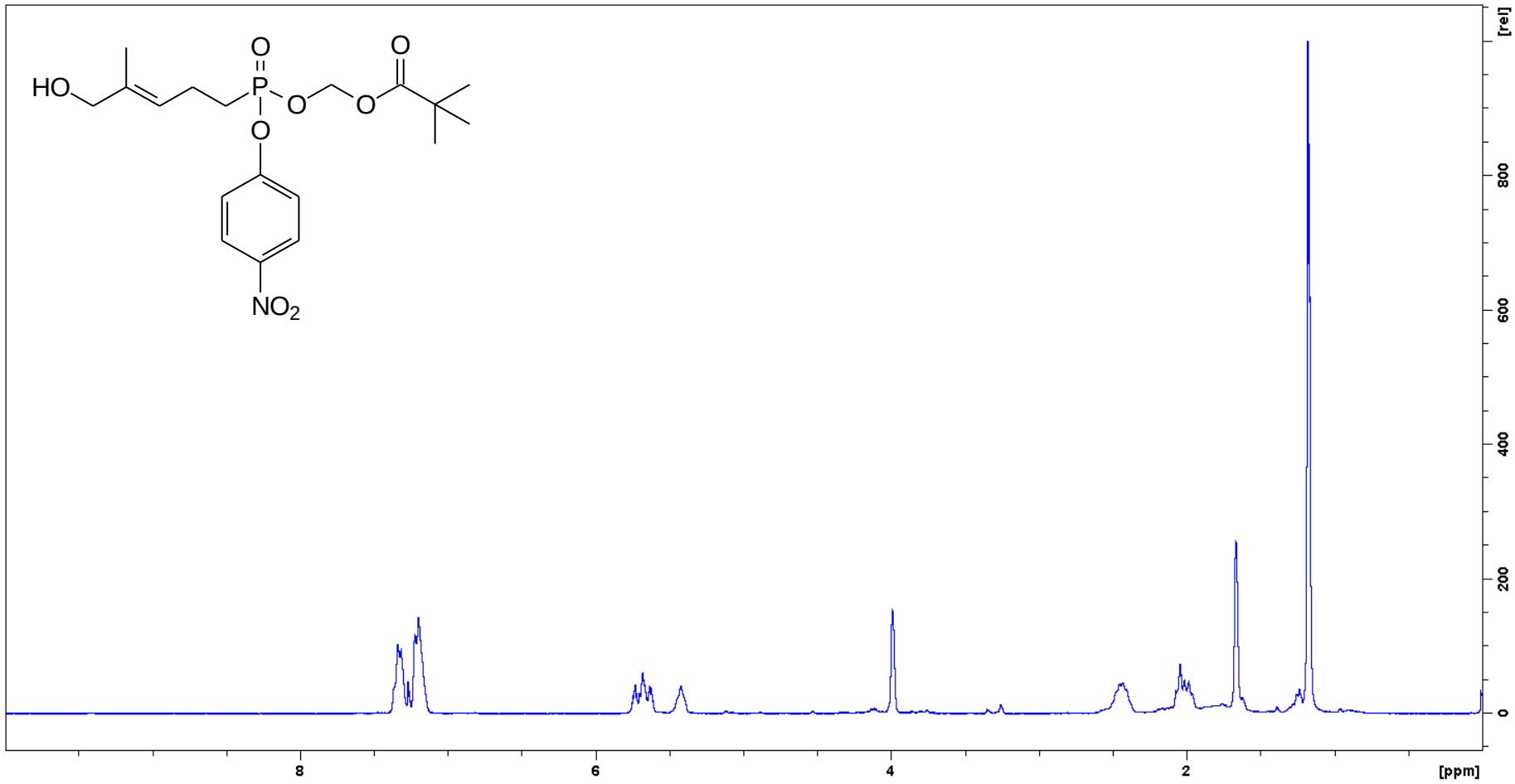

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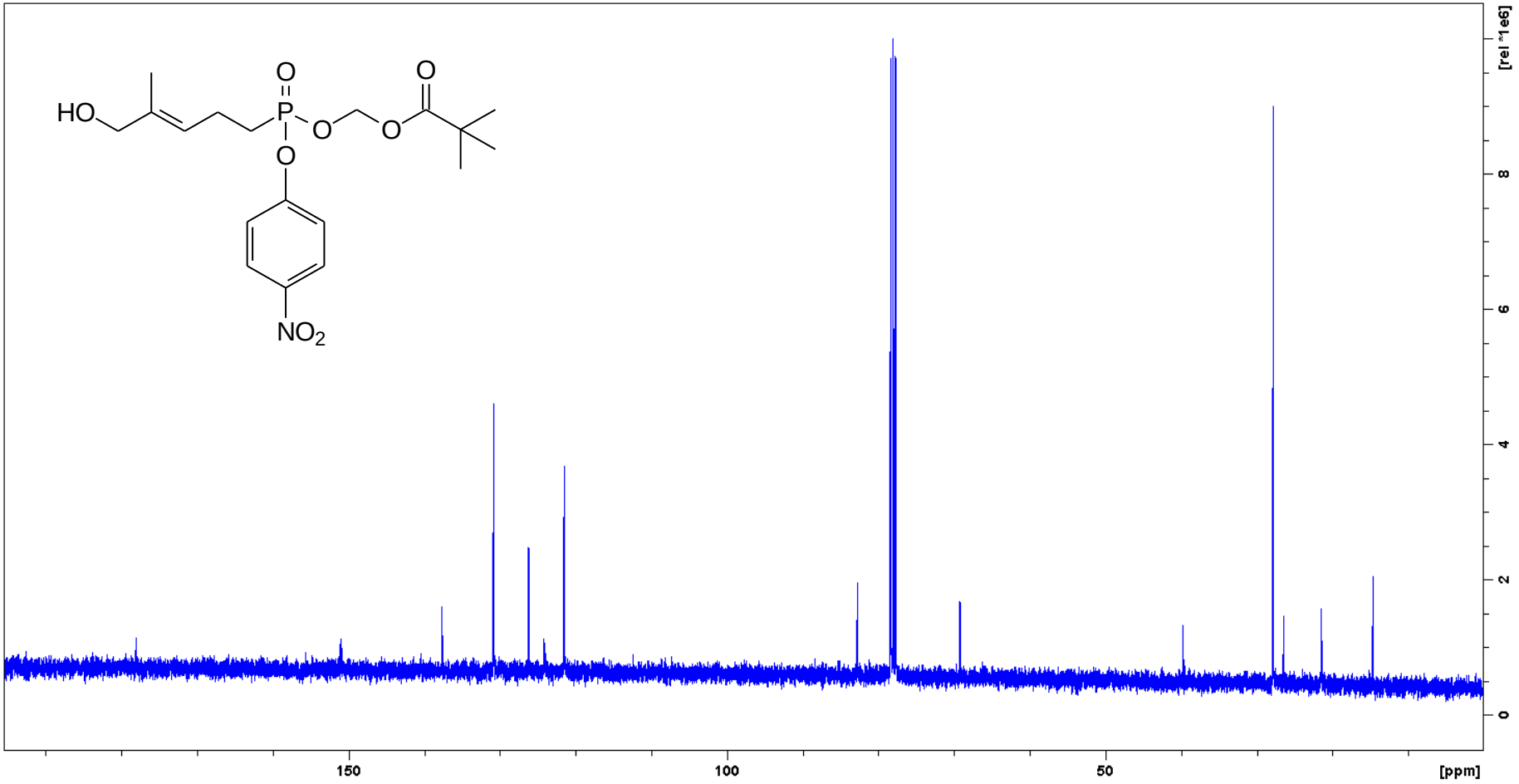
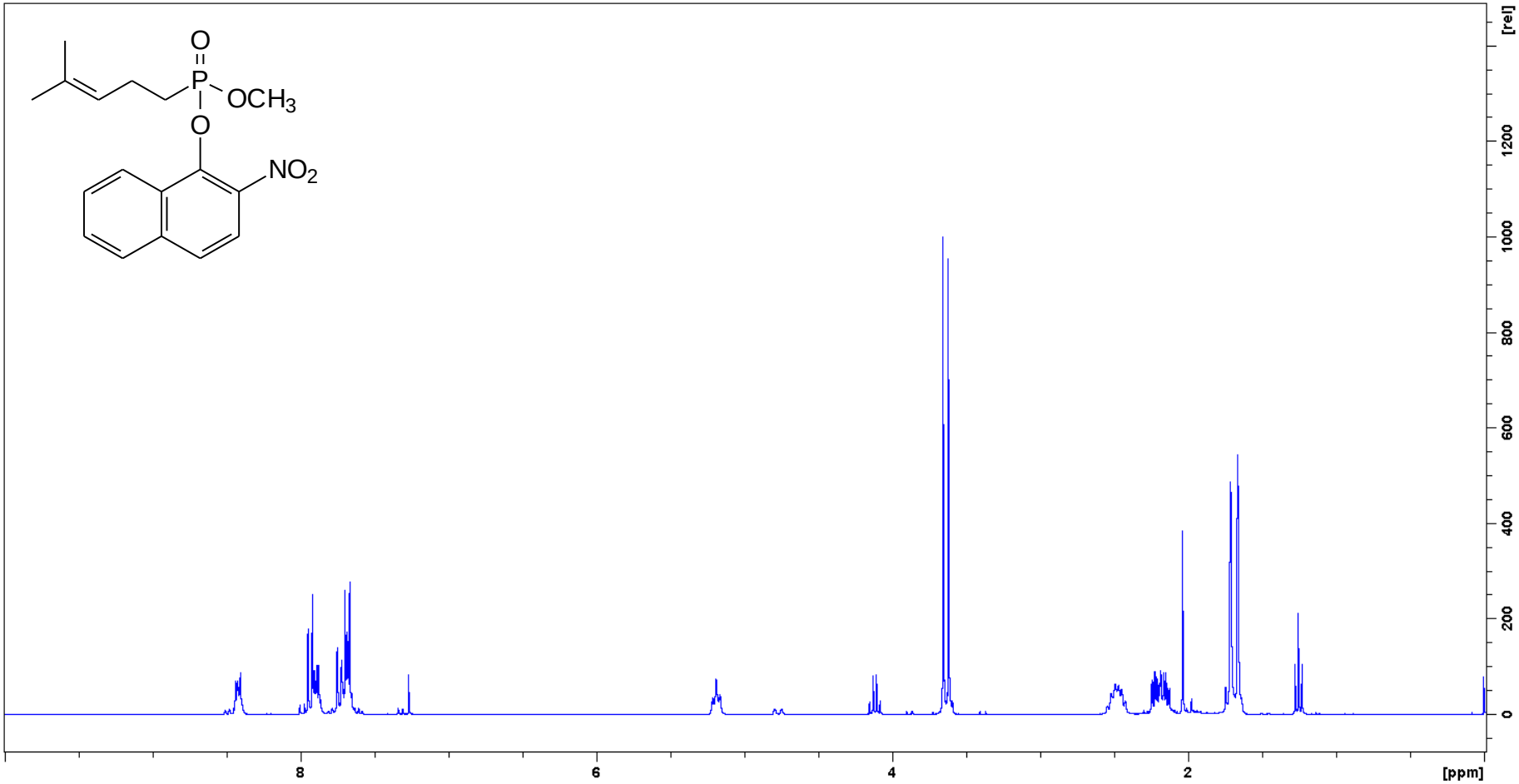


100 MHz ^{13}C NMR Spectrum of Compound **14**



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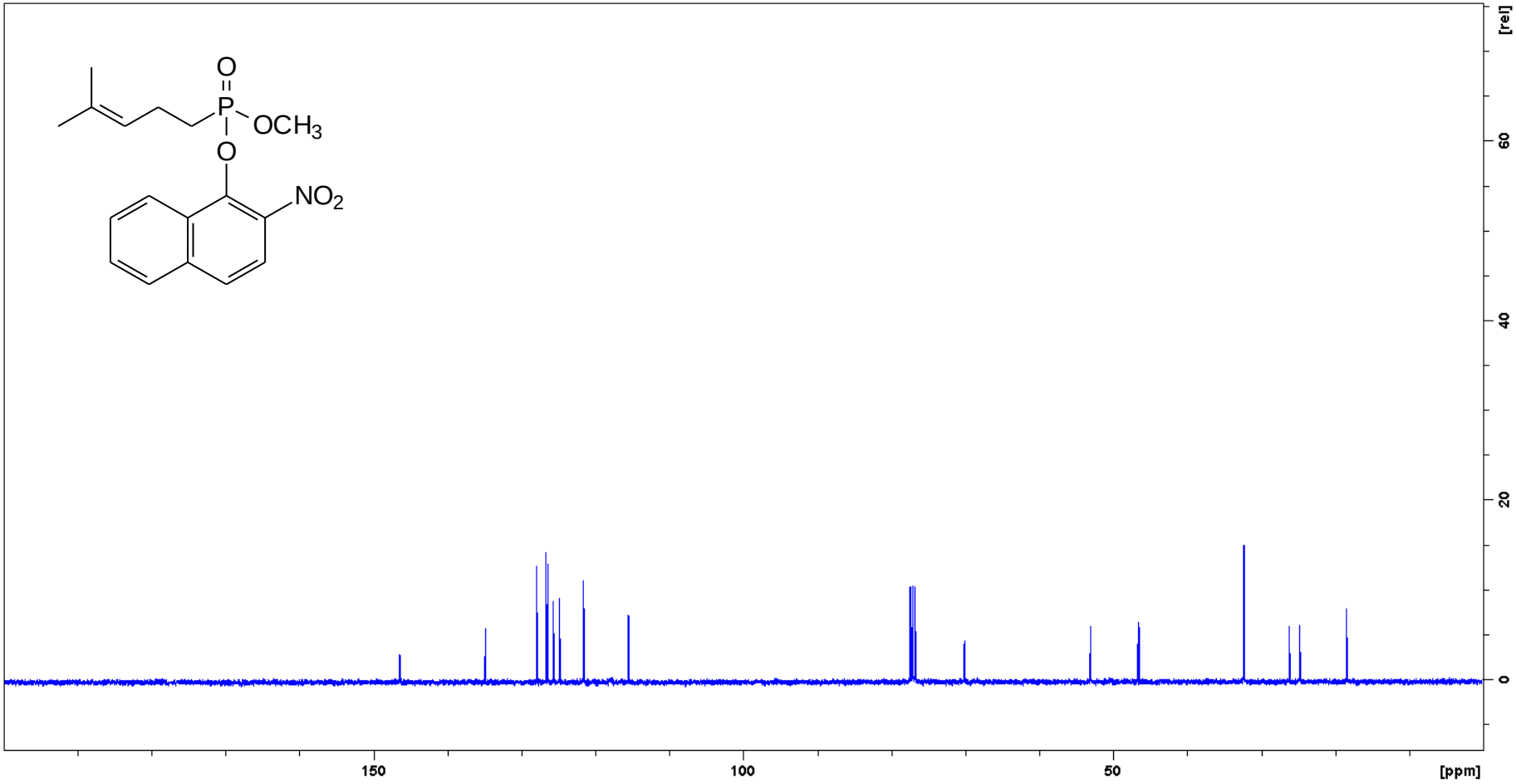
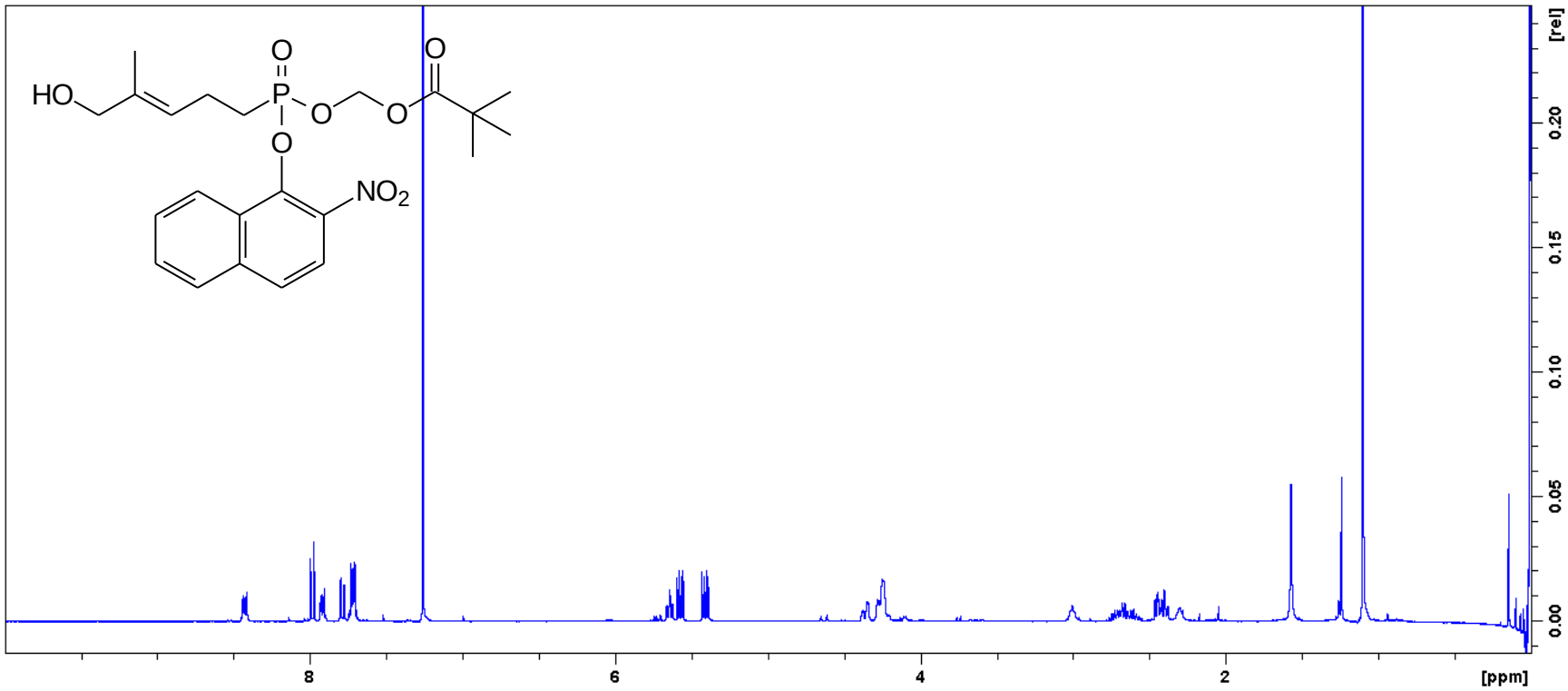


100 MHz ^{13}C NMR Spectrum of Compound **16**



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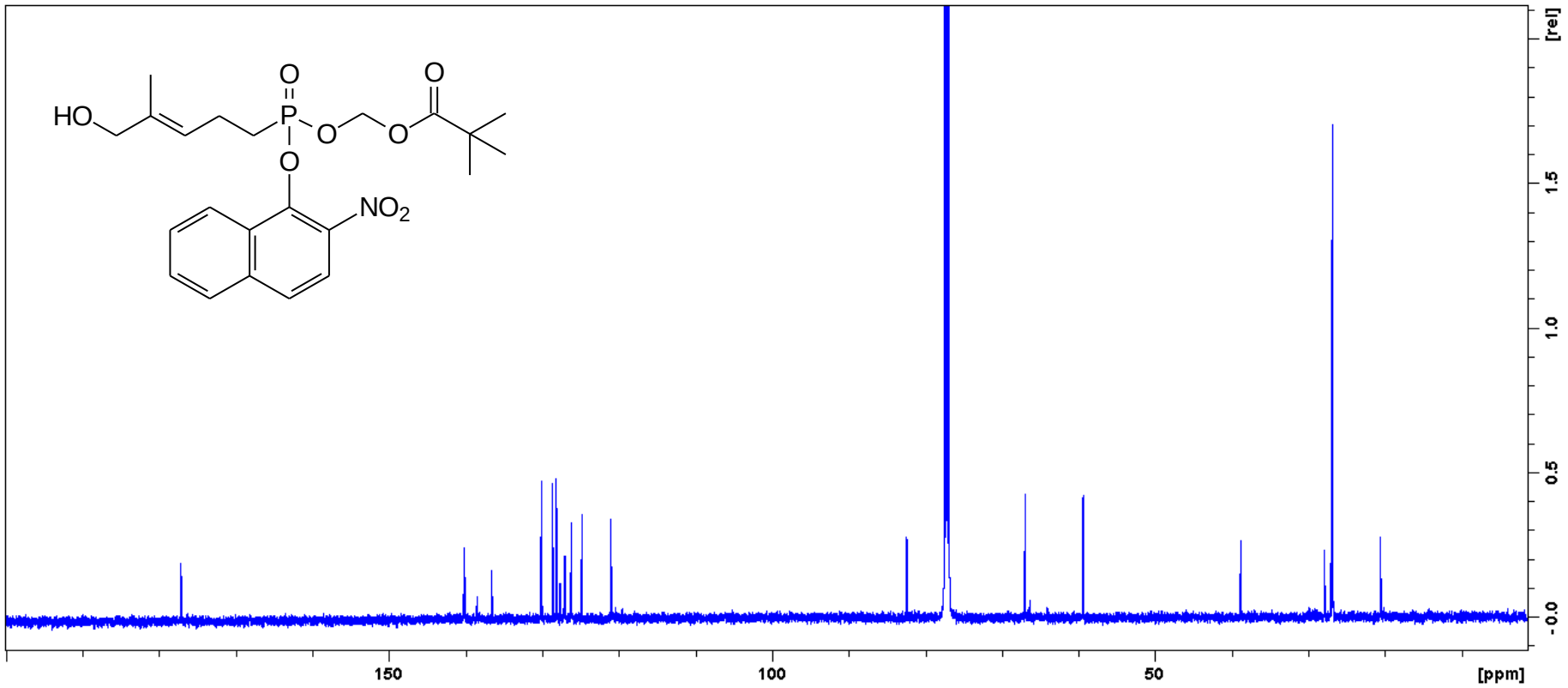

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