N-phosphorylation of amino acids by trimetaphosphate in aqueous solution—learning from prebiotic synthesis

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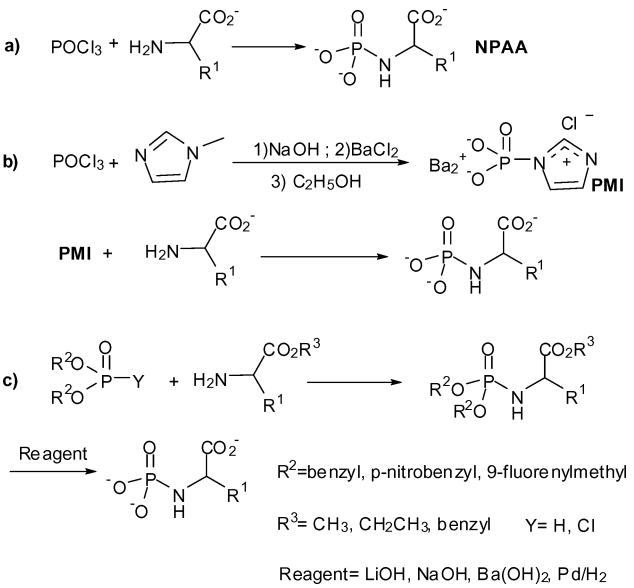
Inspired by a reactivity study between sodium trimetaphosphate (P_3m) and amino acids in prebiotic chemistry, a one-step reaction with efficient purification procedure in aqueous media has been developed for the synthesis of *N*-phosphono-amino acids (**NPAA**). P_3m was used to phosphorylate amino acids to **NPAA** with yields of 60~91%. The by-products, inorganic polyphosphates, were recycled to regenerate the phosphorylation reagent P_3m.

Introduction

Amino acid derivatives of phosphoramidates are an important class of compounds. *N*-Dialkyloxyphosphoryl amino acids showed the ability for both peptide and nucleic acid oligomerization and have been proposed as a coupling species in prebiotic chemistry.[1] *N*-Mononucleoside phosphoryl amino acids are multifunctional prodrugs of nucleotide analogues.[2] *N*-Phosphono-amino acids (**NPAA**) are non-esterified phosphoramidates. It has been found that they are potential inhibitors of some enzymes[3] and phosphoryl donors to biomolecules.[4] Additionally, **NPAA** are one of the suggested species existing in the prebiotic reaction of polyphosphates with amino acids.[5] Elucidating the similarities and differences between **NPAA** and their counterparts, *N*-dialkyloxyphosphoryl amino acids, will be of great interest in aspects of peptide and nucleic acid oligomerization related to prebiotic chemistry.

Because of their acid-lability, syntheses of **NPAAs** have been limited to three categories, namely: use of highly-reactive $POCl_3$ as phosphorylation reagent followed by neutralization with strong base and repeated recrystallization for purification[6] (Scheme 1a); a mild-reactive strategy using *N*-phosphoryl *N*′-methylimidazole (**PMI**) barium salts as phosphorylation reagents (prepared using $POCl_3$ and *N*′-methylimidazole[7] (Scheme 1b); and a protection-deprotection strategy, the current prevailing method, whereby amino acid esters are phosphorylated by dialkyl phosphite/CCl_4 or dialkyl phosphorochloridate in organic solvent and deprotection by base or hydrogenolysis[8] (Scheme 1c).

Trimetaphosphate (P_3m), known to exist in volcanic products,[9] has been suggested as a probable phosphorylation reagent for bioorganic compounds and a coupling reagent for oligomerzation of amino acids and nucleosides in prebiotic chemistry.[5,10] It's worth noting that **NPAA** have been detected



Scheme 1 Literature methods for the synthesis of *N*-phosphono-amino acids.

in the reaction of P_3m with amino acids.[5] In this paper, we would like to report the green chemistry-related direct synthesis of **NPAA** by amino acids and P_3m.

By optimizing the reaction of P_3m with amino acids in aqueous solution, a series of *N*-phosphono-amino acids were synthesized with yields of 60~91% and inorganic polyphosphate was recycled to regenerate P_3m.

Results and discussion

The general procedure is as follows: each amino acid was reacted with P_3m (1:1.2) in distilled water at 35–45 °C and at a fixed pH (Table 1), maintained by a real-time pH controller coupled to a sodium hydroxide solution dropping device. Taking serine as an example, in the ^{31}P NMR spectra P_3m shows a distinct signal at –21.01 ppm, which will disappear as it is converted into other products, *N*-phosphono-serine (8.46 ppm), pyrophosphate (–5.18 ppm), and tripolyphosphate (–4.76 and –19.03 ppm). The ^{1}H-coupled ^{31}P NMR spectra showed a

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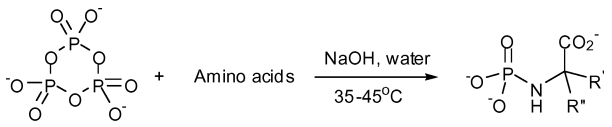
Table 1 Synthesis of *N*-phosphono-amino acids, **NPAA**



1 2 3 NPAA

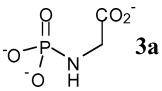
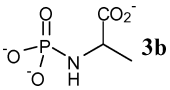
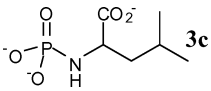
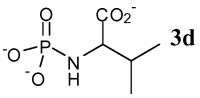
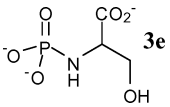
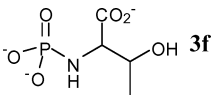
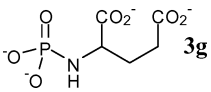
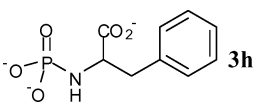
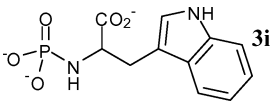
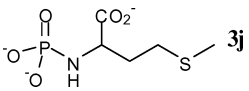
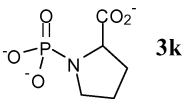
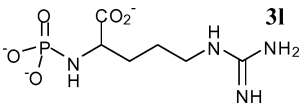
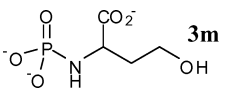
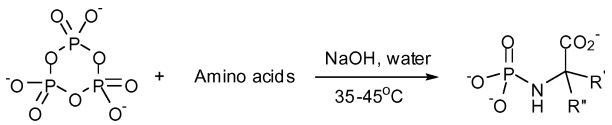
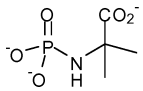
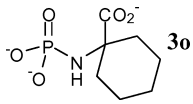
2	3	Time (h)	Temp. (°C)	pH (±0.1)	Yield (%)[a]
Gly	 **3a**	16	45	11.4	60
Ala	 **3b**	24	45	11.3	63
Leu	 **3c**	20	40	11.2	84
Val	 **3d**	30	40	11.2	86
Ser	 **3e**	30 15	40 35	11.2 10.7	84[b] 90
Thr	 **3f**	16	35	11.8	75
Glu	 **3g**	20	35	11.3	91
Phe	 **3h**	35	40	10.8	64
Trp	 **3i**	40	45	11.0	89
Met	 **3j**	15	35	10.8	88
Pro	 **3k**	10	35	12.0	60
Arg	 **3l**	18	40	11.3	80
Homo serine	 **3m**	10	35	11.0	70

Table 2 Synthesis of *N*-phosphono-amino acids, **NPAA**



1 2 3 NPAA

2	3	Time (h)	Temp. (°C)	pH (±0.1)	Yield (%)[a]
MeA	 **3n**			11.5	trace
cHex	 **3o**			11.5	no product

[a] Calculation based on amino acids. [b] Recycled P_3m was used.

doublet signal at 8.46 ppm ($J_{H,P} = 7.7$ Hz) due to H_α-P coupling of *N*-phosphono-serine. ^{13}C NMR spectra of the isolated *N*-phosphono-serine exhibited two doublet signals at 65.70 ppm (d, $^3J_{C\text{-}P} = 2.0$ Hz), 180.07 ppm (d, $^3J_{C\text{-}P} = 8.8$ Hz) attributed to C_β and the carbonyl carbon of *N*-phosphono-serine respectively.

Aliphatic amino acids (Gly, Ala, Leu, Val), aromatic amino acids (Phe, Trp), acidic amino acid (Glu), basic amino acid (Arg), hydroxy amino acids (Ser, Thr, Homoserine), sulfur-containing amino acid (Met), and Pro were each converted into the corresponding **NPAA** in reasonable yields by this method (Table 1). However, the amide amino acids (Gln, Asn) failed to give the expected products because of the instability of the amide side chain in alkali. Two sterically hindered, unnatural amino acids, 2-methylalanine (MeA) and 1-aminocyclohexanecarboxylic acid (cHex), also failed to give the expected products. To avoid peptide formation, phosphorylation of Gly and Ala was carried out at 0.1 M concentration.

Compared to the phosphorylation reagents in Scheme 1a–c, P_3m is a milder and more easily-handled reagent, being widely used as a food additive. In addition, this method is a one-step synthesis and is environmentally friendly, using water as the solvent. Consequently, it has many advantages over existing methods in the literature, especially for scale-up synthesis.

Recycling of polyphosphates

Due to the latent worldwide phosphate shortage, it is meaningful to recycle phosphates. In present work, the recycling of the by-products (pyrophosphate, triphosphate) was achieved by adjusting the mixture to pH to 4.18 and heating stepwise.[11] The regenerated P_3m with 96% purity was successfully put back into the synthesis of **3d**.

Purification and purity determination

In this paper, the isolation and purification of **NPAA** was tracked by ^{31}P NMR. For example, serine was reacted with P_3m for 15 h and the reaction quenched with NaOH (**A** in Fig. 1). Then most of the pyrophosphate (–5.18 ppm) was precipitated by cooling the reaction mixture at 4 °C (**B** in Fig. 1). For further purification, the reaction mixture was condensed and ethanol was added to the total solution (1:2 ratio) to remove the remaining triphosphate (–4.76 and –19.03 ppm) and pyrophosphate by cooling the H_2O/ethanol mixture at –10 °C[12] (C in Fig. 1). Finally, the pure *N*-phosphono-serine (8.46 ppm, D in Fig. 1) was precipitated as the sodium salt from the remaining solution by adding more ethanol, leaving unreacted amino acid in solution. The purity of the product was confirmed by ion-exchange chromatography.

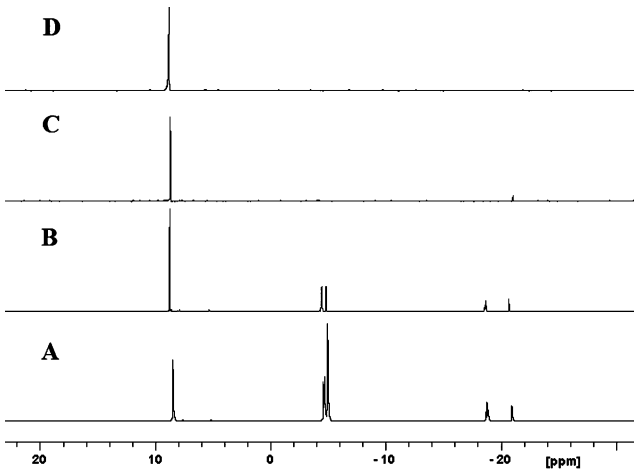
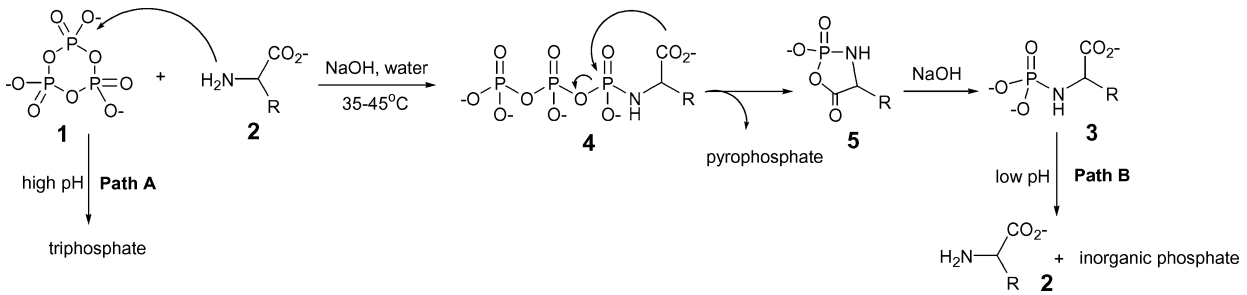


Fig. 1 ^{31}P NMR monitoring the purification of *N*-phosphono-serine. **A**: Ser + P_3m (1:1.2), 35°C, 15 h; **B**: Reaction mixture A concentrated and the precipitate removed; **C**: 1/2 volume of ethanol was added to reaction mixture B and the precipitate removed; **D**: the pure product *N*-phosphono-serine.

pH dependence of synthesis of NPAA

According to a reaction mechanism study of P_3m with amino acids,[5] the reaction starts with deprotonation of the α-amino group of amino acid **2** in alkaline media followed by nucleophilic attack of NH_2 at phosphorus in P_3m. This leads to a P_3-AA intermediate **4** which fragments into a cyclic phosphoric-amino acid anhydride **5** and pyrophosphate. Intermediate **5** is converted



Scheme 2 Mechanism for *N*-phosphorylation of amino acids by P_3m.

into **NPAA** by ring-opening (Scheme 2). Control of pH is critical and delicate because higher pH results in hydrolysis of P_3m due to the high concentration of hydroxyl anion (**Path A**) while lower pH will cause **NPAA** to decompose into inorganic phosphate and amino acids (**Path B**).

Conclusions

In this paper, a one-step synthesis method for *N*-phosphono-amino acids in aqueous solution is described. A prebiotic phosphorylation reagent P_3m is used to phosphorylate thirteen amino acids. Compared with phosphorylation reagents in Scheme 1a–c, P_3m is cheaper, milder and a more easily-handled reagent and has significant advantages for scale-up synthesis. Moreover, the by-product pyrophosphate and triphosphate can be recycled to regenerate the reagent P_3m by filtration and heating. Finally, the critical prerequisite for successful synthesis is the accurate control of pH. The present work provides an example of learning from prebiotic synthesis and is within the scope of learning from nature.

Experimental

General procedure

Amino acid (15 mmol) and P_3m (18 mmol) were added to stirred distilled water (40 cm^3) (for the synthesis of **3a** and **3b**, 150 cm^3 0.5 M pyrophosphate solution was used) at 35–45 °C. The pH was strictly maintained at the set pH (Table 1) by a real-time pH control coupled with a NaOH solution dropping device. After most of the P_3m had been consumed (monitored by ^{31}P NMR), the reaction was quenched with 4 M NaOH solution (4 cm^3). The reaction mixture was cooled to 4 °C and the precipitate (pyrophosphate) was filtered off and recovered. The filtrate was reduced to 20 cm^3 volume under vacuum, then ethanol (10 cm^3) was added and the solution stored at −10 °C for 10 h to precipitate excess triphosphate and residual pyrophosphate. According to the different properties of **NPAA**, one of the following two purification procedures was used. **a**) For the purification of **3a**, **3b**, **3e**, **3f**, **3g**, **3l** and **3m**, the filtrate comprised of two layers (underlayer was the oily crude **NPAA**), reduced to a volume of 10 cm^3 and washed with ethanol (3×10 cm^3). Finally, the oily **NPAA** was evaporated to dryness under reduced pressure to give the product as a white solid. **b**) For the purification of **3c**, **3d**, **3h**, **3i**, **3j** and **3k**, the homogeneous filtrate was reduced to a volume of 10 cm^3 and mixed with methanol (20 cm^3) to precipitate the **NPAA** which was filtered out and evaporated to dryness under reduced pressure to give the product as a white solid. All the **NPAA**s are sensitive to moisture and should be kept in a dry environment. Their melting points are all above 300 °C.

^1H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker 400 MHz NMR spectrometer. ^1H NMR chemical shifts are relative to D_2O ($\delta = 4.70$), ^{13}C NMR chemical shifts are relative to CD_3OD ($\delta = 49.50$ ppm), ^{31}P NMR chemical shifts in D_2O were externally referenced to 85% H_3PO_4 ($\delta = 0.00$ ppm).

Sodium salt of *N*-phosphono-glycine 3a. White solid; δ_H (400 MHz; D_2O; D_2O) 3.21(d, $^3J_{P\text{-}H} = 6.4$ Hz, 2H); δ_C(100 MHz; D_2O; CD_3OD) 46.27, 180.32(d, $^3J_{C\text{-}P} = 11.8$Hz); δ_P(162 MHz; D_2O; 85% H_3PO_4) 8.29; ESI-MS m/z 221.8 $[M + H]^+$.

Sodium salt of *N*-phosphono-alanine 3b. White solid; δ_H (400 MHz; D_2O; D_2O) 1.15(d, $^3J_{H\text{-}H} = 7.0$ Hz, 3H), 3.38–3.46(m, 1H); δ_C(100 MHz; D_2O; CD_3OD) 20.74(d, $^3J_{C\text{-}P} = 4.0$Hz), 52.06, 183.31(d, $^3J_{C\text{-}P} = 8.4$Hz); δ_P(162 MHz; D_2O; 85% H_3PO_4) 8.13; ESI-MS m/z 235.8 $[M + H]^+$.

Sodium salt of *N*-phosphono-leucine 3c. White solid; δ_H (400 MHz; D_2O; D_2O) 0.79–0.82(m, 6H), 1.28–1.43(m, 2H), 1.45–1.55(m, 1H), 3.37–3.43(m, 1H); δ_C(100 MHz; D_2O; CD_3OD) 21.90, 22.80, 24.58, 45.22(d, $^3J_{C\text{-}P} = 5.4$Hz), 56.47, 184.41(d, $^3J_{C\text{-}P} = 5.2$Hz); δ_P(162 MHz; D_2O; 85% H_3PO_4) 7.77; ESI-MS m/z 277.9 $[M + H]^+$.

Sodium salt of *N*-phosphono-valine 3d. White solid; δ_H(400 MHz; D_2O; D_2O) 0.77–0.80(m, 6H), 1.71–1.80(m, 1H), 3.21(dd, $^3J_{H\text{-}H} = 5.0$ Hz, $^3J_{H\text{-}P} = 10.71$ Hz, 1H); δ_C(100 MHz; D_2O; CD_3OD) 18.17, 18.27, 32.53(d, $^3J_{C\text{-}P} = 6.3$ Hz), 62.66, 183.08(d, $^3J_{C\text{-}P} = 3.7$ Hz); δ_P(162 MHz; D_2O; 85% H_3PO_4) 8.35; ESI-MS m/z 263.9 $[M + H]^+$.

Sodium salt of *N*-phosphono-serine 3e. White solid; δ_H(400 MHz; D_2O; D_2O) 3.50–3.57(m, 2H), 3.66–3.71(m, 1H); δ_C(100 MHz; D_2O; CD_3OD) 58.99, 65.70(d, $^3J_{C\text{-}P} = 2.0$ Hz), 180.07(d, $^3J_{C\text{-}P} = 8.8$ Hz); δ_P(162 MHz; D_2O; 85% H_3PO_4) 7.93; ESI-MS m/z 251.8 $[M + H]^+$.

Sodium salt of *N*-phosphono-threonine 3f. White solid; δ_H(400 MHz; D_2O; D_2O) 1.01(d, $^3J_{H\text{-}H} = 6.3$ Hz, 3H), 3.20(dd, $^3J_{H\text{-}H} = 7.2$ Hz, $^3J_{P\text{-}H} = 8.9$ Hz, 1H), 3.57–3.64(m, 1H); δ_C(100 MHz; D_2O; CD_3OD) 18.61, 64.15, 71.40(d, $^3J_{C\text{-}P} = 3.5$ Hz), 180.61(d, $^3J_{C\text{-}P} = 6.9$ Hz); δ_P(162 MHz; D_2O; 85%H_3PO_4) 7.87; ESI-MS m/z 265.9 $[M + H]^+$.

Sodium salt of *N*-phosphono-glutamic acid 3g. White solid; δ_H(400 MHz; D_2O; D_2O) 1.62–1.80(m, 2H), 1.94–2.12(m, 2H),

3.34–3.39(m, 1H); δ_C(100 MHz; D_2O; CD_3OD) 32.13 (d, $^3J_{C\text{-}P}$ = 4.9 Hz), 33.81, 57.07, 182.89(d, $^3J_{C\text{-}P}$ = 5.3 Hz), 183.50; δ_P(162 MHz; D_2O; 85% H_3PO_4) 7.80; ESI-MS *m/z* 315.8 [M + H].$^+$

Sodium salt of *N*-phosphono-phenylalanine 3h. White solid; δ_H(400 MHz; D_2O; D_2O) 2.71–2.76(m 1H), 3.03–3.08(m, 1H), 3.66–3.71(m, 1H), 7.12–7.27(m, 5H); δ_C(100 MHz; D_2O; CD_3OD) 41.28(d, $^3J_{C\text{-}P}$ = 3.7 Hz), 59.07, 126.28, 128.31, 129.67, 138.73, 182.22(d, $^3J_{C\text{-}P}$ = 7.4 Hz); δ_P(162 MHz; D_2O; 85% H_3PO_4) 7.36; ESI-MS *m/z* 311.9 [M + H].$^+$

Sodium salt of *N*-phosphono-tryptophan 3i. White solid; δ_H(400 MHz; D_2O; D_2O) 2.91–2.97(m, 1H), 3.16–3.20(m, 1H), 3.70–3.76(m, 1H), 7.00–7.68(m, 4H), 7.16(s, 1H); δ_C(100 MHz; D_2O; CD_3OD) 30.70(d, $^3J_{C\text{-}P}$ = 3.7 Hz), 58.00, 111.09, 111.47, 118.78, 119.26, 121.43, 124.11, 127.55, 135.86, 183.01(d, $^3J_{C\text{-}P}$ = 7.2 Hz); δ_P(162 MHz; D_2O; 85% H_3PO_4) 7.68; ESI-MS *m/z* 351 [M + H].$^+$

Sodium salt of *N*-phosphono-methionine 3j. White solid; δ_H(400 MHz; D_2O; D_2O) 1.77–1.86(m, 2H), 2.01(s, 3H), 2.34–2.40(m, 1H), 2.43–2.49(m, 1H), 3.44–3.50(m, 1H); δ_C(100 MHz; D_2O; CD_3OD) 14.11, 29.24, 34.99 (d, $^2J_{C\text{-}P}$ = 3.7 Hz), 56.83, 182.44(d, $^3J_{C\text{-}P}$ = 6.4 Hz); δ_P(162 MHz; D_2O; 85% H_3PO_4) 7.78; ESI-MS *m/z* 295.9 [M + H].$^+$

Sodium salt of *N*-phosphono-proline 3k. White solid; δ_H (400 MHz; D_2O; D_2O) 1.55–1.70(m, 3H), 1.94–2.02(m, 1H), 2.96–3.07(m, 2H), 3.78–3.83(m, 1H); δ_C(100 MHz; D_2O; CD_3OD) 25.24(d, $^3J_{C\text{-}P}$ = 5.6Hz), 32.36(d, $^3J_{C\text{-}P}$ = 4.7Hz), 48.54, 62.78(d, $^3J_{C\text{-}P}$ = 3.3Hz), 185.82(d, $^3J_{C\text{-}P}$ = 4.9 Hz); δ_P(162 MHz; D_2O; 85% H_3PO_4) 10.738; ESI-MS *m/z* 261.9 [M + H].$^+$

Sodium salt of *N*-phosphono-arginine 3l. White solid; δ_H(400 MHz; D_2O; D_2O) 1.31–1.62(m, 4H), 2.96–3.03(m, 2H), 3.37–3.42(m, 1H); δ_C(100 MHz; D_2O; CD_3OD) 23.69, 31.87(d, $^3J_{C\text{-}P}$ = 3.8 Hz), 41.07, 56.55, 156.98, 182.71(d, $^3J_{C\text{-}P}$ = 7.1 Hz); δ_P(162 MHz; D_2O; 85% H_3PO_4) 7.73 ESI-MS *m/z* 320.8 [M + H].$^+$

Sodium salt of *N*-phosphono-homoserine 3m. White solid; δ_H (400 MHz; D_2O; D_2O) 1.53–1.61(m, 1H), 1.78–1.87(m, 1H), 3.45–3.55(m, 2H), 3.62–3.69(m, 1H); δ_C(100 MHz; D_2O; CD_3OD) 36.96(d, $^3J_{C\text{-}P}$ = 3.6 Hz), 54.74, 58.90, 183.07(d, $^3J_{C\text{-}P}$ = 5.2 Hz); δ_P(162 MHz; D_2O; 85% H_3PO_4) 8.11; ESI-MS *m/z* 265.8 [M + H].$^+$

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