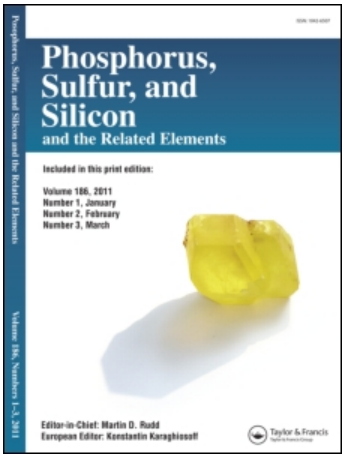

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STUDIES ON THE MECHANISM OF SOME BIOORGANIC REACTIONS FOR N-PHOSPHOAMINO ACIDS

HUA FU[a], XIANG-HONG LI[a], ZHAO-LONG LI[a], JING-ZUN WANG[b] and YU-FEN ZHAO[a*]

[a]*Bioorganic Phosphorus Chemistry Laboratory, Department of Chemistry, Tsinghua University, Beijing 100084, P. R. China and* [b]*Beijing Institute of Microchemistry, Beijing 100091, P. R. China*

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Reactions of fresh pentacoordinate phosphoric-amino acid mixed anhydrides with amine and alcohol yielded peptide analogs, N-phosphoamino acid esters, phosphodiesters and some products of ester exchange, which might provid some evidence that the formation of pentacoordinate intermediates was crucial to some bioorganic reactions of N-Phosphoamino acids.

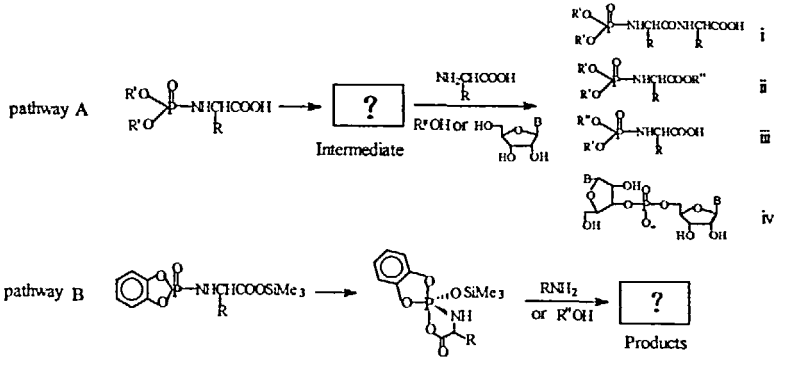
Keywords: Pentacoordinate phosphorus; amino acid; mechanism; Phosphoamino acids

INTRODUCTION

Previously, a series of N-phosphoamino acids have been synthesized,[1–5] it was found that they showed some novel bioorganic properties under mild conditions as shown in pathway A of **Scheme 1**. For example, they could autocatalyze to give N-phosphoryl peptides,[6] N-phosphoamino acid esters,[7] ester-exchanged products on phosphorus in water/alcohol solution.[8] When they were incubated with nucleosides, peptides and nucleotides were simultaneously obtained.[9] It is worthwhile to note that only the N-phospho-α-amino acids could lead to these reactions. For N-phospho-β- or -γ-amino acids, however, these phenomena did not occur under similar conditions. All these results seem to imply that the origin of life might contribute to the evolution of N-phosphoamino acids.[10] Why could

* Corresponding Author.

N-phospho-α-amino acids cause these? A possible mechanism involving a five membered cyclic pentacoordinate phosphoric-amino acid mixed anhydride intermediate was suggested. However, it is difficult to observe the pentacoordinate phosphorus intermediates because of their transient states in water/alcohol system. Later an interesting experiment involving silicon chemistry was used to trap pentacoordinate phosphoric-amino acid mixed anhydrides p(5)-aa as shown in pathway B of Scheme 1 in aprotic solvents. These structures were identified by ^{31}P, ^{1}H, ^{13}C NMR and high resolution EIMS.[11] In this paper, the pentacoordinate phosphorus compounds reacted with amine and alcohol respectively, some biochemical products including analogues of N-phosphopeptides, N-phosphoamino acid esters and nucleotides were yielded indicating the existence of pentacoordinate phosphorus intermediates in pathway A.



SCHEME 1 Pathway A: Autocatalyzed reactions of N-phosphoamino acids; Pathway B: Synthesis of pentacoordinate phosphoric-amino acid mixed anhydride

RESULTS AND DISCUSSION

1. Reaction of P(5)-aa with amine

Amino acids are zwitterions, which can be poorly dissolved in all aprotic solvents. Pentacoordinate phosphorus compounds are labile in protic solvents. In order to study the above mechanism, reactions of benzyl amine with P(5)-aa were chosen in an anhydrous benzene. The final products

N-(N-benzyl)phosphoamino acid N-benzyl amides were obtained, their structures were identified by ^{31}P, ^{1}H, ^{13}C NMR and negative ion FAB-MS. These data are shown in **Table I – II**. Figure 1 shows the negative ion FAB-MS of N-(N-benzyl)phospholeucine N-benzylamide. According to these reaction products, the following reaction mechanism is suggested (**Scheme 2**). The p(5)-aa have two active sites: the carbonyl group of the mixed anhydride and phosphorus. When it was added to the p(5)-aa benzene solution, a nucleophilic attack by benzyl amine on the carbonyl group and the phosphorus occurred, a new pentacoordinate phosphorus intermediate (**2**), which was very labile, was formed, and transferred **3** leaving a catechol, N-(N-benzyl)phosphoamino acid N-benylamides **4** were obtained after hydrolysis. Their ^{31}P NMR showed single peaks, and ^{1}H NMR of methylene on benzyl of (N-benzyl)phosphoamidate showed a larger chemical shift than reported in the literature[12,13] which might imply **4** as the titrating species.

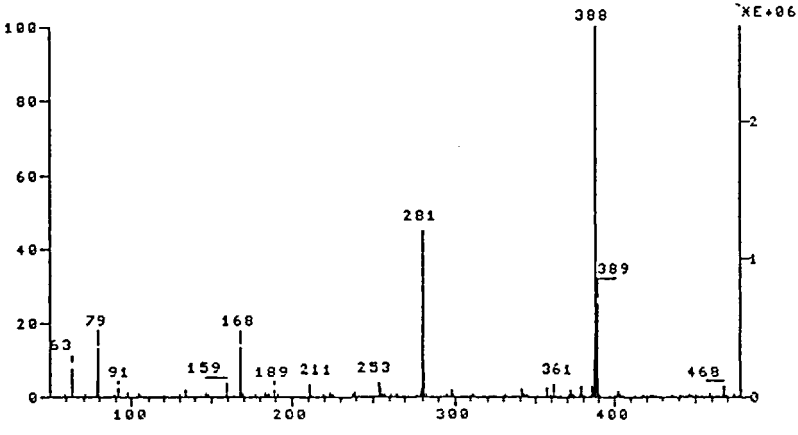


FIGURE 1 The negative ion mass spectrum of the reaction product of p(5)-Leu with benzyl amine

TABLE I The negative ion FABMS main fragment ions of reaction products of p(5)-aa with benzyl amine (relative intensity in parentheses) (R′=CH_2Ph)

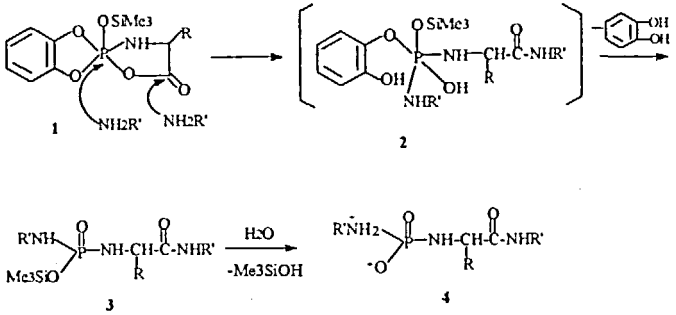
aa	*[M-H]⁻*	*[M-H-NH_2R']⁻*	*[M-H-HCONHR′]⁻*	*[M-H-NH_2CH RCONHR′]⁻*
Ala	346 (100)	239 (48)	211 (10)	168 (12)
Val	374 (100)	267 (63)	239 (5)	168 (11)
Leu	388 (100)	281 (44)	253 (5)	168 (12)
iso-Leu	388 (100)	281 (42)	253 (8)	168 (16)
Phe	422 (100)	315 (54)	287 (10)	168 (14)

aa= amino acid residue.

TABLE II The NMR data of reaction products of p(5)-aa with benzyl amine

aa	*NMR*	*chemical shift δ (ppm)* *
Ala	^1H	1.11 (d, 3H), 4.10 (m, 1H, $^3J_{P-H}$=9.3Hz), 4.25 (s, 2H), 4.56 (d, 2H, $^3J_{P-H}$=6.8Hz), 7.42–7.76 (m, 10H)
	^{13}C	20.4, 44.11, 44.59, 49.3, 127.53, 128.15, 128.16, 129.44, 129,77, 130.09, 131.81, 132.86, 133.56, 134.34, 135.12, 136.86, 167.16
	^{31}P	10.4
Val	^1H	1.12 (d, 3H), 1.16 (d, 3H), 2.23 (m, 1H), 3.64 (m, 1H, $^3J_{P-H}$=9.8Hz), 4.26 (s, 2H), 4.58 (d, 2H, $^3J_{P-H}$=7.2Hz), 7.41–7.75 (m, 10H)
	^{13}C	18.15, 20.01, 33.14, 44.14, 44.61, 62.74, 128.20, 128,24, 128.55, 129.16, 129.93, 130.14, 131.76, 132.31, 133.54, 134.38, 135.31, 136.76, 165.18
	^{31}P	11.2
Leu	^1H	0.94 (d, 3H), 1.02 (d, 3H), 1.76 (m, 1H), 2.42 (m, 2H), 3.87 (m, 1H, $^3J_{P-H}$=9. 1Hz), 4.24 (s, 2H), 4.57 (d, 2H, $^3J_{p-H}$=-6.9Hz), 7.43–7.72 (m, 10H)
	^{13}C	21.72, 23.03, 25.20, 42.86, 44.23, 44.61, 53.36, 128.21, 128.26, 128.54 129.17, 129.94, 130.15, 131.79 132.33 133.57, 134.37, 135.33, 136.77, 166.23
	^{31}P	10.8
iso-Leu	^1H	0.88 (t, 3H), 1.02 (d, 3H), 1.86 (m, 2H), 2.12 (m, 1H), 3.92 (m, 1H, $^3J_{P-H}$=9.3Hz), 4.23(s, 2H), 4.58 (d, 2H, $^3J_{P-H}$=7.1Hz), 7.44–7.72 (m, 10H)
	^{13}C	11.84, 15.41, 24.15, 38.02, 44.18, 45.10, 59.86, 128.22, 128.25, 128.53 129.18, 129.93, 130.14, 131.78 132.34 133.58, 134.36, 135.33, 136.76, 166.25
	^{31}P	11.5
Phe	^1H	3.01 (d,2H), 4.21(s, 2H), 4.51 (m, 1H, $^3J_{P-H}$=8.9Hz), 4.56 (d, 2H, $^3J_{P-H}$=7.7Hz), 7.42–7.76(m, 15H)
	^{13}C	39.8, 44.22, 45.17, 55.31, 127.23–132.12 (m), 168.18
	^{31}P	10.4

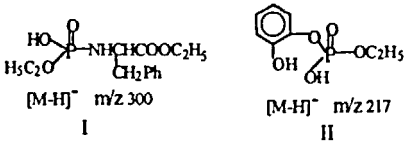
* CD_3OD as solvent, ^1H, ^{13}C NMR CD_3OD (δ=3.50, 49.00ppm) being used as internal reference, ^{31}P NMR 85%H_3PO_4 (δ=0.00ppm) as external reference.



SCHEME 2 Reaction of p(5)-aa with amine

2. Reaction of p(5)-aa with alcohol

Figure 2 shows the in situ negative ion FAB-MS of the crude reaction products of p(5)-Phe with ethyl alcohol. Two important peaks at m/z 300 and 217 were found, the following structures are proposed:



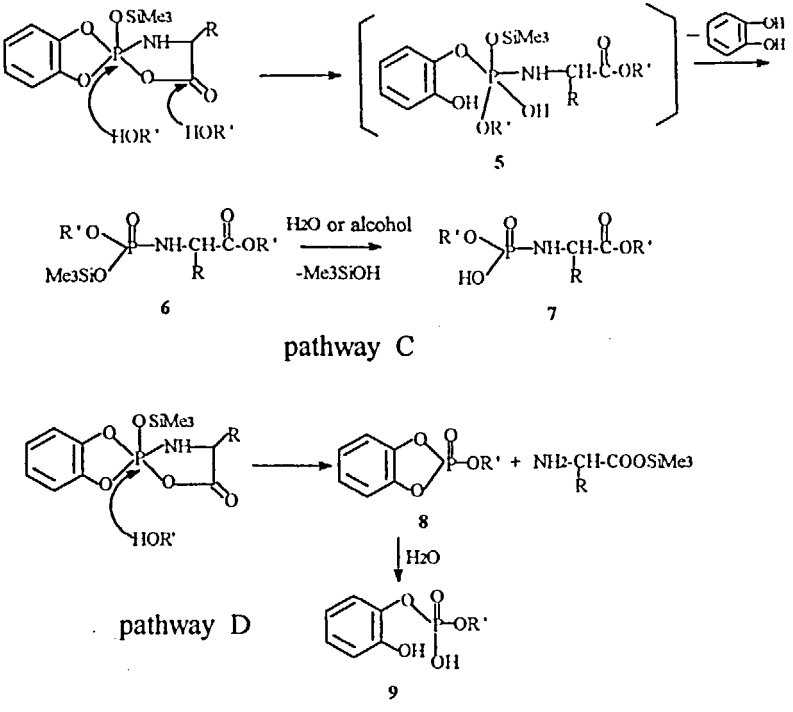
[M-H]⁻ m/z 300 I

[M-H]⁻ m/z 217 II

It is difficult to prepare compound **I** for small amounts of sample, its metastable ion mass spectrometry was examined by B/E linked-scan technique. The precursor ion at m/z 300 produced daughter ions 272, 254 and 224, which were assigned to $[M\text{-}H\text{-}C_2H_4]^-$, $[M\text{-}H\text{-}C_2H_5OH]^-$ and $[M\text{-}H\text{-}C_2H_5OCOH]^-$. High resolution mass spectrometry gave compound **I** the exact mass number and the corresponding elemental composition of $[M\text{-}H]^-$ as shown in **Table IV.**

The corresponding compound at m/z 217 in Figure 2 was prepared by HPLC. Its structure was identified by negative ion FAB-MS, ^1H, ^{13}C and ^{31}P NMR. FABMS: m/z 217 ($[M\text{-}H]^-$), 189 ($[M\text{-}H\text{-}C_2H_4]^-$), 171 ($[M\text{-}H\text{-}C_2H_5OH]^-$), 109 deprotonated ion of catechol, 79 (PO_3^-), 63 (PO_2^-), ^1H NMR: δ 1.19 (t, 3H, $-CH_3$), 4.02 (m, 2H, $^3J_{P\text{-}H}$=12Hz, $-CH_2$), 6.71–7.15 (m, 4H, $-C_6H_4$). ^{13}C NMR: δ 18.22 ($-CH_3$), 62.63 ($-OCH_2-$, $^2J_{P\text{-}C}$ =18Hz), 117.19, 119.61, 121.07, 124.60, 139.07, 148.10 ($-C_6H_4$);

^{31}P NMR: δ - 3.11ppm. These data confirmed structure **II**. These in situ negative ion FAB-MS data of reaction products of other p(5)-aa with ethyl alcohol are listed in **Table III** and **Table IV**. The possible reaction mechanism of p(5)-aa with alcohol is shown in **Scheme 3**. Pathway **C** is similar to that of p(5)-aa with amine in **Scheme 2**. A nucleophilic attack by alcohol on the carbonyl group of mixed anhydride and the phosphorus occurred, a pentacoordinate phosphorus intermediate 5 was formed, it quickly changed into 6 leaving a catechol. However, when alcohol reacted with the p(5)-aa, a substantial difference was found. Through pathway **D** in **Scheme 3**, a large amount of cyclophosphotriester (^{31}p NMR) was obtained leaving an amino acid, which was consistent with the literature.[14]



SCHEME 3 Reaction of p(5)-aa with alcohol

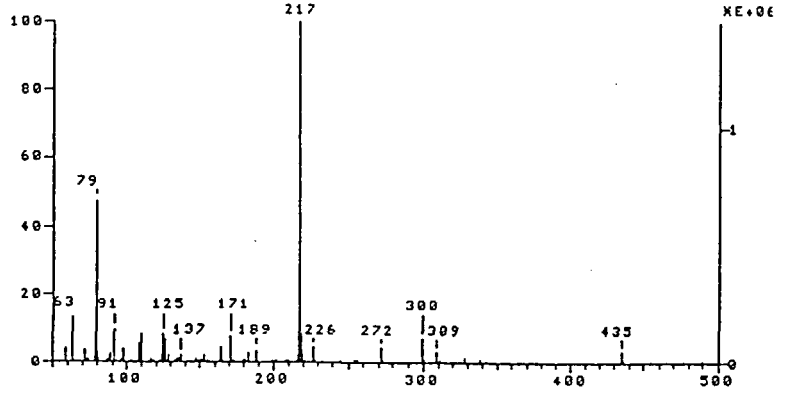


FIGURE 2 The negative ion mass spectrum of the crude reaction products of p(5)-Phe with ethyl alcohol

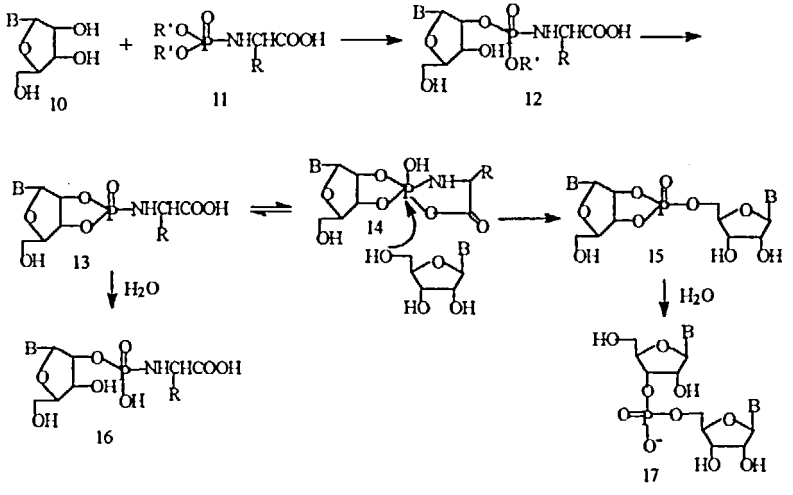
TABLE III The negative ion FABMS and ^{31}P NMR main data of the crude reaction products of p(5)-aa with ethyl alcohol (relative intensity in parentheses)

aa	*FABMS m/z (%)*	*^{31}P NMR (ppm)*
Ala	63 (17), 79 (45), 109 (8), 196 (7), 217 (100), 224 (12)	8.56, –3.07
Val	63 (13), 79 (49), 109 (3), 217 (100), 224 (7), 252 (10)	8.95, –3.08
Leu	63 (21), 79 (42), 109 (6), 217 (100), 238 (6), 266 (15)	8.37, –3.13
iso-Leu	63 (18), 79 (52), 109 (9), 217 (100), 238 (8), 266 (17)	8.66, –3.12
Phe	63 (14), 79 (46), 109 (5), 217 (100), 272 (5), 300 (8)	8.36, –3.11

aa=amino acid residue.

Pathway **D** might provide a model for formation of the nucleotide from the interaction of N-phosphoamino acid and a nucleoside. N-(O, O-dialkyl)phosphoamino acid (**11**) was incubated with the nucleoside (**10**) in anhydrous pyridine by Zhou et al., final products **12, 16** and **17** after hydrolysis were determined by capillary electrophoresis-electrospray ionization mass spectrometry (CE-ESIMS),[9] and Zhou also observed ^{31}P NMR signal at 19ppm corresponding to N-cyclophosphoamino acid (**13**). Here 2′- and 3′-OH of nucleoside correspond to two hydroxyl groups of catechol as shown in pathway **B** of **Scheme 1.** is proposed. The possible

mechanism of formation of nucleotide as shown in **Scheme 4, 13** might be isomerized to pentacoordinate phosphoric-amino acid mixed anhydride (**14**). When 5′-OH of another nucleoside attacked the phosphorus, the clyclophosphotriester (**15**) was obtained, and became phosphodiester (**16**) after hydrolysis.



SCHEME 4 The possible mechanism of the nucleotide formation from the interaction of N-phosphoamino acid and nucleoside

TABLE IV The high resolution data of various N-(O-ethyl)phosphoamino acid ethyl esters $[M-H]^-$ (m/z)

aa	*Calculated*	*Found*	*Elemental composition*
Ala	224.0688	224.0684	$C_7H_{15}O_5NP$
Val	252.1001	252.1003	$C_9H_{19}O_5NP$
Leu	266.1157	266.1152	$C_{10}H_{21}O_5NP$
iso-Leu	266.1157	266.1160	$C_{10}H_{21}O_5NP$
Phe	300.1001	300.1002	$C_{13}H_{19}O_5NP$

aa = amino acid residue.

CONCLUSION

Reaction of fresh pentacoordinate phosphoric-amino acid mixed anhydrides with benzyl amine led to N-(N-benzyl)-phosphoamino acid N-benzylamide with O-N displacement reaction occurring. Reaction with ethyl alcohol produced N-(O-ethyl)phosphoamino acid ethyl esters and phosphodiester with N-O displacement reaction occurring, the formation of phosphodiester provided evidence of formation of the nucleotide from interaction of N-Phosphoamino acid and nucleoside. All these implied that N-phosphoamino acid really underwent pentacoordinate phosphoric-amino acid mixed anhydride intermediate and produced the biochemical products.

EXPERIMENTAL

General procedure

Negative ion FAB mass spectra were recorded on a Finnigan MAT 90 double-focusing instrument (Finnigan MAT, Bremen, Germany) of BE geometry. The standard saddle gun was operated at 5mA current and 20KeV energy using Cs^+ ion as a bombarding ion. All the samples were measured in a glycerol matrix. The scan rate was 5 s/d, and conventional resolution of the instrument was adjusted to 1000 (10% valley definition). The ^1H, ^{13}C NMR spectra were recorded on a Bruker AM 500 spectrometer at 500MHz equipped with ASPECT-3000 computer. The ^{31}P NMR spectra were determined on a Bruker AC 200p spectrometer at 200MHz, CD_3OD as solvent. The reaction products of p(5)-aa with benzyl amine were purified by DEAE-cellulose (D-32) column chromatography, eluting with a linear gradient of 0.005~0.05M NH_4HCO_3 buffer. The main reaction product of p(5)-Phe with ethyl alcohol was prepared by HPLC. HPLC preparation was performed with a Shimadzu LC-9A system and a Shimadzu SPD-6AV detector. Data were acquired on a Shimadzu CR-4A chromatography. The mixture was fractionated on a Zorbax Bio series oligo column (6.2 mm ID × 8 cm) with a curve (curve 4 in Shimadzu SCL-6B controller) elution gradient of 0~100% B (elution buffer: A, 15mM NH_4HCO_3; B, 1.5M NH_4HCO_3) over 35 min with a flow rate of 1.2 ml/min. The elu-

tion profile showed a peak with a retention time of 2.424 min and several peaks with shorter retention time. The elute corresponding to the peak at 2.424 min was collected for 30 cycles, lyophilized, and determined by negative FABMS, ^{1}H NMR, ^{13}C NMR and ^{31}P NMR.

Reaction of p(5)-aa with amine: To a stirred solution of p(5)-aa (5mmol) in anhydrous benzene (15ml) at room temperature under a nitrogen atmosphere was added dropwise dry benzyl amine (11mmol). A white precipitate gradually appeared over 24 hours. The product was filtered and isolated by ion exchange column chromatography. A white solid was obtained after lyophilization. Yield: 54 ~ 63%.

Reaction of p(5)-aa with alcohol: To a stirred solution of p(5)-aa (5mmol) in anhydrous benzene (15ml) at room temperature under a nitrogen atmosphere was added dropwise dry ethyl alcohol (11mmol) over 24 hours. The solvent was removed by rotary evaporation. The oily crude product was identified by in situ negative ion FAB-MS, and isolated by HPLC, an oily liquid was obtained. (Yield: 42%).

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