Supporting Information For

Discovery of phosphorodiamidate mustard-based O^2-phosporylated diazeniumdiolates with potent anticancer activity

Yu Zou,[†] Chang Yan,[†] Edward E. Knaus,[‡] Huibin Zhang,[†,*] Yihua Zhang,[†,*] Zhangjian Huang[†,*]

[†] State Key Laboratory of Natural Medicines, Jiangsu Key Laboratory of Drug Discovery for Metabolic Diseases, Jiangsu Key Laboratory of Drug Screening, China Pharmaceutical University, Nanjing 210009, PR China

[‡] Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta T6G 2E1, Canada

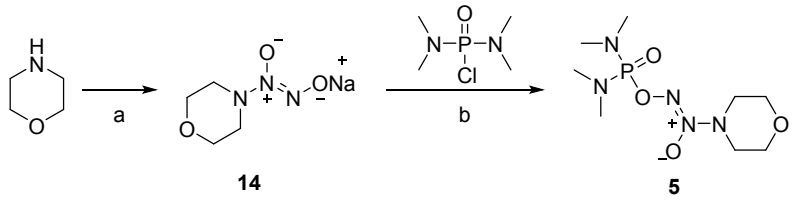
Contents

1. General information

^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectra were recorded with a Bruker Avance 300 MHz spectrometer at 300 K, using TMS as an internal standard. MS spectra were recorded on a Mariner mass spectrometer (ESI) and high resolution mass spectrometry (HRMS) spectra on an Agilent Technologies LC/MSD TOF instrument. Analytical and preparative TLC was performed on silica gel (200−300 mesh) GF/UV 254 plates, and the chromatograms were visualized under UV light at 254 and 365 nm. All solvents were reagent grade and, when necessary, were purified and dried by standard methods. The purity of all compounds tested was characterized by high resolution mass spectrometry (Agilent Technologies LC/MSD TOF). Individual compounds with a purity of > 95% were used for biological experiments. Compounds **10-12** were prepared according to references 1-3.

2. Experimental information and characterization Data

Scheme S1. The synthetic route for compounds **5**.



Reagents and conditions: a) NO gas, 50 psi, anhydrous ether, NaOMe/MeOH, rt, 36h; b) N_2, THF, -28°C, 20%.

***O*^{2}-[Bis(dimethylamino)phosphoryl]-1-morpholine-diazen-1-ium-1,2-diolate (5)**

To a solution of bis(dimethylamino) phosphorodiamidic chloride (6 mmol) in dry THF (20 mL) at -28℃ under nitrogen was added 1-morpholine-diazen-1-ium-1,2-diolate sodium salt (6 mmol) which was prepared by using a previously reported method.[1] The reaction mixture was stirred at -28℃ for 6~8h until the starting material was totally consumed as indicated by TLC. The solvent was evaporated and then the crude product was purified by column chromatography [4% (v/v) MeOH-CH_2Cl_2] to give **5** (472 mg, 28% yield) as a colorless oil. ^{1}H NMR (300 MHz, CD_3OD), δ(ppm): 2.66~2.77 (m, 12H, 4×NCH_3), 3.47 (t, J = 10.9 Hz, 4H, CH_2NCH_2), 3.84(t, J = 11.8 Hz, 4H, CH_2OCH_2), ESI-MS 304.1 $[M + Na]^+$.

Preparation of *N*, *N*-bis (2-chloroethyl) phosphorodiamidic chloride (10)

A 100 mL, three-neck, roundbottom flask was charged with phosphorus oxychloride (1.04 mL, 11.2 mmol), bis(2-chloroethyl)amine hydrochloride (2.0 g, 11.2 mmol), and toluene (25 mL). Then triethylamine (3.28 mL, 23.5 mmol) was added to the reaction mixture over 10 min. The reaction mixture was then stirred for 26 h at room temperature. The crude product (**10**) was used without further purification to the next step.[1,2] The proton MS spectrum was consistent with the proton MS spectrum of a known compound online (CAS NO. 127-88-8).

Preparation of *N*, *N*, *N*', *N*'-tetrakis (2-chloroethyl) phosphorodiamidic chloride (11)

To this crude product (**10**) mixture was charged bis(2-chloroethyl)-amine hydrochloride (2.0 g,

11.2 mmol) and triethylamine(3.3 mL, 23.5 mmol) over 10 min. The tan suspension was heated to toluene reflux (110 °C) and stirred for 20 h. The mixture was then cooled to room temperature and filtered to remove the triethylamine hydrochloride salt. The solvent was removed under reduced pressure (30 mmHg, final bath temperature at 50 °C) on a rotary evaporator to produce crude *N*,*N*,*N*',*N*'-tetrakis (2-chloroethyl) phosphorodiamidic chloride (**11**) as a reddish-brown oil (3.3 g, 81% yield), which was used without further purification to the next step. TLC R_f 0.71 (ethyl acetate/hexanes, 1:1). The proton NMR spectrum ($CDCl_3$) was consistent with the proton NMR spectrum of a commercially available batch of this material (Aldrich, lot no. 11608 TZ).[1,2]

Preparation of *N*, *N*-bis (2-chloroethyl) –*P*-(4-methyl-1-piperazinyl)-phosphorodiamidic chloride (12)

To a solution of crude **10** (11.2 mmol) in anhydrous DCM (25 mL) was charged *N*-methyl piperazine (1.24 mL, 11.2 mmol) and triethylamine (3.3 mL, 11.5 mmol) over 10 min. The reaction mixture was stirred for 8 h at room temperature. When the reaction was complete, the solvent was evaporated and anhydrous diethyl ether was added to dissolve the intermediate product, while the undissolved triethylamine hydrochloride was separated by filtration. The crude product (**12**), which was obtained by evaporation of the diethyl ether, was used without further purification to the next step. The proton MS spectrum was consistent with the proton MS spectrum of a known compound online (CAS NO. 1333491-96-5).[3]

3. Stability evaluation for compound 7

Considering that compound **7** showed better cytotoxicity against human cancer cell lines, we evaluated the stability of compound **7**. The stability of 7 in PBS (50mM) at pH 7.4 was evaluated by HPLC method. As shown in Figure S1, the remaining percentage of 7 did not decline nearly, even after incubation of 28h, generating good stability in the above condition.

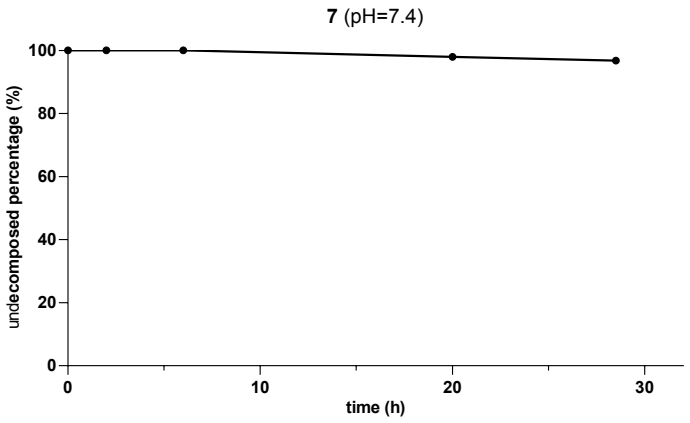


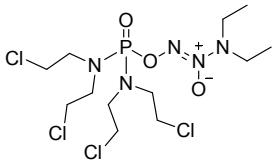
Figure S1. 50 μM Compounds **7** were incubated in pH 7.4 PBS buffer (50mM) at 37 °C in 28 hours.

3. References:

(1) M. H. Lyttle, A. Satyam, M. D. Hocker, K. E. Bauer, C.G. Caldwell, H. C. Hui, A. S. Morgan, A. Mergia and L. M. Kauvar, *J. Med.Chem.*, **1994**, *37*, 1501-1507.

(2) A. Satyam, M. D. Hocker, K. A. Kane-Maguire, A. S. Morgan, H. O. Villar and M. H. Lyttle, *J. Med.Chem.*, **1996**, *39*, 1736-1747.
(3) X.L. Chen, J.W. Yuan, L.B. Qu, Z.B. Qu, S.H. Xu, F.J. Wang and Y.F. Zhao, *Phosphorus, Sulfur, and Silicon*., **2012**, *187*, 245–254.

4. Spectral copies of ^{1}H NMR ,^{13}C NMR, ^{31}P NMR and HRMS of compounds 6-9



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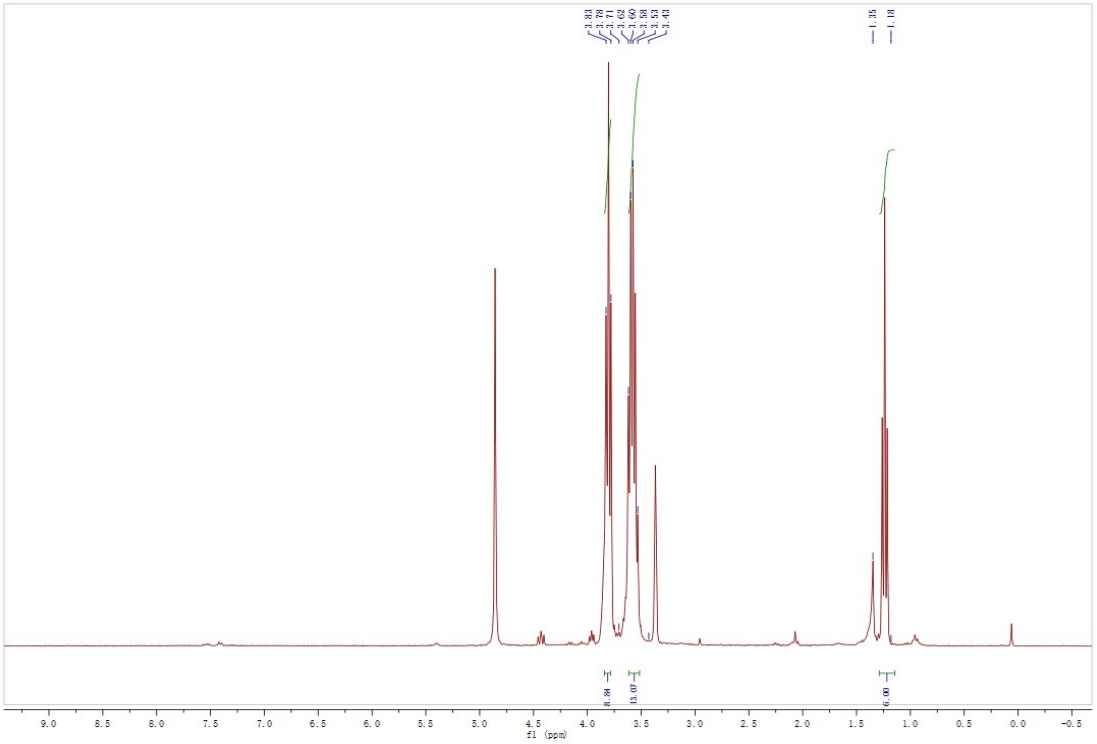
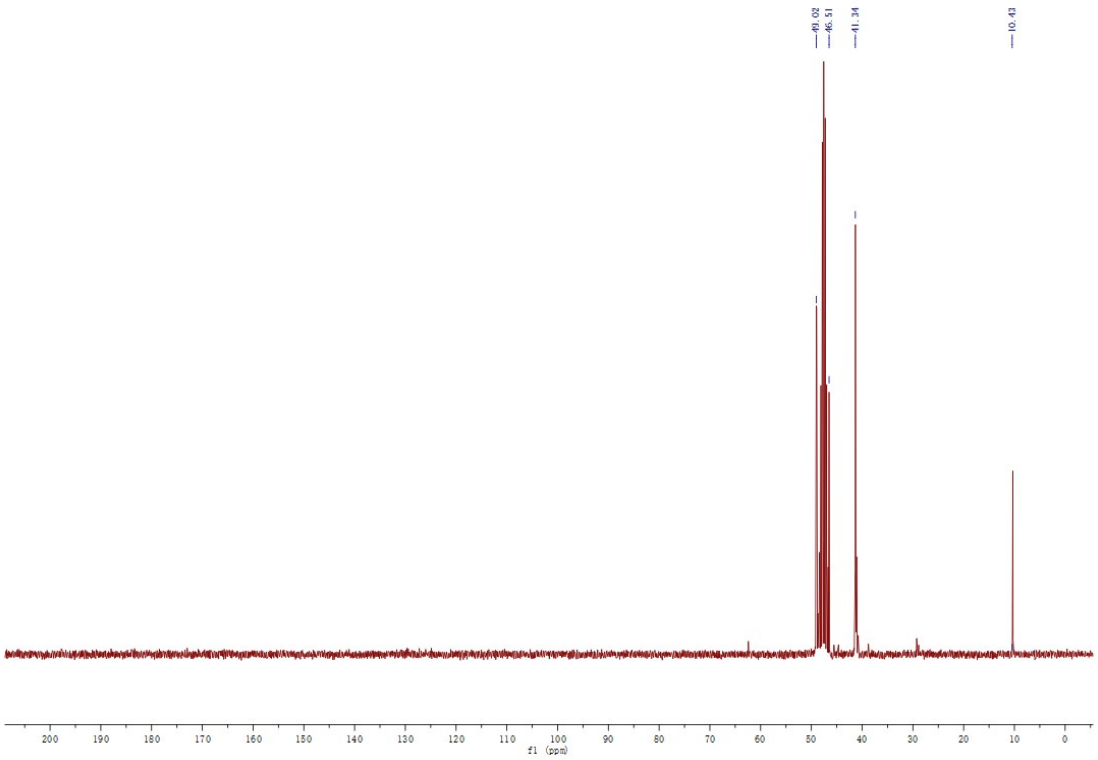


^{1}H NMR spectrum of **6**



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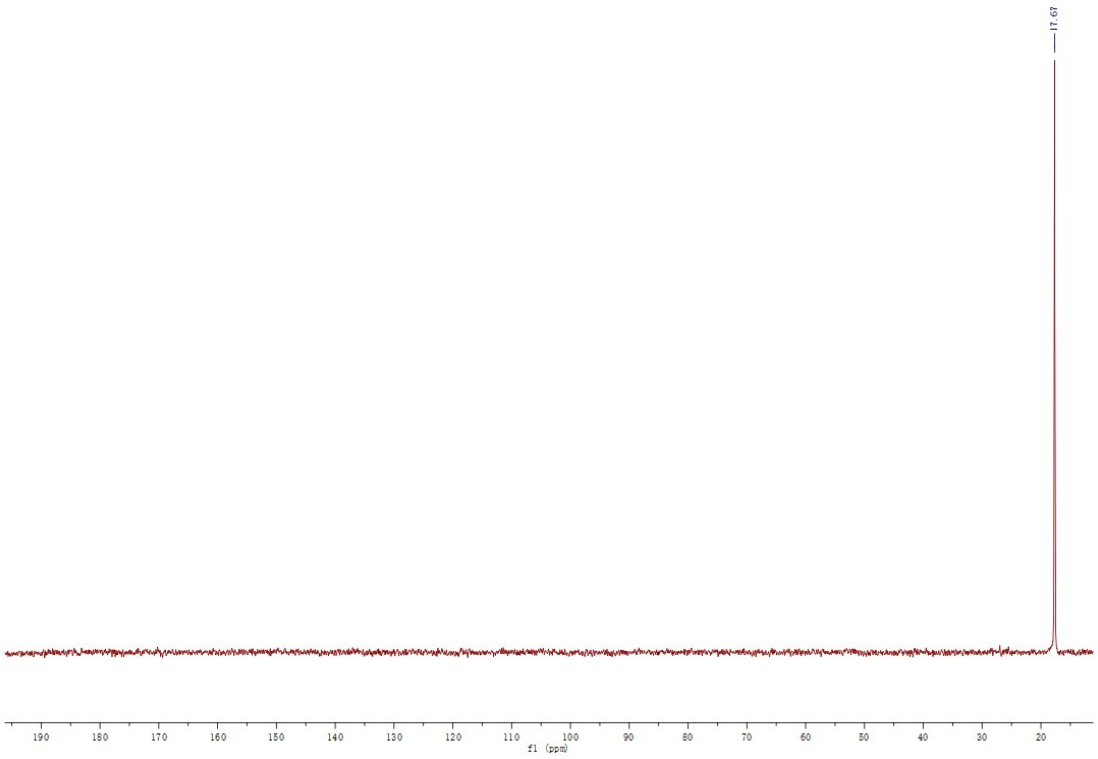

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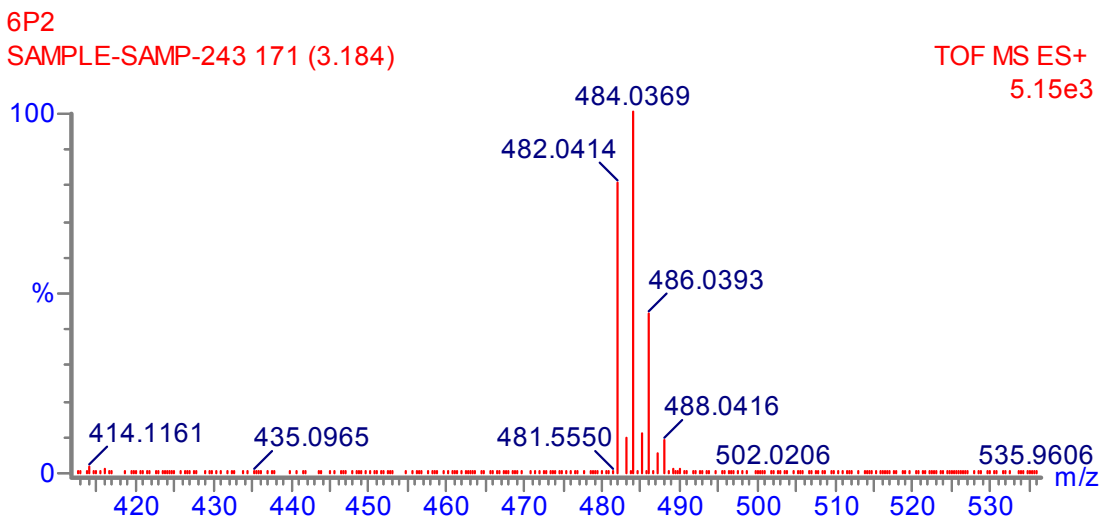
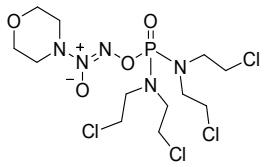


HRMS of **6**



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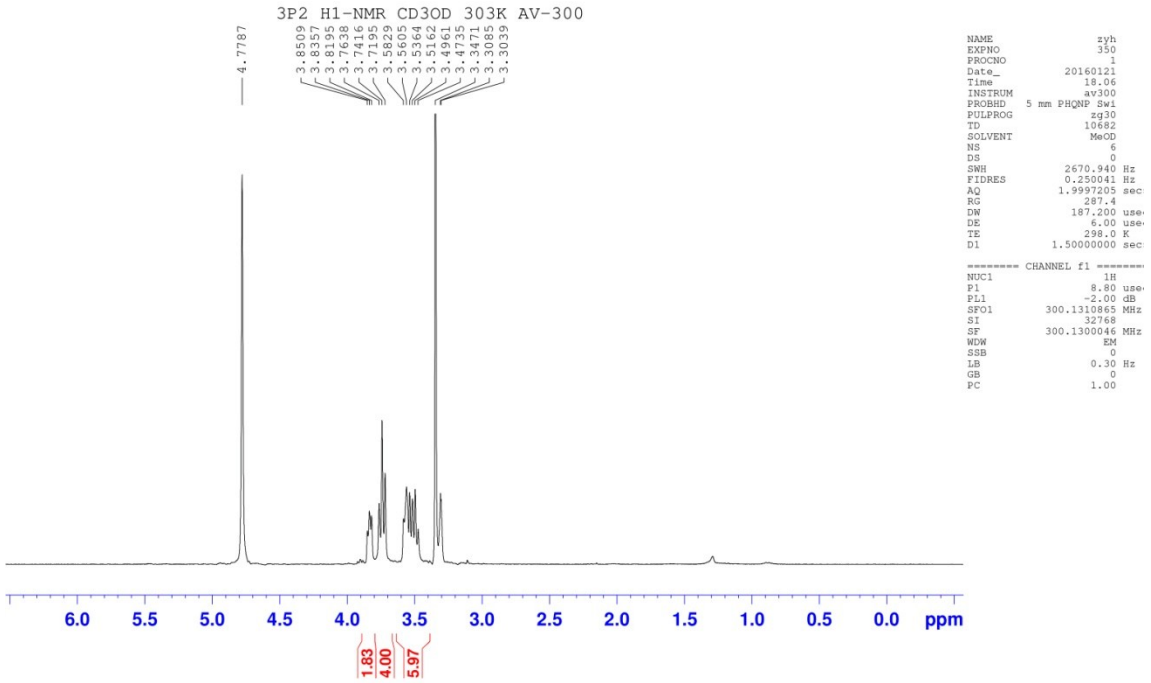

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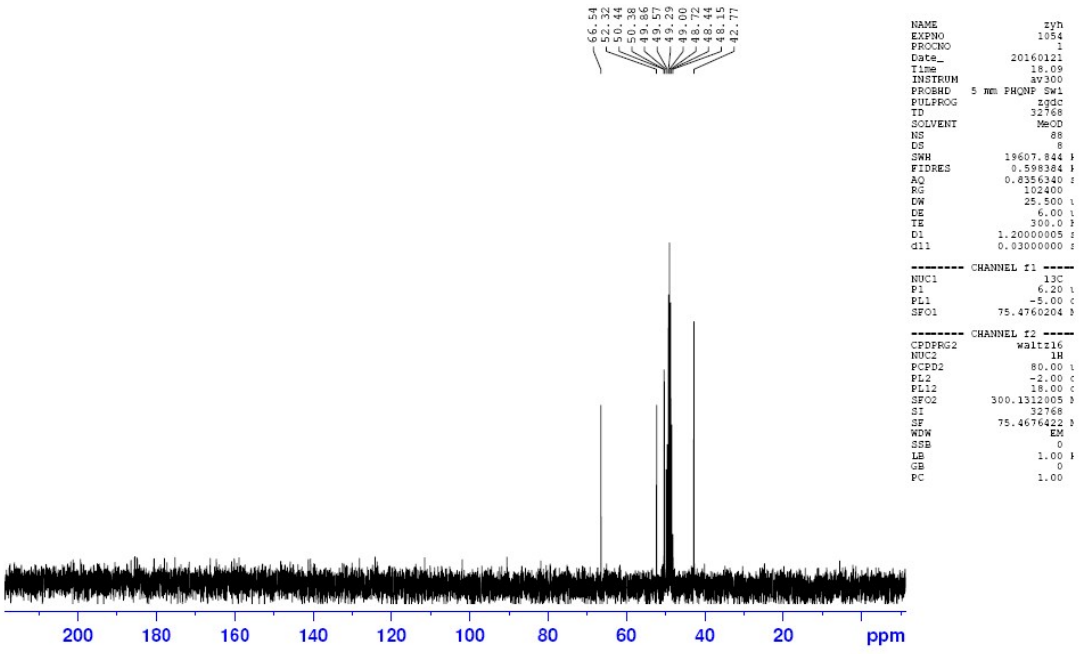

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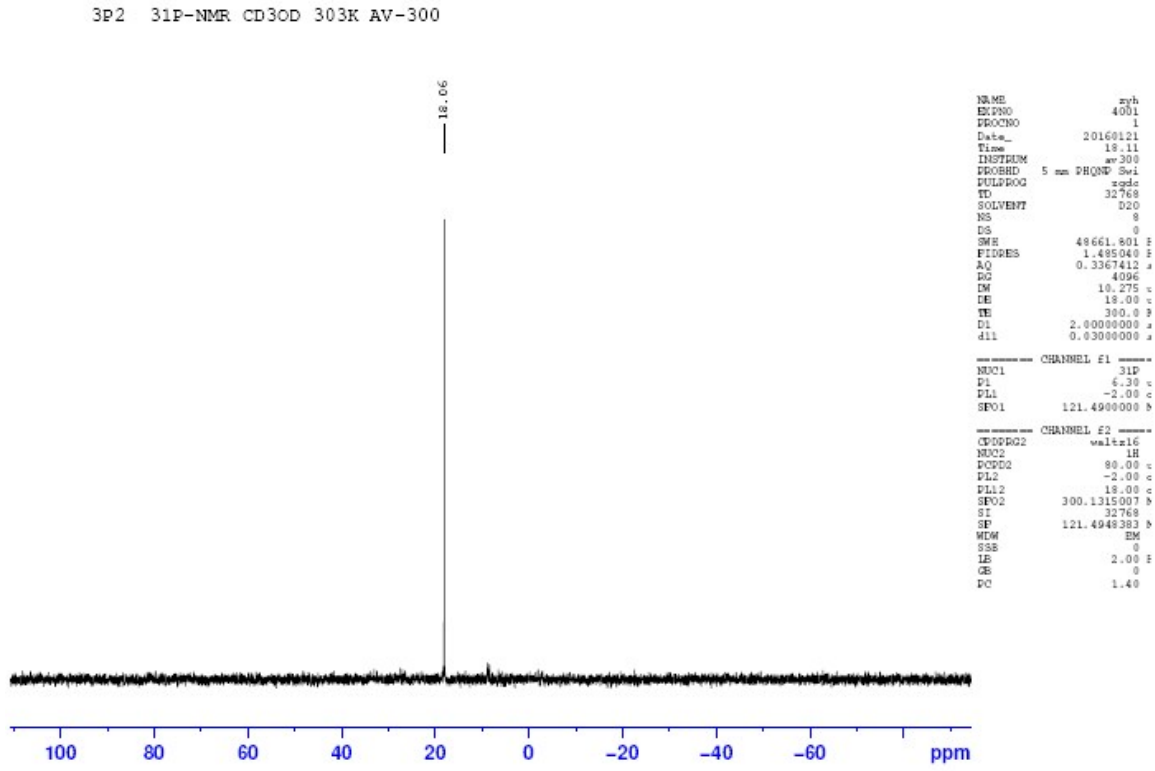

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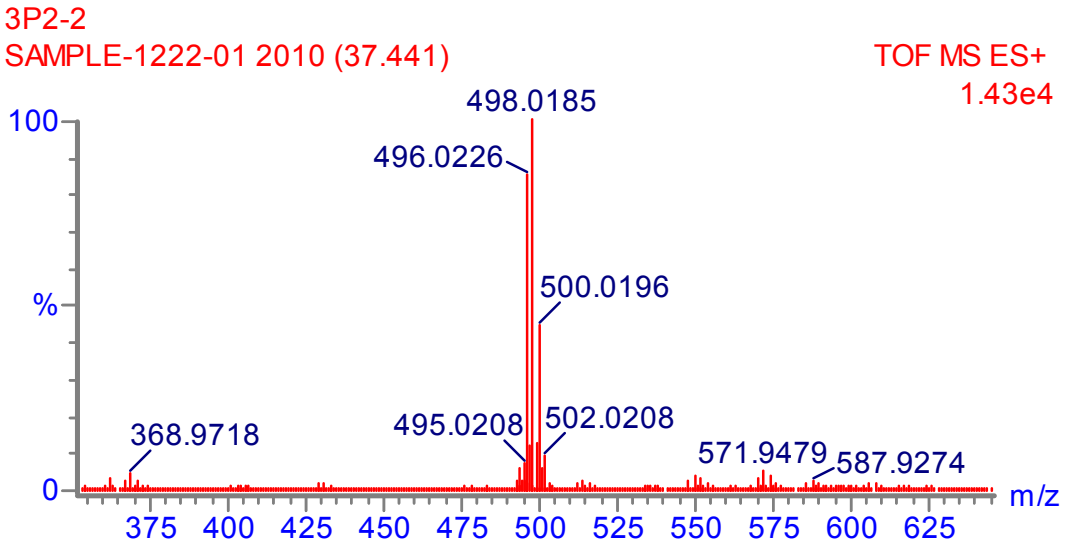
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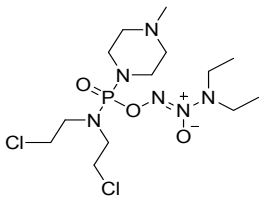

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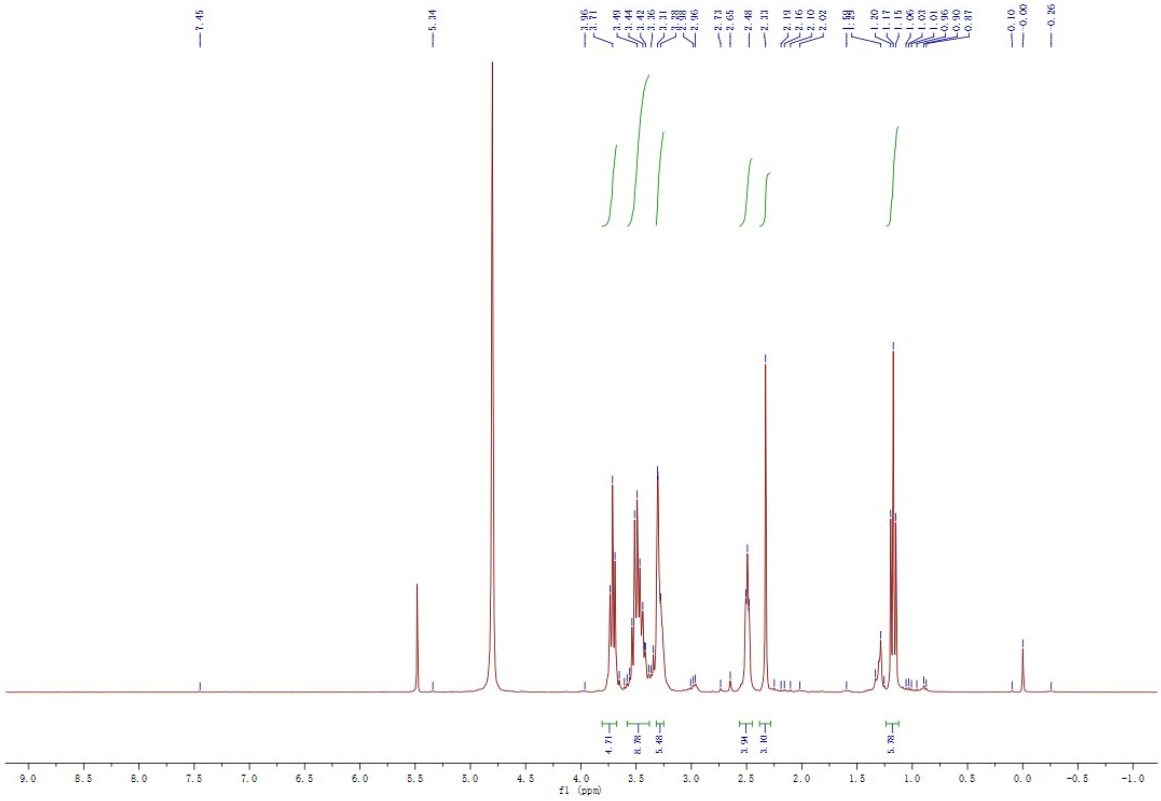

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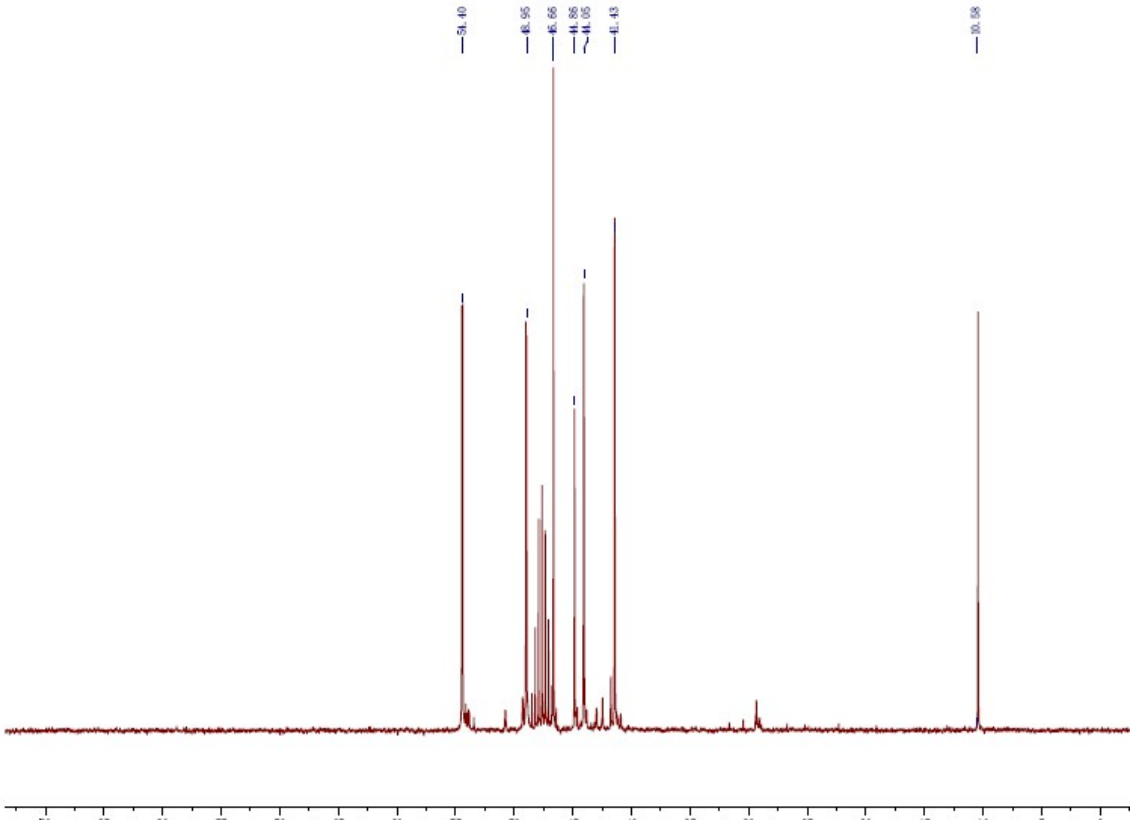

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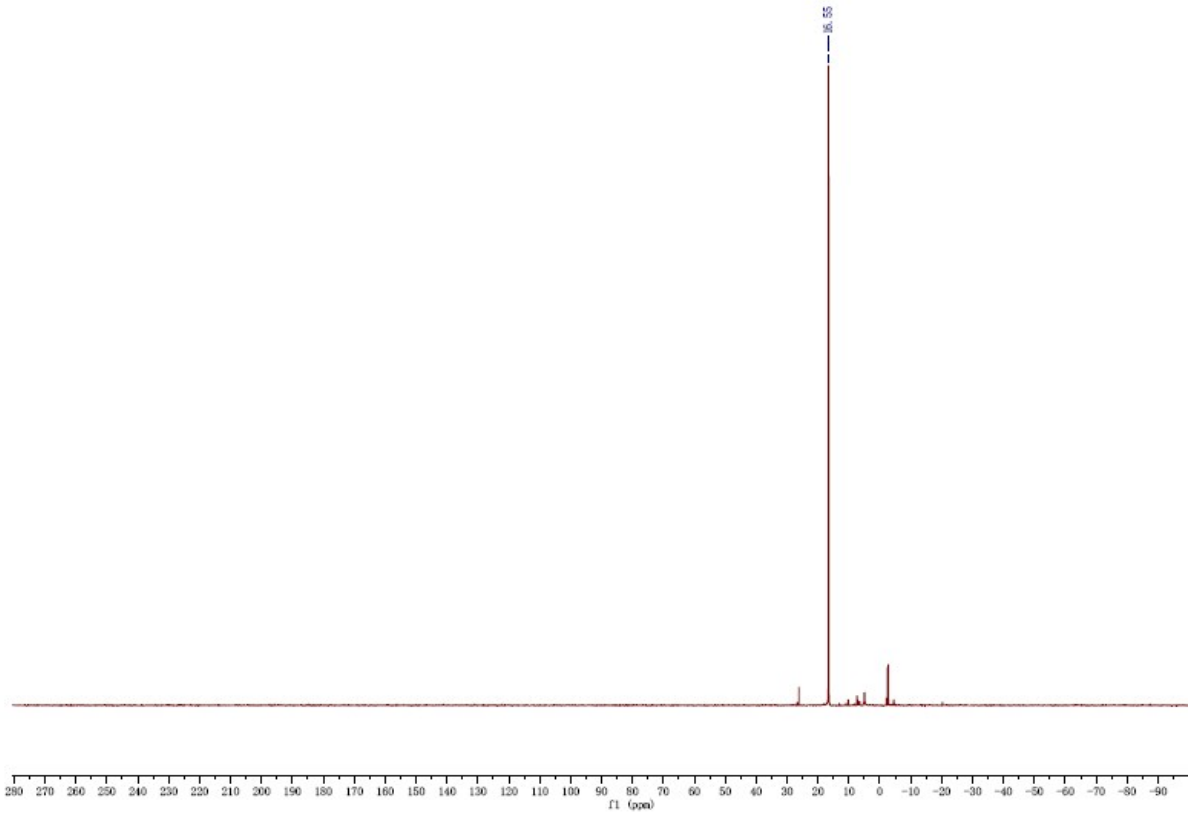

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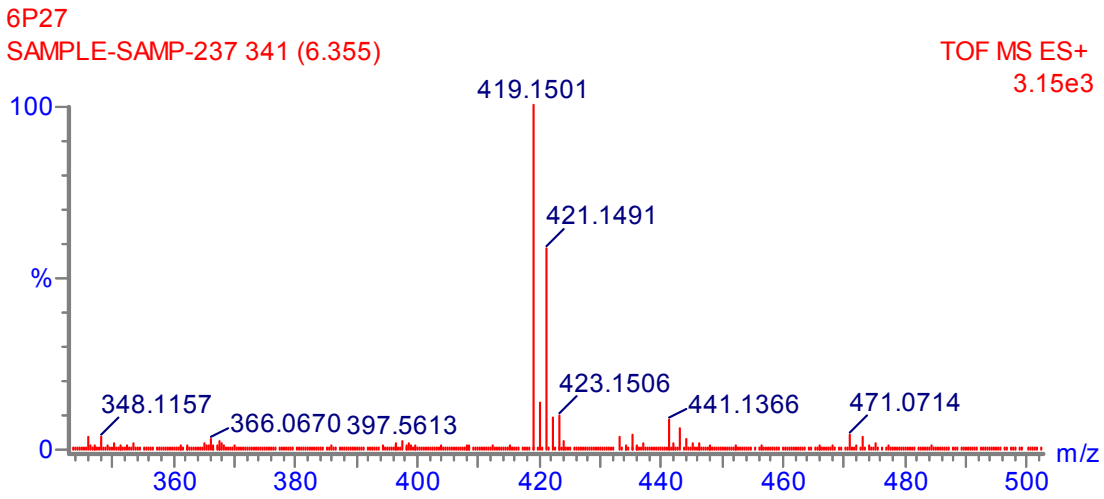

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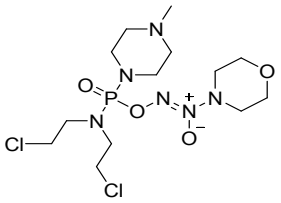

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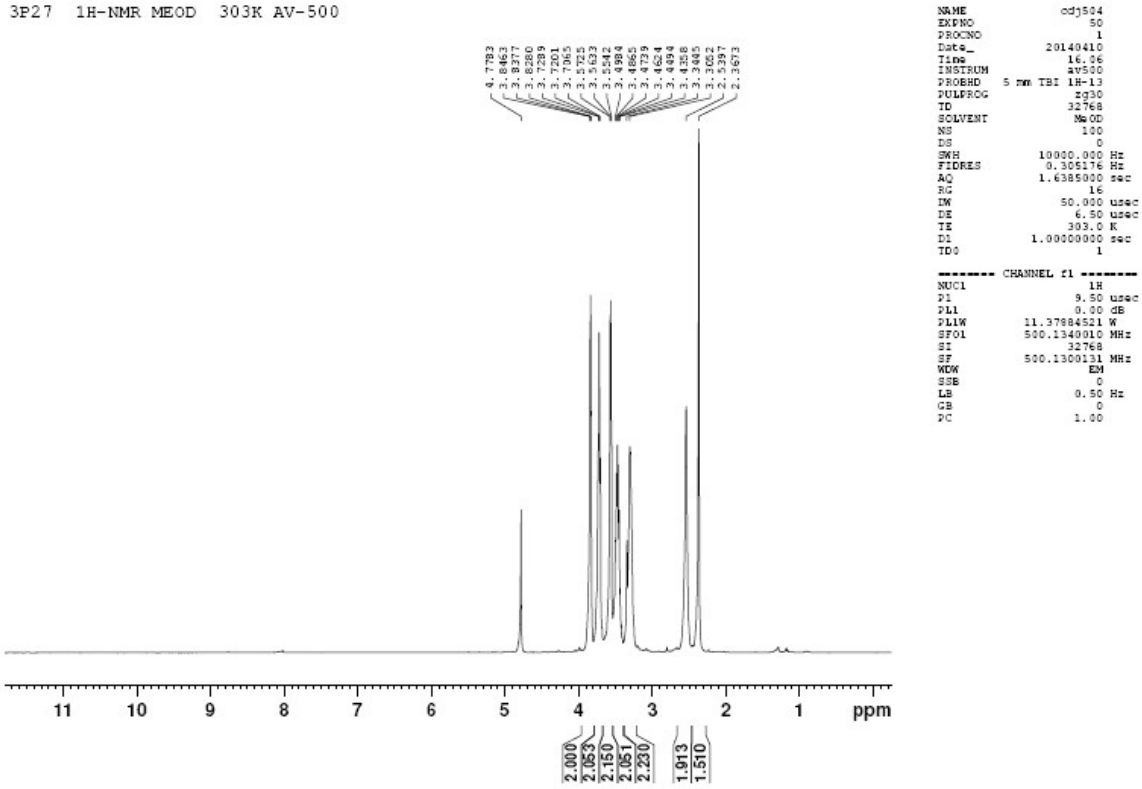
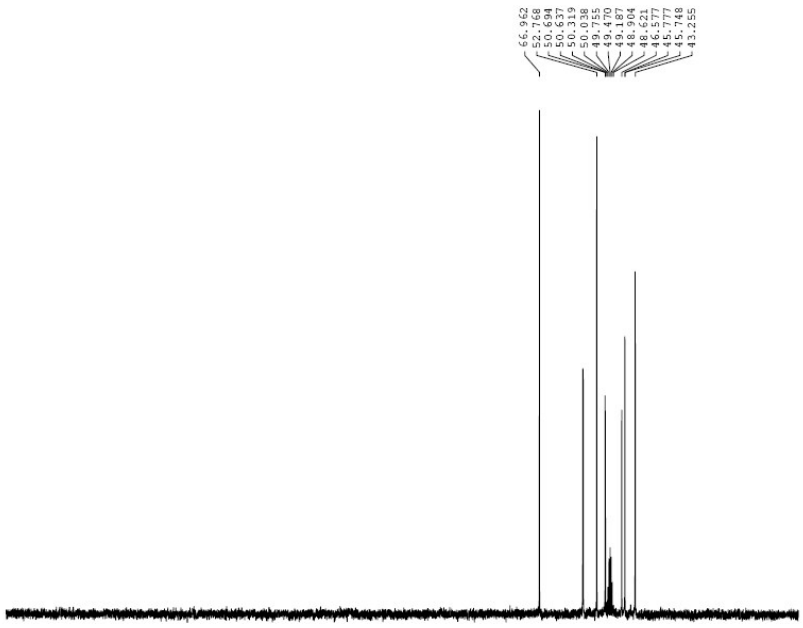


^{1}H NMR spectrum of **9**

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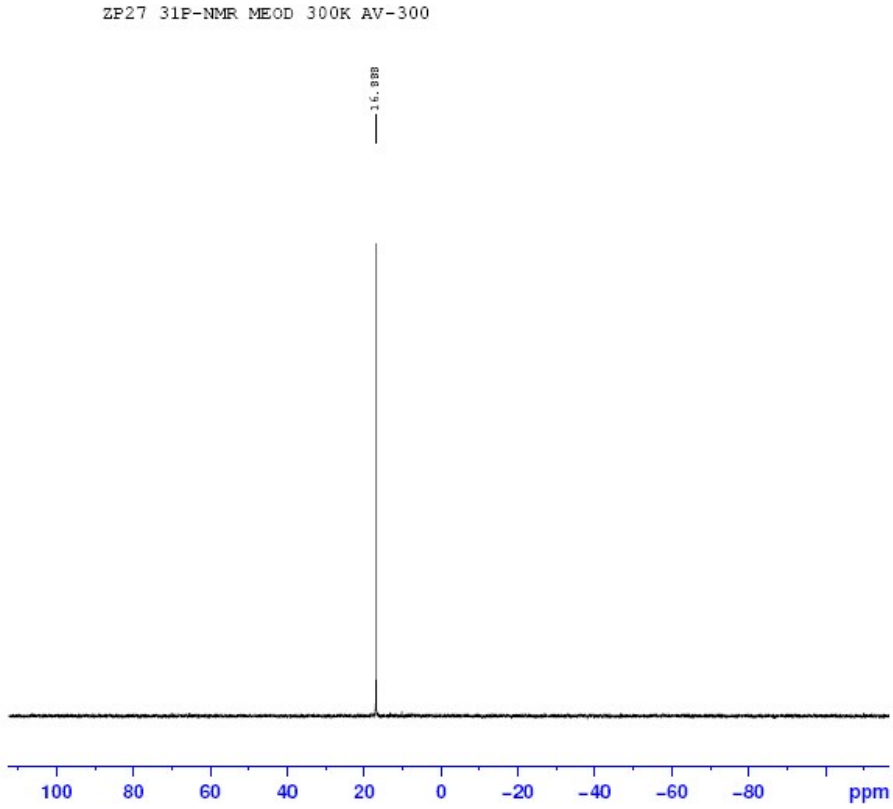
^{13}C NMR spectrum of **9**

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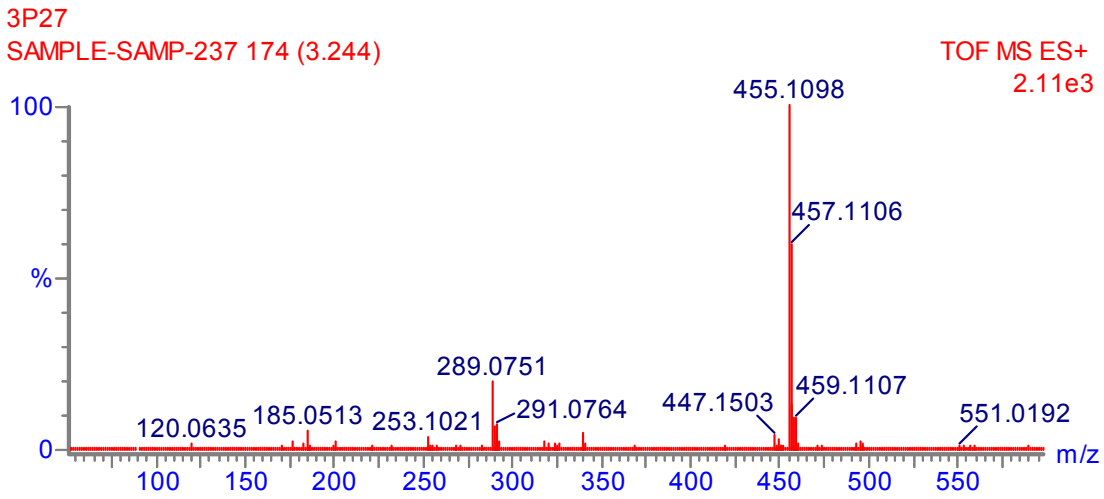
^{31}P NMR spectrum of **9**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HRMS of **9**