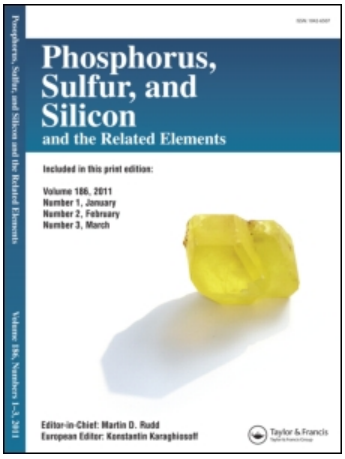

Phosphorus, Sulfur, and Silicon and the Related Elements
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713618290

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Online publication date: 27 October 2010

To cite this Article Jiang, Yang , Zhong, Ru-Gang , Tan, Bo , Li, Yan-Mei and Zhao, Yu-Fen(2003) 'Recognition of α-Amino Acid from β- and γ-Amino Acid by N -Phosphorylation', Phosphorus, Sulfur, and Silicon and the Related Elements, 178: 4, 831 — 837
To link to this Article: DOI: 10.1080/10426500307806
URL: http://dx.doi.org/10.1080/10426500307806

Phosphorus, Sulfur and Silicon, 2003, Vol. 178:831–837

1042-6507/03 $12.00 + .00
DOI: 10.1080/10426500390197995

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RECOGNITION OF α-AMINO ACID FROM β- AND γ-AMINO ACID BY *N*-PHOSPHORYLATION

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(Received June 9, 2002; accepted September 10, 2002)

The reactivity of N-*phosphoryl* α-*alanine differs from* N-*phosphoryl* β-*alanine and* N-*phosphoryl* γ-*amino butyric acid enormously.* N-*phospho-*α-*alanine could self-activate to yield* N-*phosphopeptides either in aqueous or nonaqueous media, but not* β- *or* γ-*amino acid analogues. The mechanism of the reaction is discussed.*

In prebiotic chemistry there were many possible candidates for the building block of protein. A variety of compounds, such as α-amino acids and β-amino acids were obtained in many experiments simulating the prebiotic earth.[1] The problem is why the α-amino acid is nature's final choice. In the literature,[2,5] there are some studies about the reactivity differences among α-, β- and γ-amino acids. For example, J. Rabinowitz et al. demonstrated that dipeptides were produced in the reaction of trimetaphosphates with glycine or L-α-alanine, but not with β-alanine.[2,3] B. M. Rode reported that in salt-induced peptide formation (SIPF) reactions, α-amino acids were much more favorable than other kinds of amino acids due to the presence of copper ions.[4] The work of H. Fu in our group showed that *N*,O-bis(trimethylsilyl)-α-amino acids, mediated by o-phenylene phosphorochloridate, could oligomerize into polypeptides, while the β-alanine analogues could not.[5]

The authors thank the National Natural Science Foundation of China (No. 39870415), the Ministry of Science and Technology, the Chinese Ministry of Education and Tsinghua University for financial support.

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RESULTS AND DISCUSSION

Our previous work showed that *N*-(O,O′-dialkyl)phosphoryl α-amino acids could self-activate through an intramolecular phosphoric-carboxylic mixed anhydride, in which the carbonyl group was activated and can be attacked easily by a nucleophilic reagent.[6,7] For example, the amino group of the derivatives of amino acid could attack it to form an amide bond. In order to understand the intrinsic relationship between the structures of phosphoric-carboxylic mixed anhydride and reactivity of α-, β- and γ-amino acid, the activation effects of the phosphoryl group among *N*-(O,O′-diisopropyl)phospho-(L)-α-alanine (DIPP-α-Ala), *N*-(O,O′-diisopropyl)phospho-β-alanine (DIPP-β-Ala) and *N*-(O,O′-diisopropyl)phospho-γ-amino butyric acid (DIPP-γ-Ala) were compared by experimental and theoretical approaches.

Different solvents, pyridine, chloroform, and water were chosen for the comparison. After incubation in each solvent, MS and the ^{31}P NMR spectra showed that neither DIPP-β-Ala nor DIPP-γ-Aba showed any transformation and implied that these compounds were relatively stable under such conditions. However, the ^{31}P NMR showed that DIPP-α-Ala **1** underwent complicated reactions as incubated in each of the three solvents (Figure 1).

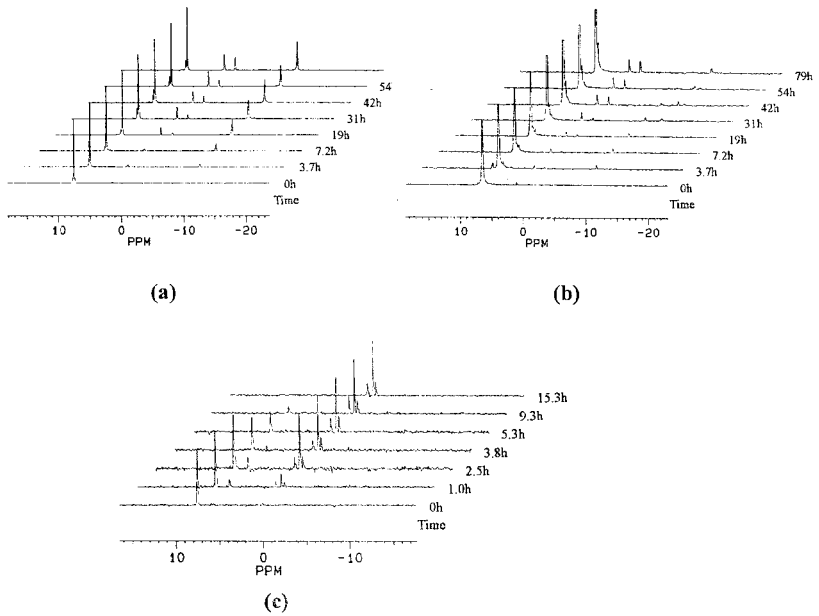


FIGURE 1 The ^{31}P NMR stacked profiles of DIPP-α-Ala incubated in different solvents: (**a**) pyridine, 48°C; (**b**) chloroform, 48°C; (**c**) water, 40°C.

N-phosphodipeptide and tripeptide of (L)-α-alanine (DIPP-Ala_2 and DIPP-Ala_3) were produced from DIPP-α-Ala in all three solvents, which were characterized by both electrospray (ESI) and fast-atom-bombardment (FAB) mass spectrometry. In ESI-MS the ions at m/z 325 and 396, corresponding to protonated DIPP-Ala_2 and DIPP-Ala_3, were identified by multistage mass spectrometry. A particular fragmentation pattern of stepwise extrusion of water, carbon monoxide, and imine was observed.[8] However, there was no peptide product observed in the reaction mixtures of DIPP-β-Ala or DIPP-γ-Aba in each solvent.

According to the ^{31}P NMR stacked spectra, a mechanism for the peptide formation reaction of DIPP-α-Ala was proposed (Scheme 1). In either solvent, a phosphoric-carboxylic mixed anhydride **3** formed from the reactant **1** via an intermediate **2** with a cyclic mixed anhydride moiety, i.e., the phosphoryl group on the nitrogen transferred to the oxygen of the carboxyl group via a five-membered ring intermediate. The transference must be intramolecular, otherwise DIPP-β-Ala and DIPP-γ-Aba must undergo similar reaction under the same conditions. Then the amino group of **3** attacked the carbonyl group of another molecule of **2** to form an *N*-phosphodipeptidyl-phosphoric anhydride **4** and water. The water would react immediately with the mixed anhydride group at the C-terminal of **4** to yield the *N*-phospho-dipeptide **5** and diisopropyl phosphate **6**. Simultaneously, a small amount of dipeptide alanylalanine **8** also was generated by the reaction between two linear anhydride **3**. The amino group of dipeptide **8** attacked **2** to produce *N*-phosphotripeptide **10**.

In pyridine (Figure 1a), a new peak at 1.50 ppm appeared first, corresponding to diisopropyl phosphate **6** as proved by authentic samples in ^{31}P NMR. Then a peak at −10.08 ppm appeared, which might be attributed to the phosphoric-carboxylic mixed anhydride **3**. After 19 h, a peak at 7.79 ppm appeared close to the peak of DIPP-α-Ala at 7.53 ppm, corresponding to *N*-(O,O′-diisopropyl)phosphoryl alanylalanine (DIPP-Ala_2) **5**. From MS results, *N*-phosphotripeptide **10** also was formed. While in ^{31}P NMR, because the chemical shift of **10** is the same as **5** on the Bruker ACP200 NMR spectrometer, no new signal was observed.

In chloroform (Figure 1b), similar reactions occurred, but two differences were apparent. First, the mixed anhydride **3** (at −9.10 ppm) was more reactive so that it was converted to tetraisopropyl pyrophosphate **9** at −12.25 ppm, which was identical to an authentic compound by ^{31}P NMR. It was interesting that in pyridine, **3** was relatively stable and **9** was not produced. The reason might be that the anionic form of diisopropyl phosphate **6** was less reactive than its neutral counterpart present in chloroform. Also, the ^{31}P NMR signals of reactant **1** and product **5** were inverted in these two solvents. The inversion could

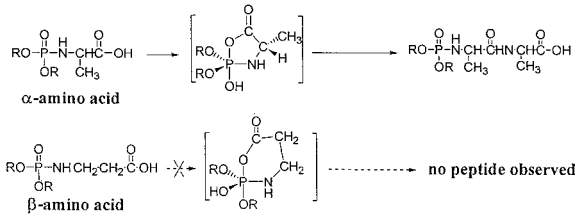
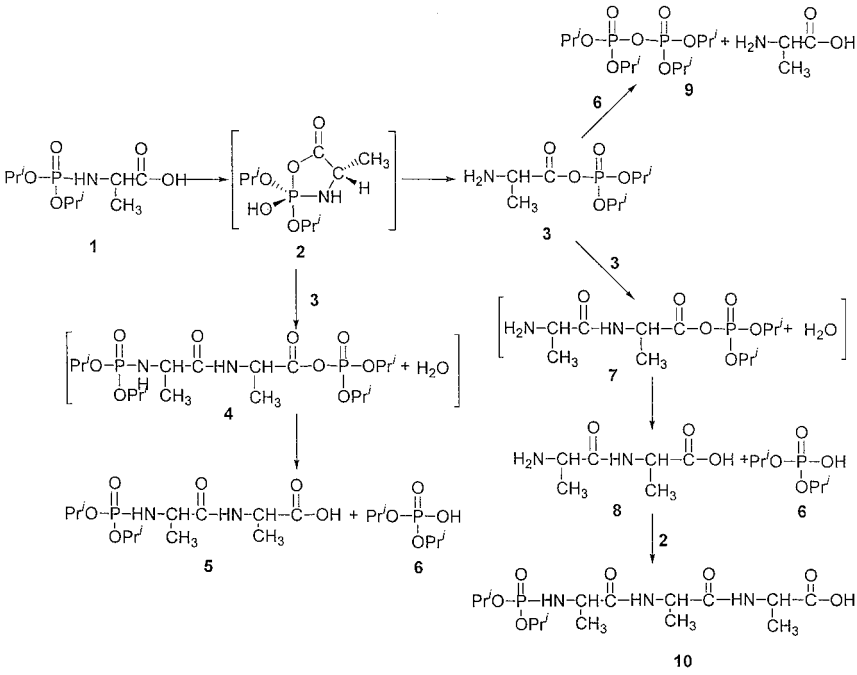


Table of Contents Graphic

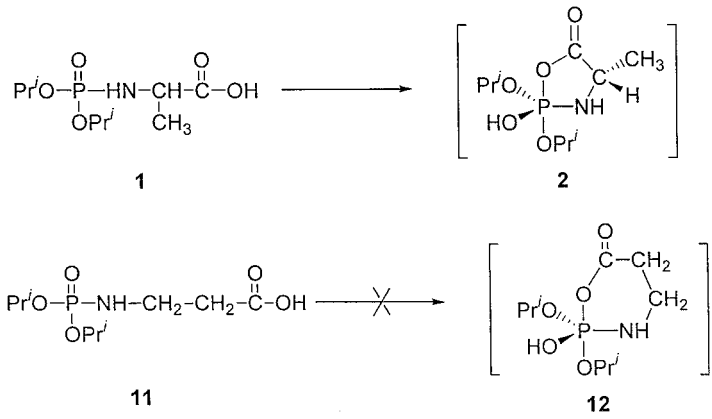


SCHEME 1 The mechanism of peptide formation reactions of DIPP-α-alanine.

also be interpreted by the basicity of pyridine. In pyridine, reactant **1** was partially ionized to form pyridine salt, while in **5** the amide group —CONH— replaced the carboxylic anion —COO^-. As the former was more electro-withdrawing, the signal of DIPP-Ala_2 **5** was at lower field. But in chloroform the reactant **1** was mostly in the form of the acid. Compared with amide group of **5**, the carboxylic group —COOH is more electro-withdrawing. Therefore, the signal of **5** was at higher field.

In water, DIPP-β-Ala or DIPP-γ-Aba did not show any change, but the *N*-phosphodipeptide and *N*-phosphotripeptide of alanine also were

produced from DIPP-α-Ala as well as hydrolysis products. The signal at 5.96 ppm was *N*-(O-isopropyl)phosphoryl alanine, and the three peaks at 0.65, 0.06 and −0.33 ppm were attributed to the hydrolysis products phosphoric acid, diisopropyl phosphate, and mono-isopropyl phosphate, as proved by addition of authentic compounds in ^{31}P NMR determination (Figure 1c).[9] These results support the concept that the mixed anhydride **3** also formed, although no corresponding signal was observed, because the peptide formation and hydrolysis reactions result from the formation of **3**. But because of the existence of large amount of water, the mixed anhydride **3** hydrolyzed too quickly to be observed by ^{31}P NMR.



SCHEME 2 The comparison of the reactivity between *N*-(O,O′-diisopropyl)-phospho-α-Ala and *N*-(O,O′-diisopropyl)phospho-β-Ala by computational method.

In order to understand the reactivity difference between the *N*-phospho-α-amino acid and *N*-phospho-β-amino acid, the proposed two pentacoordinate phosphorus intermediates **2** and **12** (Scheme 2) were investigated by density function theory. The structures and relative potential energies of the two intermediates were obtained by full optimization under B3LYP/6-31G** level by Gaussian98 package.[10] As showed in Figure 2, the relative potential energy of intermediate **2** was 45.61 kJ mol^{-1} lower than that of the intermediate **12** (Table I). This is consistent with the experimental results and the proposed mechanism, in which DIPP-α-Ala could form a five-membered ring pentacoordinate phosphorus intermediate and then undergo intramolecular P—N to P—O transfer. However DIPP-β-Ala forms an energetically unfavorable six-membered ring intermediate. It is reasonable that a

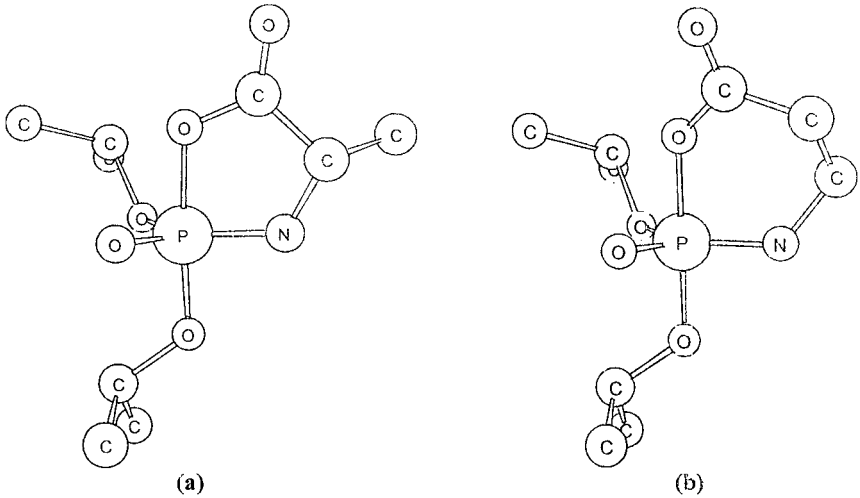


FIGURE 2 The computational structure of the two proposed phosphoric-carboxylic mixed anhydride intermediates: (**a**) intermediate **2** formed by *N*-(O,O′-dimethyl)phosphoryl α-alanine; (**b**) intermediate **12** formed by *N*-(O,O′-dimethyl)phosphoryl β-alanine. (Atoms without label are hydrogens).

seven-membered ring is much more unfavorable in intramolecular reactions compared with five- or six-membered rings. Thus the carboxyl group of *N*-phospho-γ-amino butyric acid is energetically not able to be activated by the phosphoryl group to form a P(V) intermediate.

EXPERIMENTAL

General Procedures

The amino acids were purchased from Sigma Co., Switzerland, *N*-(O,O′-diisopropyl)phosphoryl amino acids were synthesized by the method reported previously.[9] For the reaction in water, 0.4 mmol of each *N*-phospho-α-, β- or γ-amino acids was dissolved in 2.5 mL water respectively and incubated at 40°C for 12 h. Then each solution was

TABLE I Relative Potential Energies of the Two Intermediates **2** and **14** Calculated by DFT Method in Different Media (unit: kJ mol^{-1})

	DIPP-α-Ala	Intermediate **2**	DIPP-β-Ala	Intermediate **12**	ΔE between **2** and **12**
Relative energy	0.00	29.28	0.00	74.89	45.61

freeze-dried. The residue was extracted by methanol and separated by a 1×25 cm Sephadex gel LH20 column. The components with larger molecular weight, which were eluted first, were collected and analyzed by a Brucker ESQUIRE-LC ESI mass spectrometer. For the reaction in organic solvents, 0.2 mmol of each *N*-phospho-α-, β- or γ-amino acids was dissolved in 0.6 mL dry pyrimidine or chloroform respectively and incubated at 48°C. These reactions were monitored by ^{31}P NMR on a Bruker ACP200 NMR spectrometer, and the reaction mixtures were analyzed by FAB-MS on a KYKY ZhP-5a double-focusing mass spectrometer.

***N*-(O,O′-diisopropyl)phosphorylalanylalanine (DIPP-Ala$_2$):** IR and ^{1}H, ^{13}C NMR data were reported previously.[10] LRMS [ESI$^+$, methanol] $m/z = 325$ $[M+H]^+$, $m/z = 307$ $[M-H_2O+H]^+$, $m/z = 283$ $[M-C_3H_6+H]^+$, $m/z = 279$ $[M-H_2O-CO+H]^+$, $m/z = 236$ $[M-Ala+H]^+$.

***N*-(O,O′-diisopropyl)phosphorylalanylalanylalanine (DIPP-Ala$_3$):** LRMS [ESI$^+$, methanol] $m/z = 396$ $[M+H]^+$, $m/z = 378$ $[M+H-H_2O]^+$, $m/z = 354$ $[M-C_3H_6+H]^+$, $m/z = 378$ $[M+H-H_2O-CO]^+$, $m/z = 307$ $[M-Ala+H]^+$.

The Calculations of the Relative Potential Energy

The calculations of the potential energies for compounds **1**, **2**, **11**, and **12** were performed on a SGI12000 workstation. The structures were optimized by Gaussian98 package at B3LYP/6-31G** level.

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