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N^5-Phosphonoacetyl-L-ornithine (PALO): A convenient synthesis and investigation of influence on regulation of amino acid biosynthetic genes in *Saccharomyces cerevisiae*

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Abstract

A scalable four-step synthesis of the ornithine transcarbamylase inhibitor N^5-phosphonoacetyl-L-ornithine (PALO) is achieved through boroxazolidinone protection of ornithine. Investigations in the model organism *Saccharomyces cerevisiae* found that, in contrast to a previous report, PALO did not influence growth rate or expression of genes involved in arginine metabolism.

Keywords: PALO, Ornithine transcarbamylase, *Saccharomyces cerevisiae,* Arginine biosynthesis

N^5-Phosphonoacetyl-L-ornithine (PALO, **1**) is a bisubstrate transition-state analog which competitively inhibits ornithine transcarbamylase (OTC) in vitro, blocking biosynthesis of citrulline in both the urea and the arginine biosynthetic pathways (Figure 1). [1, 2] Competitive inhibitors of amino acid metabolism have been key components in studies ranging from arginine starvation in fungi and bacteria to studies on OTC deficiency. [3, 4]

PALO has been tested as a potential tool for in vivo metabolic studies. It failed to inhibit citrulline biosynthesis in isolated rat mitochondria or intact rat hapatocytes,[5] and did not inhibit growth of the bacterium *Escherichia coli*.[6] These failures were attributed to the inability of PALO to cross cell membranes. In contrast, Kinney and Lusty showed that PALO induced arginine limitation in the yeast *Saccharomyces cerevisiae*.[4] The regulation of arginine metabolism in fungi is particularly important to understand because of the role of this amino acid in protein synthesis, as a nitrogen source and in signaling. [2, 7–10] We now report a short and scalable synthesis of PALO, as well as results of investigations into the influence of PALO on growth and arginine metabolism in *S. cerevisiae*.

The first syntheses of PALO employed the direct functionalization of copper-complexed ornithine. This strategy, while rapid, requires a significant set of purification steps. [1, 2, 5, 11] Several more traditional routes begin with partially protected derivatives of ornithine, [3, 5] some of which are no longer commercially available. As illustrated in Scheme 1, we found that temporary masking of the amino acid of ornithine as the boroxazolidinone derivative enables a rapid and scalable synthesis of PALO in highly pure form. Reaction of L-ornithine with 9-borabicyclononane (9-BBN) furnished the corresponding boroxazolidinone, [12, 13] which was directly condensed with dibenzylphosphonoacetic acid (**2**) to furnish amide **3**.[14] We were unable to achieve deprotection of the boroxazolidinone with aq HCl or methanolic ethylenediamine,[12] even though these conditions were successfully modeled on the corresponding adduct of phenylalanine.[15] Fortunately, dissolution of **3** in MeOH/$CHCl_3$ resulted in slow but clean deprotection to amino acid **4**.[16] Hydrogenolysis of the benzyl esters proceeded nearly quantitatively to furnish PALO (**1**) as a neutral molecule which was pure by ^1H, ^{13}C, and ^{31}P NMR. The PALO·NH_4 salt could be prepared by elution from an ion-exchange column.

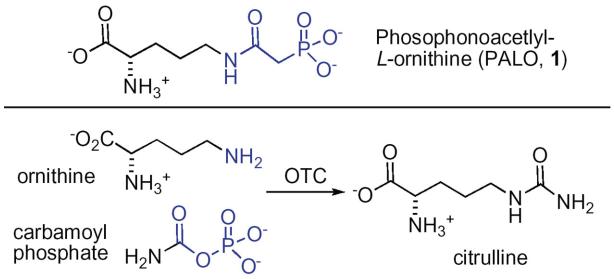
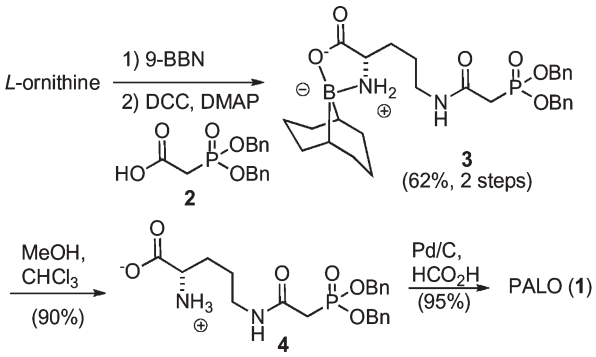


Figure 1. PALO; OTC-catalyzed synthesis of citrulline.



Scheme 1. Synthesis of PALO (**1**).

Kinney and Lusty reported that PALO increased expression of *CPA1*, *CPA2*, *HIS3*, and *TRP5* reporter constructs in the yeast *S. cerevisiae*.[4] The data were consistent with limitation of arginine leading to specific up regulation of arginine biosynthetic genes (*CPA1* and *CPA2*), and a general amino acid starvation response typified by up regulation of *HIS3* and *TRP5*.

We tested whether treatment with PALO resulted in changes in mRNA accumulation and/or translation of genes involved in amino acid biosynthesis. We examined expression of two genes required for arginine biosynthesis; *ARG5,6*, which encodes a protein involved in the second and third steps of arginine biosynthesis from glutamate, and *CPA1*, which encodes a subunit of arginine-specific carbamyl-phosphate synthetase. Both of these genes are up regulated specifically in response to arginine limitation. Expression of *TRP5*, which encodes an enzyme that catalyzes the final step in tryptophan biosynthesis, was also examined as an indicator of the general amino acid starvation response. Quantitative northern analysis was performed to measure mRNA abundance, a measure of the combined rates of transcription and mRNA decay. Western analysis was used to look at the level of protein synthesis for these genes. This combined approach enabled direct evaluation of effects on transcription, mRNA stability, and translation.

Although the yeast strains from the earlier studies were not available,[4] they were derived from the W303 genetic background, which usually carry the *can1-100* mutation.[17] As *CAN1* encodes an arginine permease required for efficient uptake of arginine, the *can1-100* mutation is not well suited for the current experiments. As a compromise, and to assess if PALO will be generally useful as a metabolic tool in *S. cerevisiae*, the current data was obtained using the BY4741 strain of *S. cerevisiae*.[18] This strain is derived from S288C, the strain used in the systematic sequencing project, and it is the most commonly used genetic background for genome-wide functional analyses.

If PALO affects transcription or mRNA stability, we expected to observe an increase in expression of *ARG5,6*, *CPA1*, and *TRP5* mRNA transcripts in the presence of PALO. We also expected that addition of arginine would relieve the arginine limitation induced by PALO. Cells were grown and treated with either 15 μM of the synthesized PALO, 1 mg/mL L-arginine, or 15 μM of the synthesized PALO and 1 mg/mL L-arginine as described by Kinney and Lusty.[4] An untreated culture was grown in the same media as a control. The abundance of *ARG5,6*, *CPA1*, and *TRP5* mRNAs was measured by quantitative northern analysis (Figure 2A). The abundance of *ARG5,6*, *CPA1*, and *TRP5* mRNAs in the PALO treated cells was not significantly different from the untreated cells. The abundance of *ARG5,6* and *CPA1*, but not *TRP5* mRNAs was lower in cells treated with arginine regardless of whether the cells were treated with PALO or not. Additionally, the abundance of the *TRP5* transcript did not show any variation in response to addition of PALO, arginine or both. In summary treatment with PALO had no effect on the abundance of *ARG5,6*, *CPA1* or *TRP5* mRNAs.

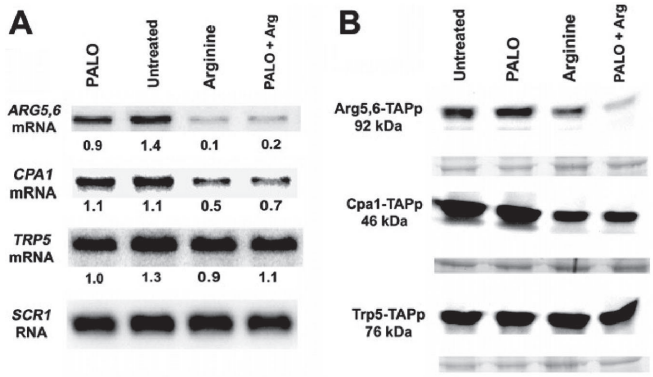


Figure 2. PALO has no effect on expression of the *ARG5,6*, *CPA1*, and *TRP5* genes in *Saccharomyces cerevisiae*. *S. cerevisiae* strains [18, 19] were inoculated from a saturated culture into minimal medium supplemented with leucine, methionine, uracil and histidine. Cultures were treated with 15 μM PALO in a potassium phosphate pH 6.8 buffer, potassium phosphate buffer pH 6.8 alone (untreated), 1 mg/mL L-arginine, or both 15 μM PALO and 1 mg/mL L-arginine at the time of inoculation. Cells were incubated at 30 °C and then harvested at mid-log growth phase (OD_{600} 0.4–0.6). (A) Northern blots were prepared with total RNA extracted from BY4741 [18, 19] and probed with oligolabeled DNA probes.[20] DNA probes were generated using primer sets for amplifying yeast open reading frames based on the sequences available from the Saccharomyces Genome Database. Shown are representative phosphorimages of a northern blot probed with radioactive *ARG5,6*, *CPA1*, *TRP5*, and *SCR1* DNAs. *SCR1* was used as a loading control. Quantitative results depicted are the average of three replicate trials and are normalized to the *SCR1* loading control. (B) Western blots were prepared using protein extracts from *S. cerevisiae* YSC1178-7500224, YSC1178-7502950 and YSC1178-7500415 strains[19] expressing TAP-fusion proteins Arg5,6-TAP, Cpa1-TAP, and Trp5-TAP, respectively, as well as BY4741 (untagged control). [21, 22] The TAP-tagged proteins were detected using an anti-TAP antibody. No proteins bound the anti-TAP antibody in the untagged control. Duplicate polyacrylamide gels were stained with Coomassie blue for use as loading controls. The corresponding loading controls are shown beneath the western blots.

The possible effect of PALO on translation was investigated by western analysis (Figure 2B). If PALO affects translation, we expected to see an increase in the production of Arg5,6p, Cpa1p, and Trp5p in the presence of PALO. We also expected that addition of L-arginine would reduce protein levels of Arg5,6p and Cpa1p, but not Trp5p. We used cell extracts from strains (YSC1178-7500224, YSC1178-7502950 and YSC1178-7500415, respectively) carrying TAP-tagged alleles of *ARG5,6*, *CPA1* and *TRP5* for the western analysis. These strains produce a TAP-fusion protein for each gene which can be detected on western blots with anti-TAP antibodies. BY4741 was used as an untagged control because it is the parent strain for the TAP-tagged strains. Cells were grown and treated in the same manner as for the northern analysis. Measurement of the signal intensity of the bands detected by western blotting revealed no difference in the production of Arg5,6p, Cpa1p, or Trp5p between strains treated with 15 μM PALO and the con-

trol. As expected, addition of L-arginine reduced production of Arg5,6p and Cpa1p, but not of Trp5p regardless of whether PALO was added or not. In summary, PALO had no effect on Arg5,6p, Cpa1p, and Trp5p protein levels.

PALO did not affect the growth rate of BY4741 in minimal media or nitrogen-limiting medium (data not shown), used in an attempt to increase sensitivity to PALO by lowering intracellular arginine concentrations. Growth in nitrogen-limiting medium induces cells to use amino acids, including arginine, as a source of nitrogen. These findings were consistent with the observations reported by Kinney and Lusty.[4]

In conclusion, we have developed an efficient synthesis of very pure PALO by a route suitable for both analytical and preparative applications. In contrast to a previous report describing PALO-induced arginine starvation,[4] we observed no response in *S. cerevisiae* strain BY4741 to treatment with PALO (Figure 2). The discrepancy suggests either that not all yeast strains are sensitive to PALO or that the earlier indications of arginine starvation resulted from impurities in the samples of PALO employed. The current results are consistent with observations that PALO had no effect in isolated rat mitochondria, intact rat hepatocytes or *E. coli*.[5,6]

Acknowledgments — We thank Dr. Steven Fields (Dow Agro, Indianapolis, IN) for useful discussions and a preprint describing investigations of PALO synthesis. Support was provided by the University of Nebraska-Lincoln Research Council and by NIH INBRE scholarships to R.S. and J.B. This work was conducted in part, in facilities remodeled with support from the NIH (RR0116544-01) and employing instrumentation purchased with NSF support (MRI 0079750 and CHE 0091975).

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Supplementary data, including experimental procedures, characterization data, and NMR (^{1}H, ^{13}C, ^{31}P) spectra for PALO and synthetic precursors, follows.

N-δ-phosphonoacetyl-*L*-ornithine (PALO): a convenient synthesis and investigation of influence on regulation of amino acid biosynthetic genes in *Saccharomyces cerevisiae*

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Supporting Information: Experimental Procedures **Page**

General Experimental Conditions: 2

L-ornithine, 9-borabicyclononyl coordination compound: 2

Dibenzylphosphonoacetic acid (**2**): 2

N_5-([2-Dibenzyloxyphosphinyl)acetyl])-L-ornithine, 9-BBN adduct (**3**): 2

N_5-([2- Dibenzyloxyphosphinyl)acetyl])-*L*-ornithine (**4**) 2

N_5-(2-Phosphonoacetyl)- L-ornithine (PALO) (**1**): 3

References: 3

Supporting Information: NMR Spectra

Dibenzyl methyl phosphonate (^{1}H, ^{13}C, ^{31}P) p 4-6

Dibenzylphosphonoacetic acid (**2**; ^{1}H, ^{13}C, ^{31}P) p 7-9

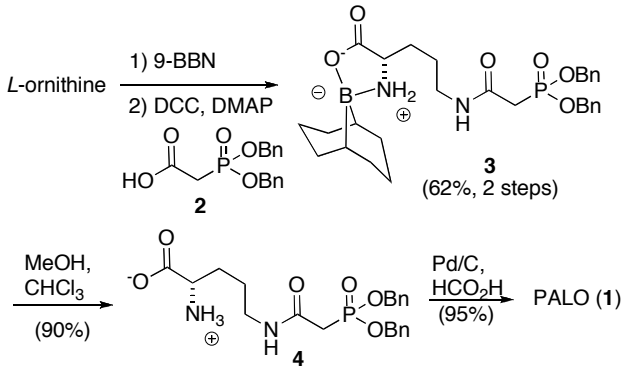
N_5-([2-Dibenzyloxyphosphinyl)acetyl])-L-ornithine, 9-BBN adduct (**3**; ^{1}H, ^{13}C, ^{31}P) p 10-12

N_5-([2- Dibenzyloxyphosphinyl)acetyl])-*L*-ornithine (**4;** ^{1}H, ^{13}C, ^{31}P) p 13-15

N_5-(2-Phosphonoacetyl)- L-ornithine (PALO) (**1**; ^{1}H, ^{13}C, ^{31}P) p 16-18

General Experimental Conditions: Reagents and solvents were used as supplied commercially, except THF, which was distilled from Na/Ph_2CO, and methanol, which was distilled from Mg/I_2. ^1H, ^{13}C, and ^{31}P spectra were recorded in $CDCl_3$ at 400, 100, and 121.5 MHz, respectively, unless noted. TLC was monitored via one or more of the following: UV; vanillin (charring); ninhydrin; bromocresol green.[1] Mass spectra were obtained at the Nebraska Center for Mass Spectrometry.

Synthesis of PALO:



L-ornithine, 9-borabicyclononyl coordination compound was prepared using a reported procedure,[2] except that *L*-ornithine•HCl (4.22 g, 25 mmol) was added before the reaction was brought to reflux. The crude product was often obtained as a viscous yellow-brown oil rather than the reported gummy solid. Washing of the crude product with ether, followed by drying under high vacuum, furnished a white powder (5.82 g, 92 %) which was used without further purification. HRMS *m/z* calcd. for $C_{13}H_{26}BN_2O_2$ (M^+): 253.20873; found: 253.20774 (3.9 ppm); [α]: -5.2° (*c* 2.0, MeOH).

Dibenzylphosphonoacetic acid (**2**) was prepared in two steps.[3] Alkylation of dibenzylphosphite (5.23 g, 20 mmol) with MeI furnished dibenzyl methyl phosphonate (3.83 g, 69%).The residue obtained from filtration and concentration was purified by flash chromatography (R_f = 0.34, 1:1 EA/hex). Spectra were identical to the previous report,[3] with the following exceptions: ^1H signals reported at 4.90-5.2 were resolved as 5.08 and 4.98 (dd, each 2H, J = 8.3-8.9 and 11.8 Hz, $^3J_{H\text{-}P}$ coupling); ^{13}C NMR, 136.4 (d, $^3J_{CP}$ = 6.1 Hz), 128.6, 128.4, 127.9, 67.1 (d, $^2J_{CP}$ = 6.2 Hz), and 11.7 (d, $^1J_{CP}$ = 144 Hz); ^{31}P 31.7; HRMS *m/z* calcd. for $C_{15}H_{17}O_3P$ $(M+H)^+$: 277.099358; found 277.10021 (3.1 ppm).

In the second step,[3] carboxylation of the phosphonate (2.76 g, 10 mmol) with CO_2-saturated ether furnished **2** (1.85 g, 58%), which was sufficiently pure to be used directly. A sample was purified by chromatography in EA/hex (R_f = 0.70, EA). Spectral data were identical to those previously reported,[3] with the following exceptions or additions: the ^1H multiplet reported at 5.08 ppm was resolved into two dd [5.12 and 5.08, each dd, 2H, 9.1, 17.3 Hz, with the latter due to $^3J_{HP}$ coupling); ^{13}C NMR 167.8 (d, $^3J_{CP}$ = 5.3 Hz) 135.7, 128.6, 128.1, 68.6 (d, *J*CP = 6.3 Hz), 34.5 (d, $^1J_{CP}$ = 136 Hz); ^{31}P NMR 22.2; IR (film) *v* 3200-2500, 1720, 1210, 990, 730, 695 cm^{-1}; HRMS *m/z* calcd. for $C_{16}H_{21}O_3P$ $(M+H)^+$: 321.08919; found: 321.08806 (3.5 ppm).

N_5-([2-Dibenzyloxyphosphinyl)acetyl])-L-ornithine, 9-borabicyclononane adduct (**3**): In an N_2-flushed, oven-dried, 50 mL round bottom flask was added dry THF (33 mL) and 9-BBN-protected *L*-ornithine (1.31 g, 5.2 mmol). The mixture was stirred until homogeneous whereupon **2** (1.50 g, 4.7 mmol), dimethyl aminopyridine (0.574 g, 4.7 mmol), and dicyclocarbodiimide (1.07 g, 5.2 mmol) were added. The reaction was stirred overnight at rt, resulting in a suspension. The residue obtained upon concentration was re-suspended in ethyl acetate and filtered. The filtrate was sequentially washed with sat. aq. $NaHCO_3$ (2 x 15 mL), water (15 mL), 1 M HCl (2 x 15 mL), and water (15 mL). The organic layer was dried with anhydrous Na_2SO_4 and filtered. The residue obtained upon concentration was purified by flash chromatography (R_f = 0.40, 1% MeOH/EA) to yield **3** as a white solid (1.61 g, 2.9 mmol, 62%); ^1H NMR 7.30 – 7.38 (m, 10H), 6.78 (t, 1H, *J* = 5.7 Hz), 5.31 (dd, 1H, *J* = 8.0 Hz, *J* = 11.6 Hz), 4.92-5.06 (m, 5H), 3.62-3.69 (m, 1H), 3.34-3.43 (m, 1H), 3.08-3.16 (m, 1H), 2.86 (d, 2H, *J* = 21.0 Hz), 1.41-2.10 (m, 16H), and 0.59 (d, 2H, *J* = 12.0 Hz); ^{13}C NMR 174.4, 135.5, 128.8, 128.0, 68.3, 54.9, 38.9, 31.4, 28.0, 24.1; ^{31}P NMR (75.5 MHz) 23.3; IR (solid) *v* 3292, 3174, 3108, 2923, 2877, 2838, 1692, 1664, 1240, 1214, 1008, 998, 724 cm^{-1}; HRMS *m/z* calcd. for $C_{29}H_{40}BN_2O_6P$ (M^+): 555.27953; found: 555.27801 (2.7 ppm); [α]: -13.4° (*c* 2.0, $CHCl_3$); MP = 125-129° C.

N_5-([2- Dibenzyloxyphosphinyl)acetyl])-*L*-ornithine (**4**)*:* Into a ~25 mL vial was added **3** (120 mg, 0.22 mmol), methanol (200 µL), and $CHCl_3$ (10 mL).[4] The reaction was stirred at rt for 72 hours and monitored by TLC (R_f = 0.50 on C18 RP-TLC plates in 3:1 $MeOH/H_2O$, developed with ninhydrin). Once the reaction appeared to be complete, the solution was concentrated to an oil, purified by ion exchange (~2 g Dowex 50X2-200; column – 1.5 cm x 30 cm, pre-acidified with 2M HCl, eluting solvent: 1% NH_4OH), and lyophilized to

yield **4** as a fluffy, white solid (85.6 mg, 0.20 mmol, 90%). ^{1}H NMR (MeOH) 7.39-7.32 (m, 10H), 5.09 (dd, 2H, J = 11.8, 11.4 Hz; ABX w/5.04 ppm), 5.04 (dd, 2H, J = 11.8, 11.4 Hz; ABX w/ 5.09 ppm), 3.53 (dd, 1H, J = 6.6, 6.6 Hz), 3.27-3.14 (m, 2H), 3.02 (d, 2H, J = 21.7 Hz), 1.93-1.75 (m, 2H), and 1.66-1.57 (m, 2H); ^{13}C NMR (MeOD) 174.4, 137.7, 129.8, 129.8, 129.3, 69.7, 55.9, 40.4, 37.0, 35.5, 29.9, and 26.3; ^{31}P NMR (162 MHz, MeOD) 24.1; IR (solid) 3350-3250, 3150-2800, 1630, 1550, 1230, 995, 900, 825, and 670 cm^{-1}; HRMS *m/z* calcd. for $C_{21}H_{28}N_2O_6P$ $(M+H)^+$: 435.16850; found: 435.16846 (0.1 ppm); [α]: +1.2° (*c* 2.1, MeOH); MP = 167-170°C.

N_5-(2-Phosphonoacetyl)- L-ornithine (PALO, **1***):* Phosphonate **4** (133 mg, 0.3 mmol was mixed with formic acid (1.1 mL) and 10% Pd/C (54 mg, 28 mol %) in a N_2-flushed vial. The vial was capped with a rubber septum and the mixture stirred for 5 min while being flushed with nitrogen. The mixture was then placed under an atm of H_2 (balloon) and the headspace briefly vented (needle) to purge N_2. The reaction was stirred for 24 h and then filtered through a plug of Celite. The filtrate was lyophilized, yielding a white solid (72 mg, 95 %) that deformed at ~ 70 °C. ^{1}H NMR (300 MHz, D_2O) 4.00 (t, 1H, J = 5.9 Hz), 3.26 (t, 2H, J = 5.9 Hz), 2.75 (d, 2H, $^{2}J_{H\text{-}P}$ = 20.4 Hz), 2.08 (m, 2H), 1.54 (m, 2H) ; ^{13}C NMR (75.5 MHz, D_2O) 172.69, 170.22, 53.10, 38.67, 38.42 (d, J = 122 Hz), 27.24, and 24.10; ^{31}P NMR (121.5 MHz, D_2O) 15.0; IR (solid) ν 3600-2350, 1715, 1630, 1547, 1300, 1204, 1020, and 915; HRMS *m/z* calcd. for $C_6H_{13}N_2O_6P$ $(M+H)^+$: 255.074600; found: 255.074685 (0.3 ppm); [α]: +3.6° (*c* 1.0, H_2O).

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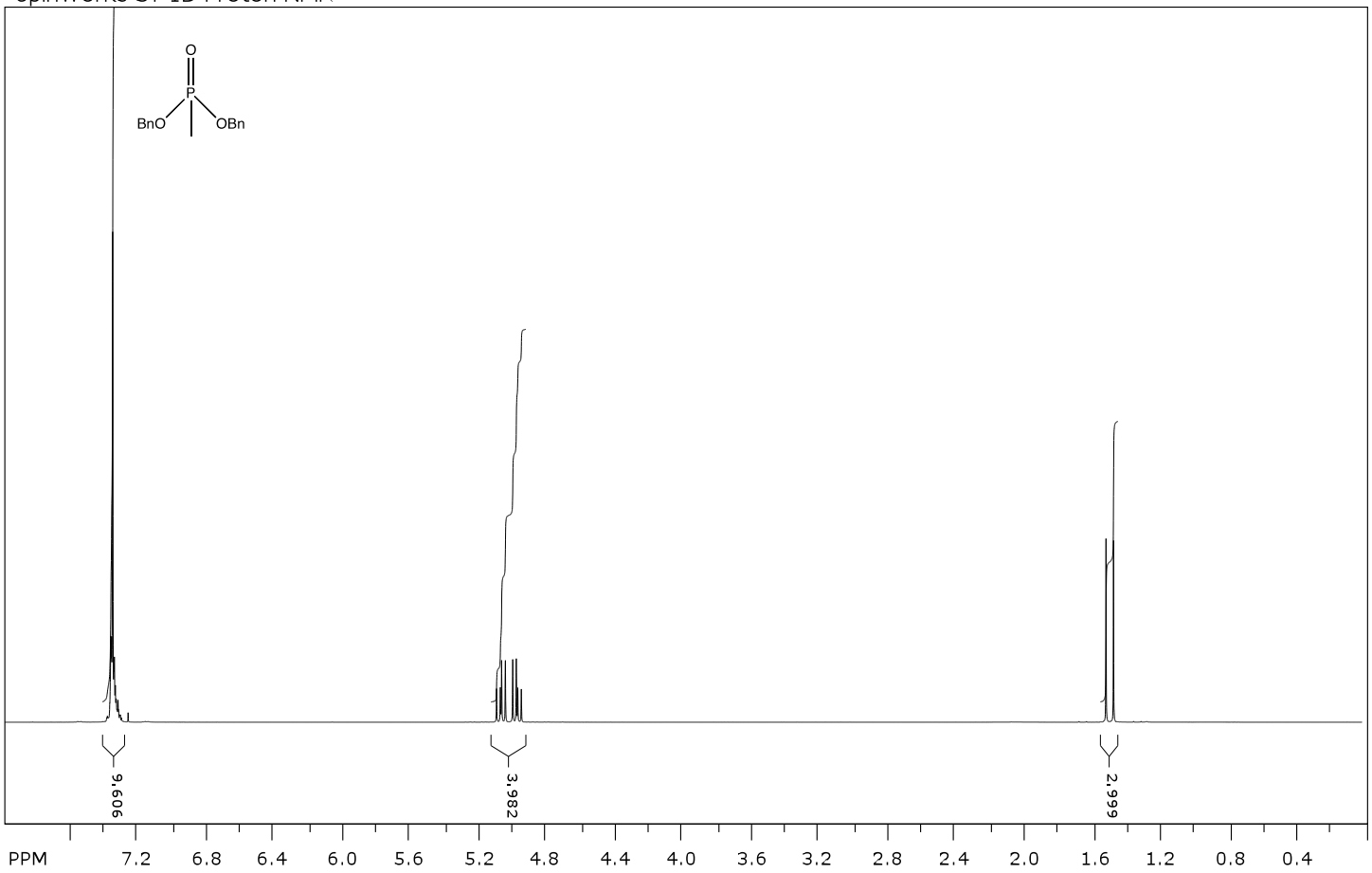
2. Fields, S. C.; Dent III, W. H.; Erickson, R. Parker, M. H., Tromiczak, E. G. *Org. Lett.* **2002**, *4*, 1249.
3. Koppel, G. A.; Kinnick, M. D. *Tetrahedron Lett.* **1974**, *9*, 711-13; Grison, C., Coutrot, P., Comoy, C., Balas, L., Joliez, S., Lavecchia, G., Oliger, P., Penverne, B., Serre, V., Herve, G. *Eur. J. Med. Chem.* **2004**, *39*, 333.
4. Walker, W. H. IV.; Rokita, S. E. *J. Org. Chem.* **2003**, *68*, 1563; Syed, B. M.; Gustafsson, T; Kihlberg, J. *Tetrahedron*, **2004**, *60*, 5571. We are also unable to explain the deprotection, but note that it is unaffected by oxygen, catalytic acid, or catalytic base.

SpinWorks 3: 1D Proton NMR



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time domain size: 65536 points
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number of scans: 16

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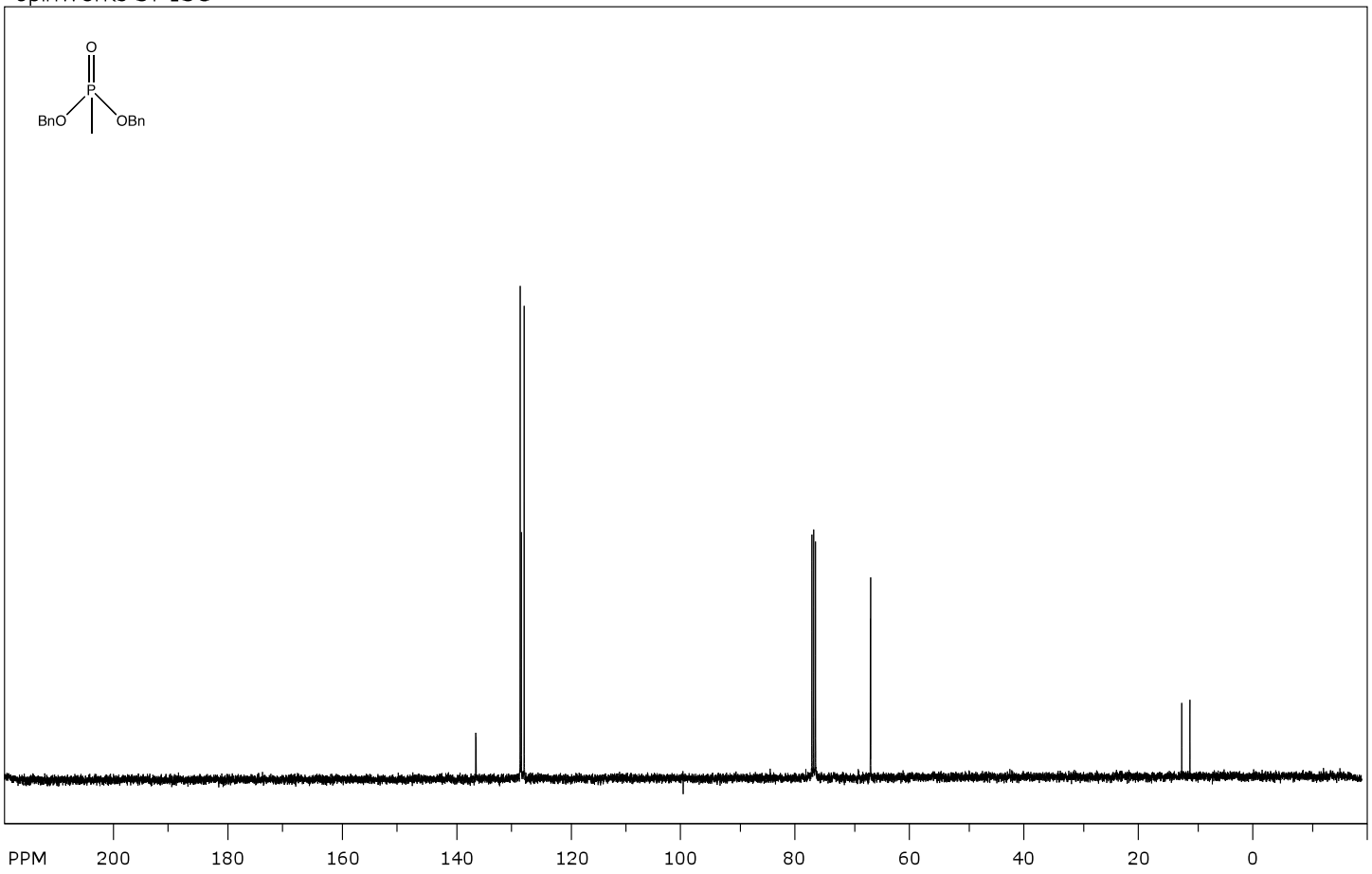
LB: 0.300 GF: 0.0000
Hz/cm: 127.967 ppm/cm: 0.31981

SpinWorks 3: 13C



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number of scans: 103

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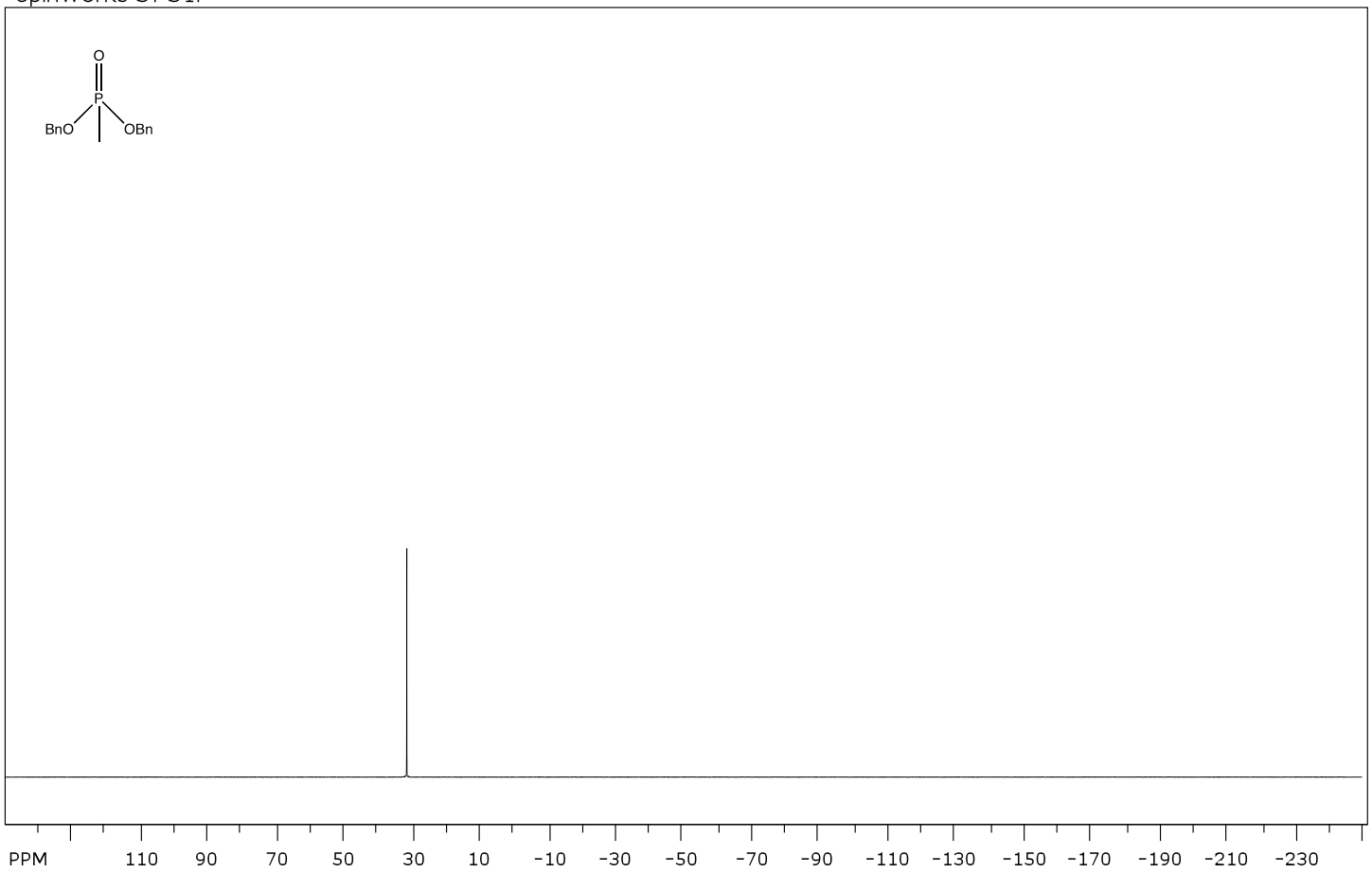
Hz/cm: 959.233 ppm/cm: 9.53295

SpinWorks 3: 31P



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number of scans: 32

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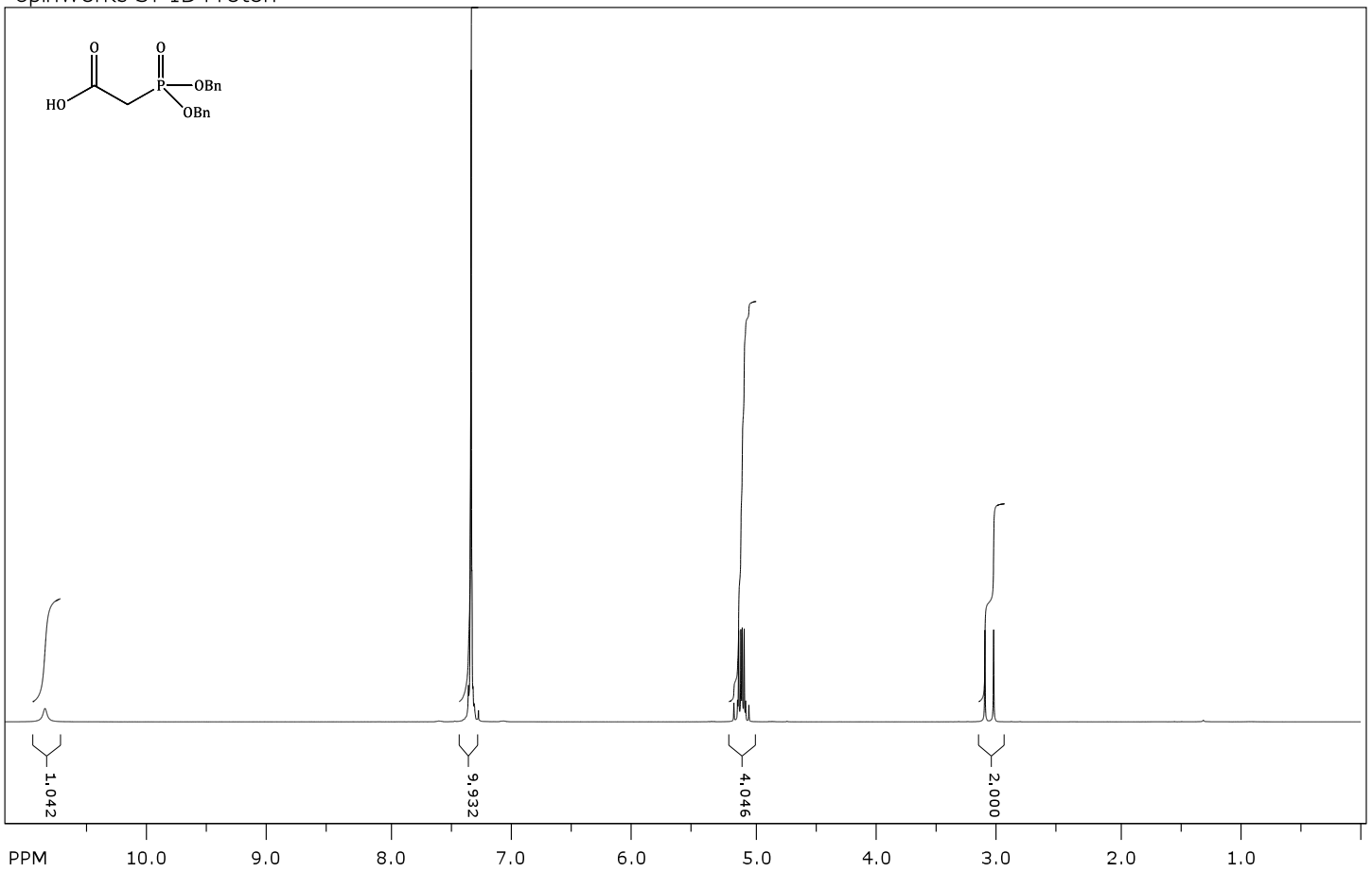
SpinWorks 3: 1D Proton



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time domain size: 65536 points
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number of scans: 16

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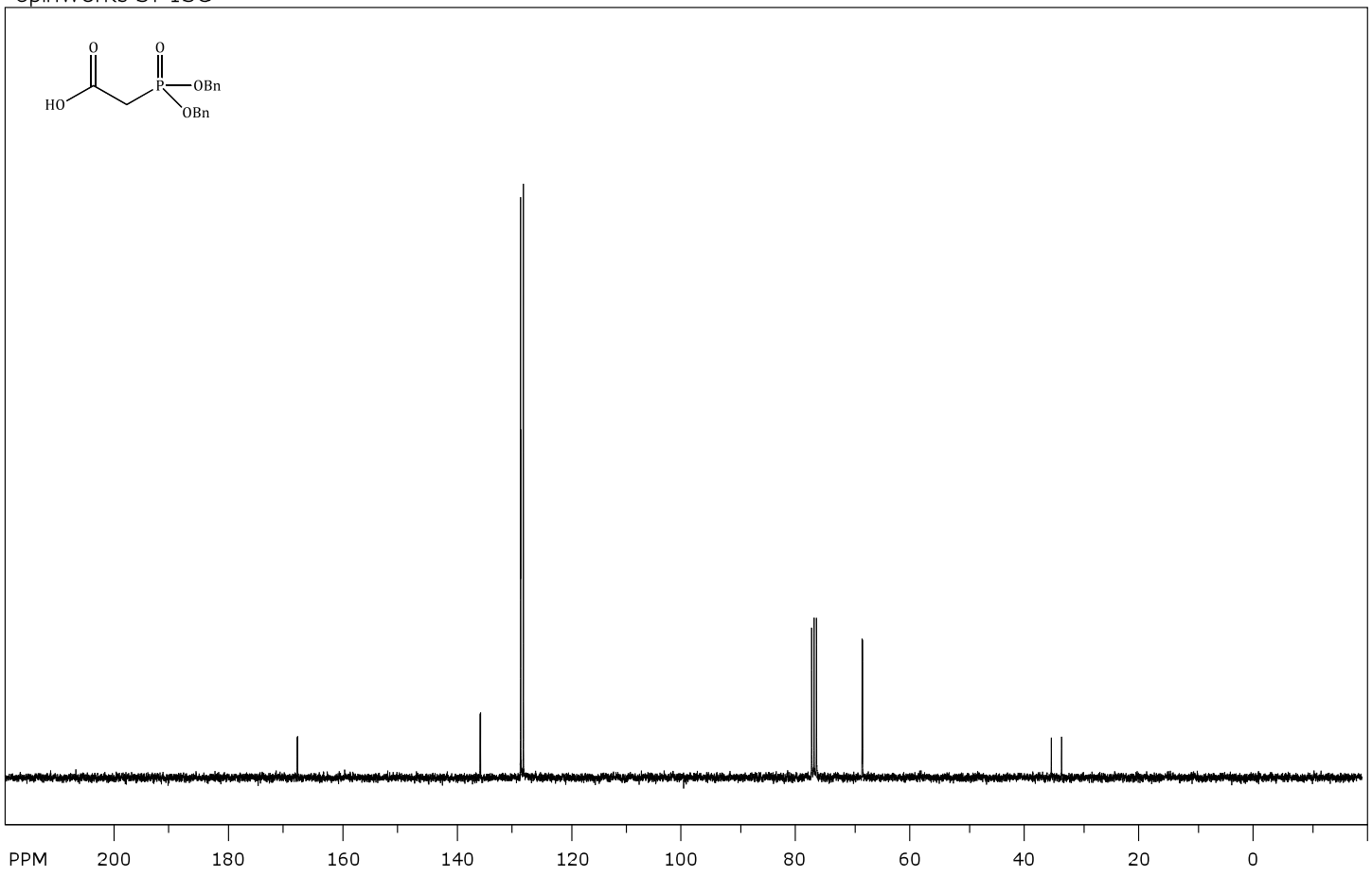
SpinWorks 3: 13C



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number of scans: 101

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processed size: 32768 complex points
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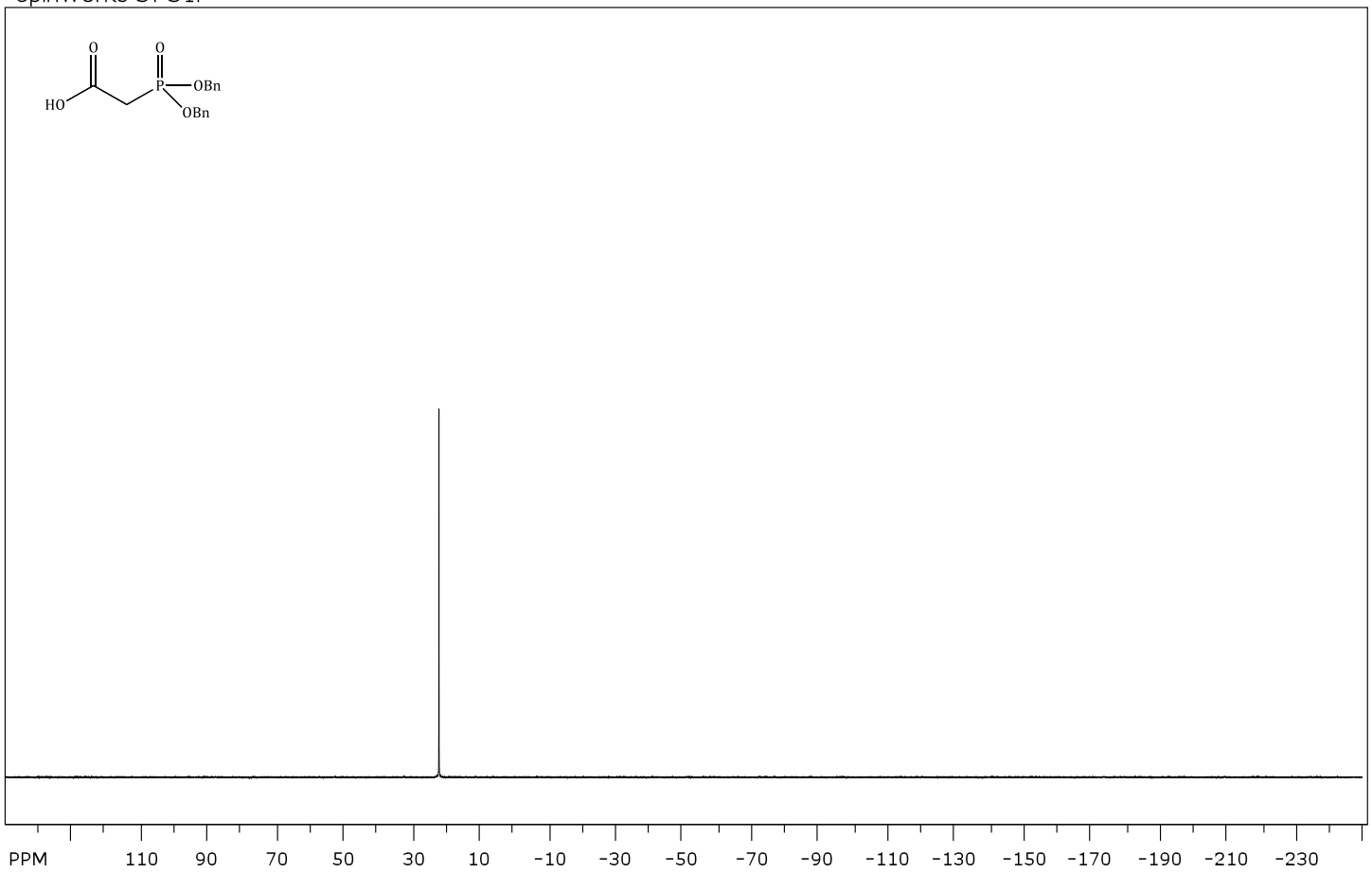
Hz/cm: 719.424 ppm/cm: 9.53192

SpinWorks 3: 31P



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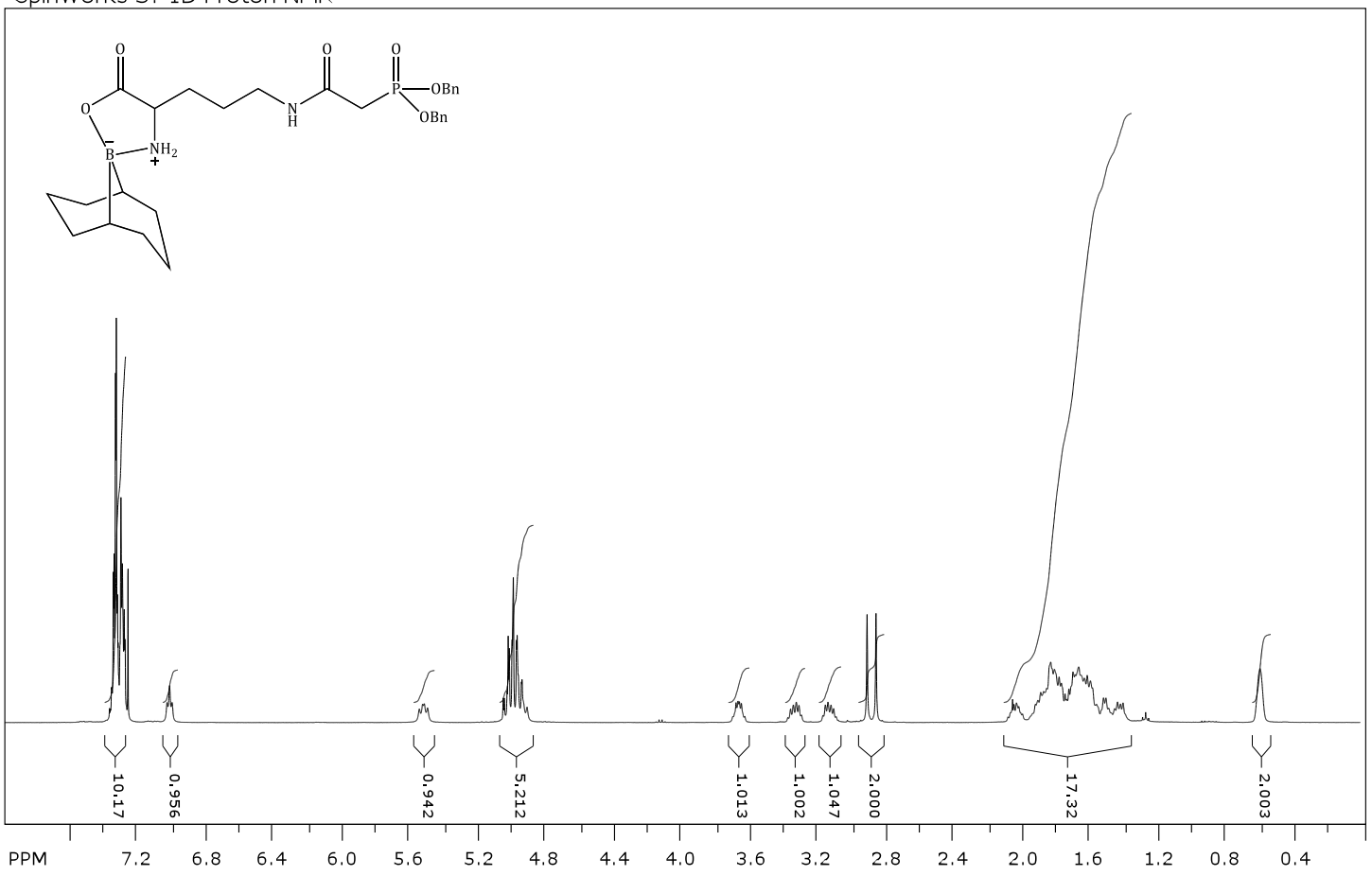
number of scans: 32

freq. of 0 ppm: 121.494851 MHz
processed size: 32768 complex points
LB: 1.000 GF: 0.0000
Hz/cm: 1946.472 ppm/cm: 16.02183

SpinWorks 3: 1D Proton NMR



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number of scans: 16

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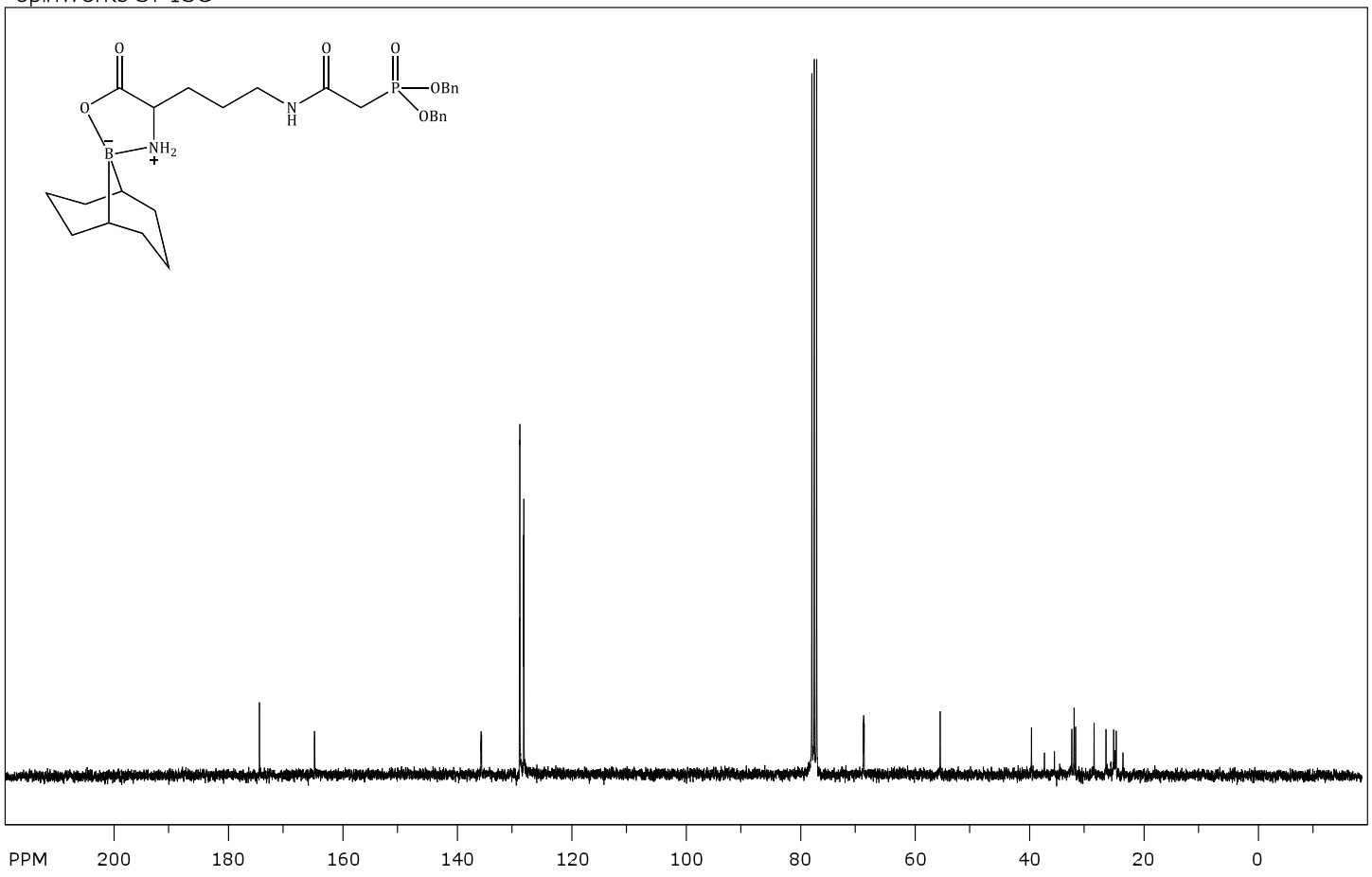
SpinWorks 3: 13C



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number of scans: 1024

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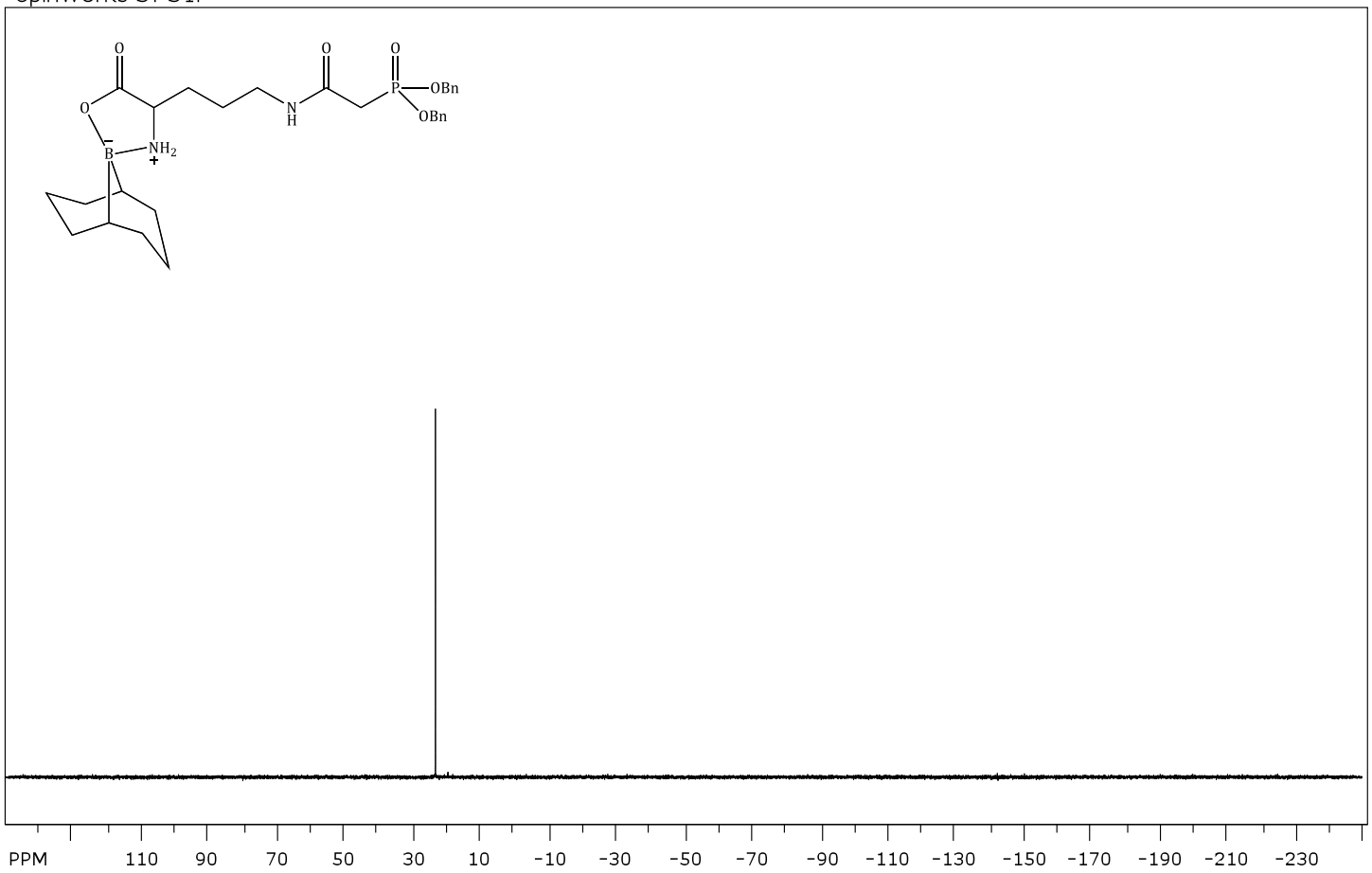
processed size: 32768 complex points
LB: 1.000 GF: 0.0000
Hz/cm: 719.424 ppm/cm: 9.53192

SpinWorks 3: 31P



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width: 64935.06 Hz = 400.9142 ppm = 0.990830 Hz/pt
number of scans: 16

freq. of 0 ppm: 161.975573 MHz
processed size: 32768 complex points
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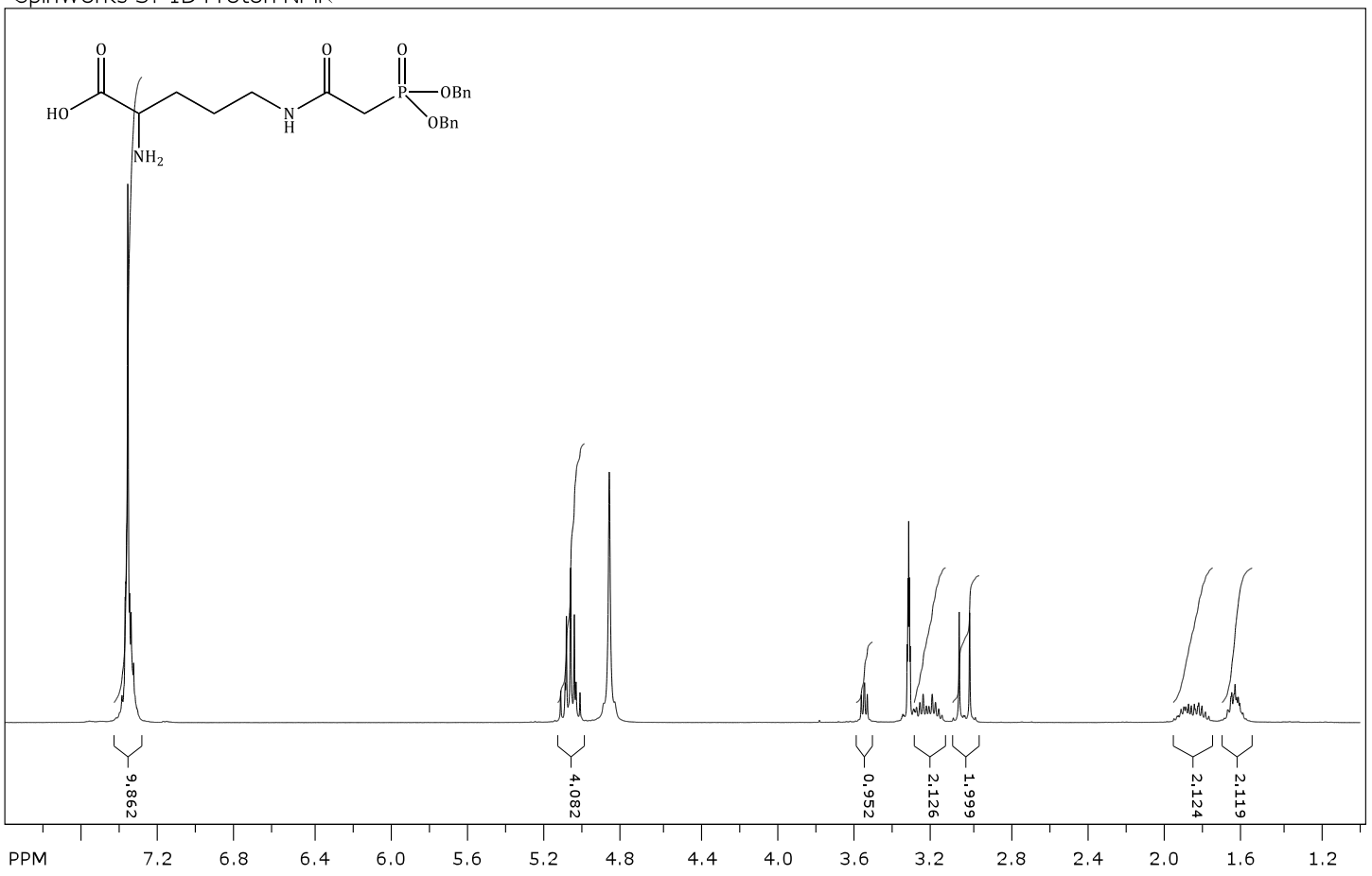
SpinWorks 3: 1D Proton NMR



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number of scans: 16

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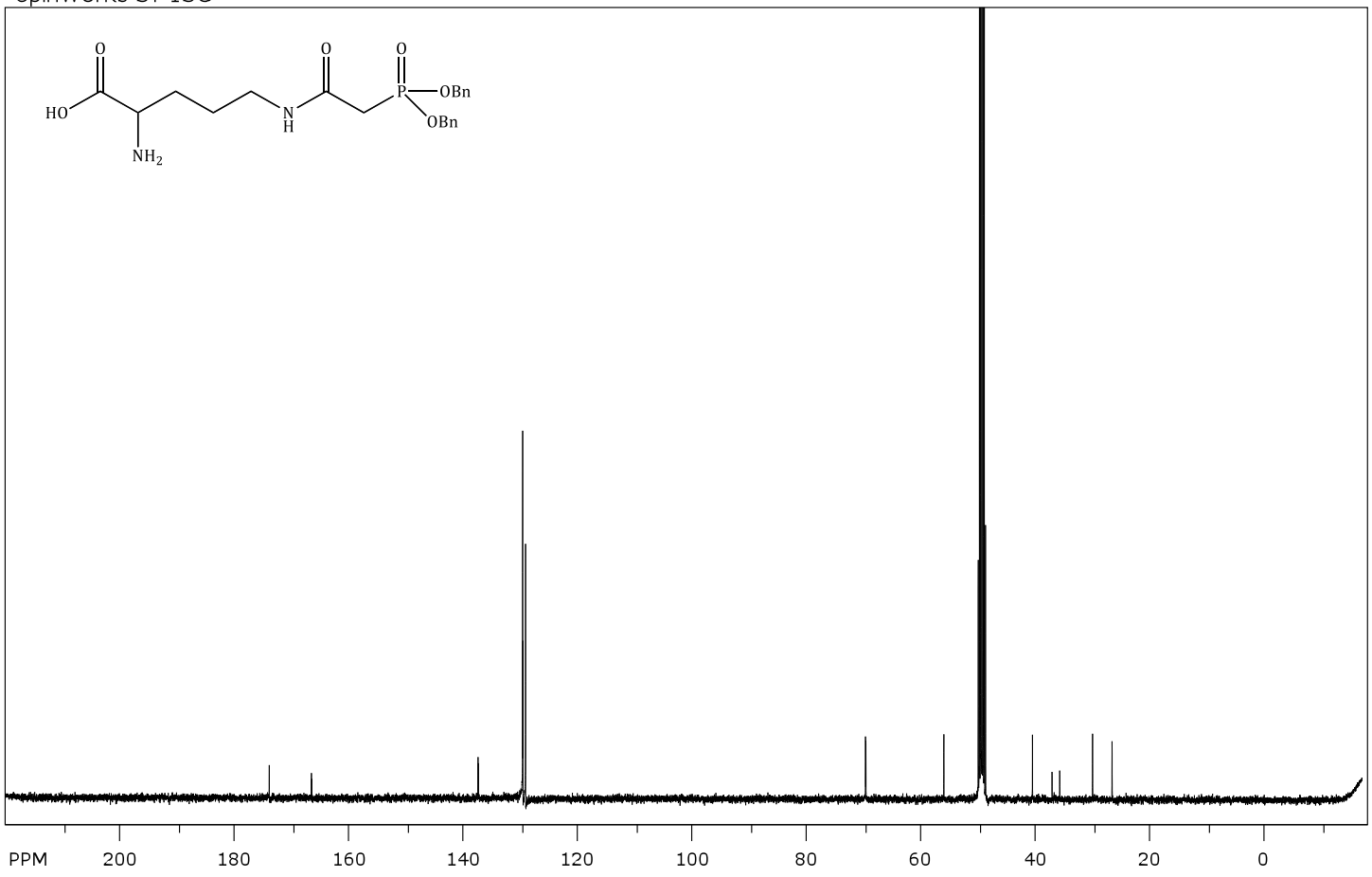
Hz/cm: 112.361 ppm/cm: 0.28081

SpinWorks 3: 13C



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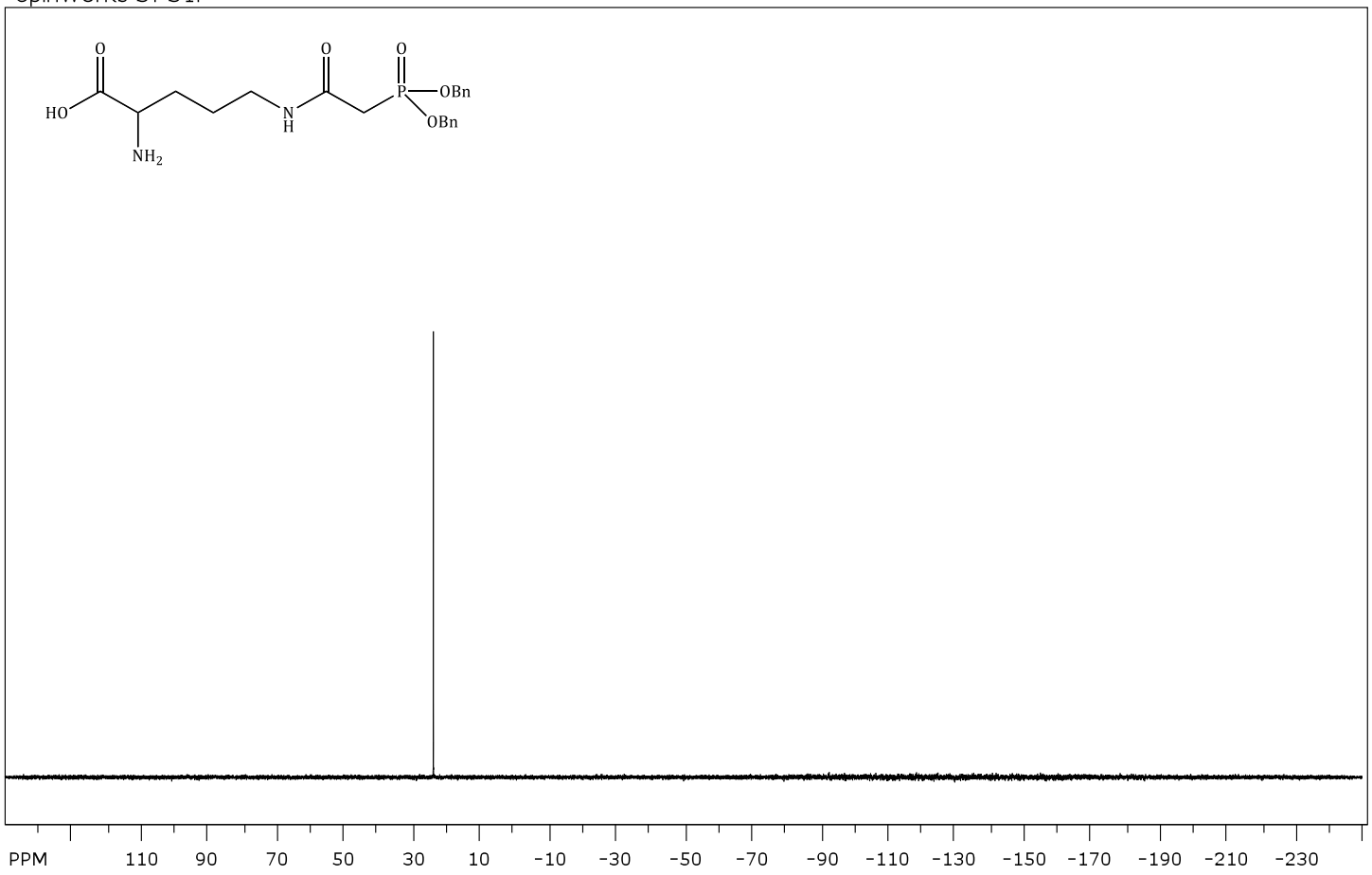
number of scans: 1024

freq. of 0 ppm: 100.612613 MHz
processed size: 32768 complex points
LB: 1.000 GF: 0.0000
Hz/cm: 959.233 ppm/cm: 9.53295

SpinWorks 3: 31P



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number of scans: 16

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processed size: 32768 complex points
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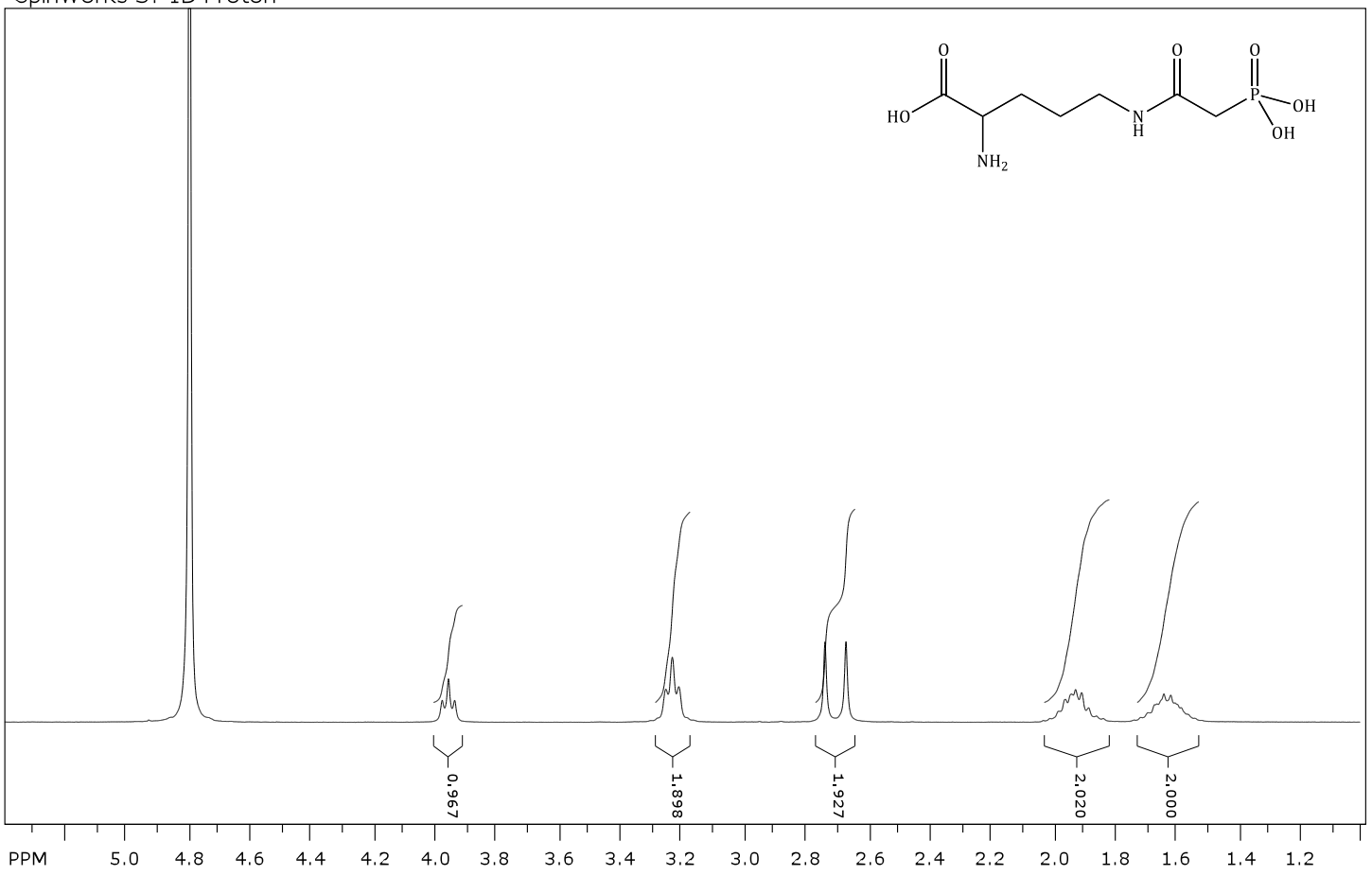
SpinWorks 3: 1D Proton



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number of scans: 20

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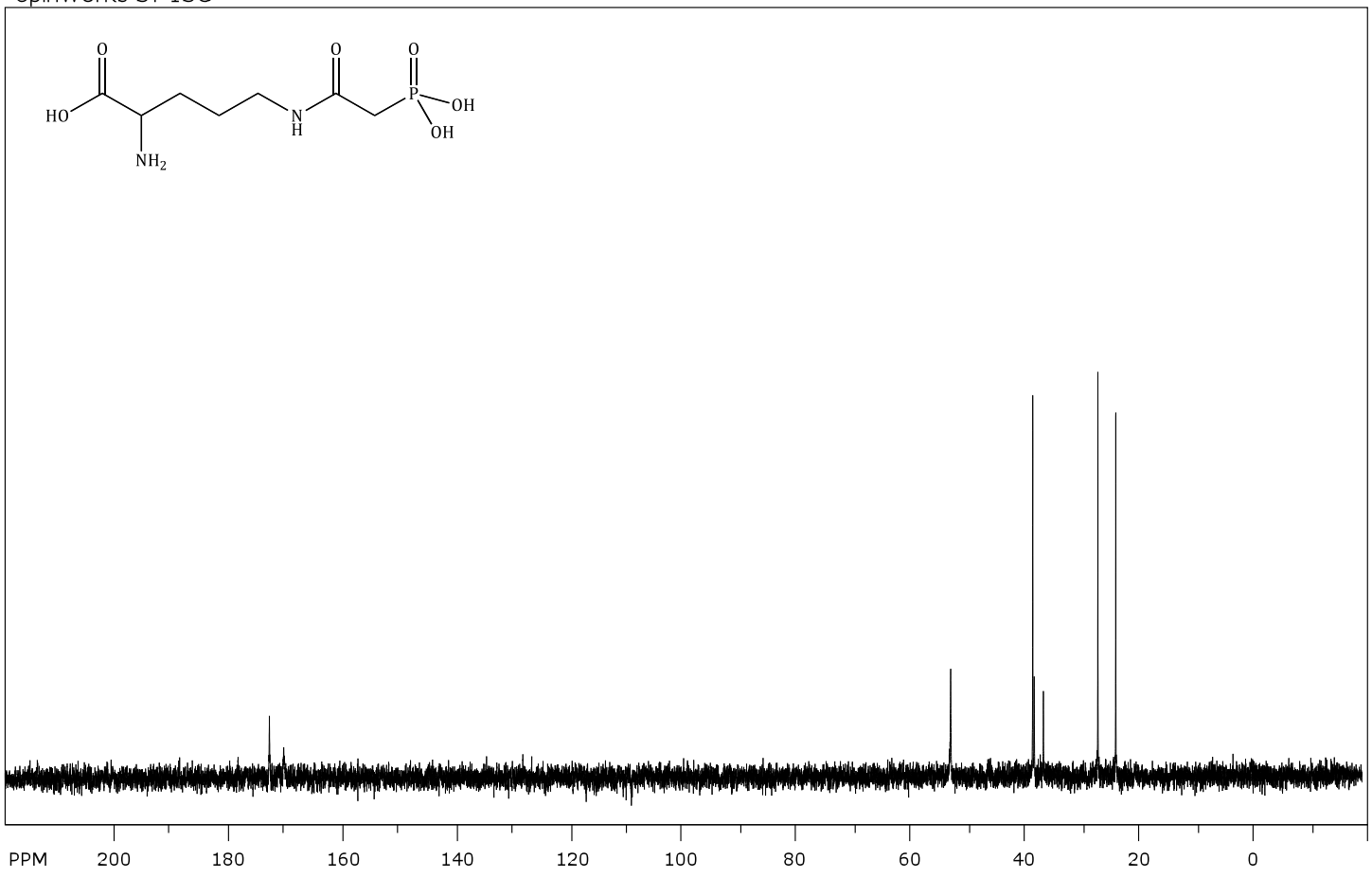
SpinWorks 3: 13C



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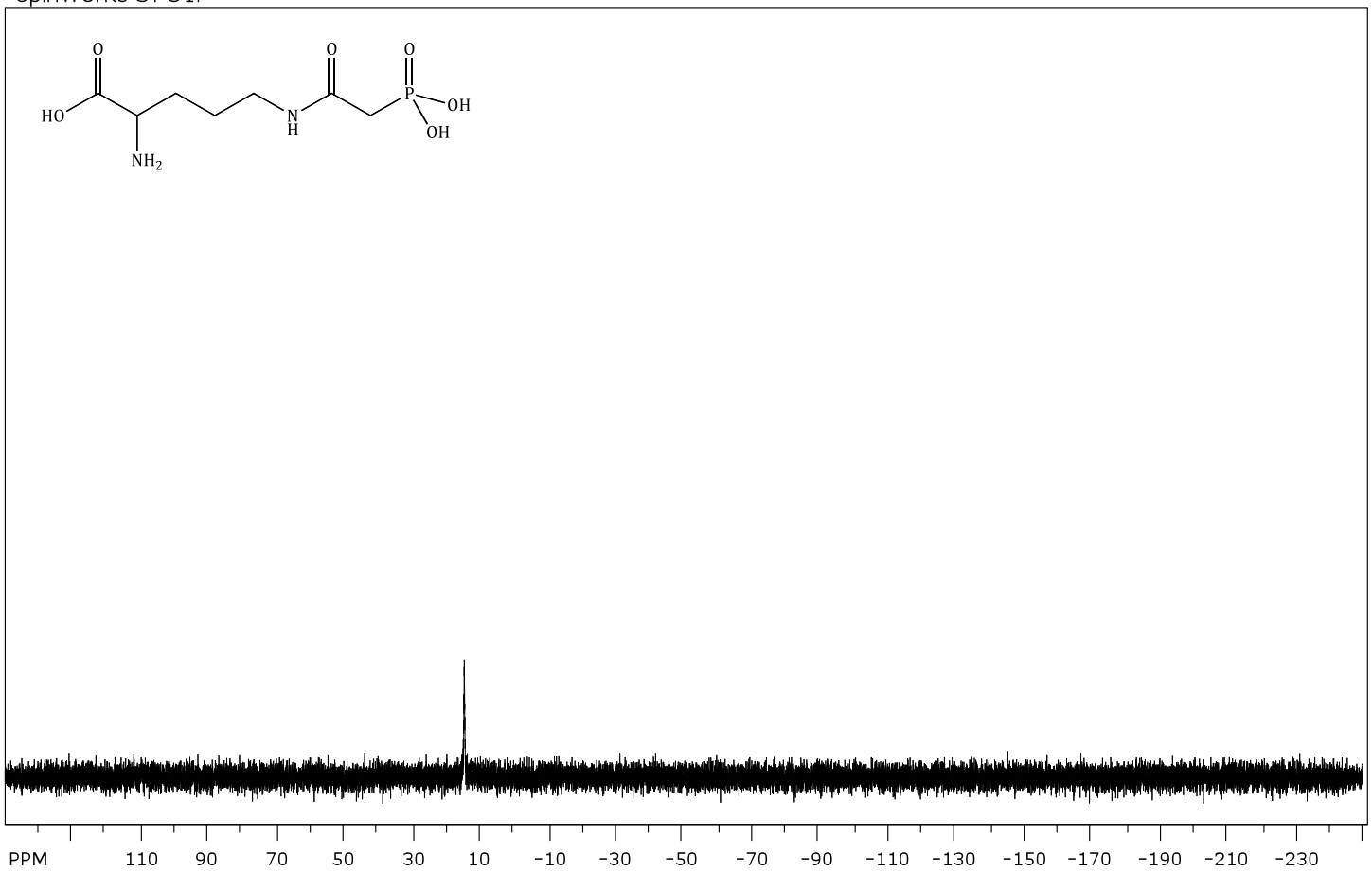
number of scans: 1024

freq. of 0 ppm: 75.467749 MHz
processed size: 32768 complex points
LB: 1.000 GF: 0.0000
Hz/cm: 719.424 ppm/cm: 9.53192

SpinWorks 3: 31P



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Hz/cm: 1946.472 ppm/cm: 16.02183