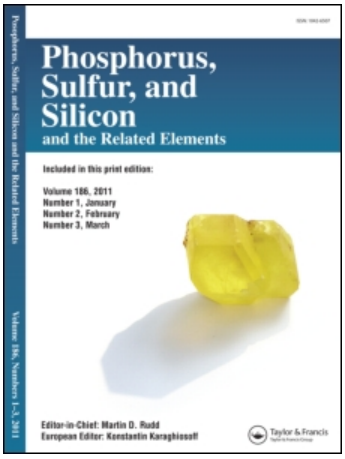

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HYDROLYSIS REACTIONS OF N-PHOSPHOAMINO ACIDS—A MODEL FOR PROTEIN DEPHOSPHORYLATION

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HYDROLYSIS REACTIONS OF N-PHOSPHOAMINO ACIDS—A MODEL FOR PROTEIN DEPHOSPHORYLATION

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Phosphoamino acids are the smallest units of phosphoprotein. In Tris-HCl buffer (pH7.5)/DMSO(v/v = 1:1) mixed solvent at 40°C, the kinetic rates of N-phosphoamino acid hydrolysis reactions were studied. The reactions were pseudo first-order, and the hydrolysis rates of the N-phosphoamino acids were much faster than those of simple phosphoamidates under the same conditions. The kinetic results suggested that the side chain groups of the amino acids played significant roles on the rate of the hydrolysis reaction. For example, the hydrolysis rate constant of a phosphohistidine that had a polar imidazole side chain group was $k = 32.5 \times 10^{-6}$ sec^{-1}, whereas for phosphoglycine without a side chain group $k = 3.3 \times 10^{-6}$ sec^{-1}. The hydrolysis products were identified. The coparticipation of the phosphoryl, carboxyl and amino acids' side chain groups to form a penta-coordinate phosphorus transition state is proposed for this reaction. The analogues of the proposed transition state were synthesized. The dephosphorylation and phosphotransfer mechanisms of phosphoprotein are proposed to occur through a penta-coordinate transition state.

Keywords: phosphoamino acids; hydrolysis reaction; kinetic study; penta-coordinate phosphorus; dephosphorylation; phosphotransfer

INTRODUCTION

It is well known that the phosphorylation and dephosphorylation of proteins play a very important role in regulating complicated biochemical processes.[1–4] How are the proteins phosphorylated or dephosphorylated by protein kinase? This problem is important for the design of artificial enzymes. However, proteins are too large to study and to understand their mechanisms by normal analytic meth-

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ods. Phosphoamino acids are the smallest units of phosphoproteins. All the functional groups of phosphoproteins could be included in twenty types of small phosphoamino acids' molecules. Insights into the mechanism of phosphorylation and dephosphorylation might be obtained partially by studying the properties of these simple phosphoamino acids. In our laboratory, N-(O,O-dialkyl)phosphoryl amino acids were synthesized,[5,6] and they were found to be labile enough to bio-mimic reactions such as the esterification, ester exchange, peptide formation and phosphotransfer reactions.[7–11] In this paper, the hydrolysis reaction of N-phosphoamino acids was studied, and the results suggest clues to the mechanism of dephosphorylation.

RESULTS

1. Kinetic Study

The kinetic of N-phosphoamino acids' hydrolysis reaction was studied by NMR spectroscopy. Each of the compounds listed in Table I was dissolved in Tris-HCl buffer (pH7.5)/DMSO(1 : 1) mixed solvent to give a concentration of 0.1M, and was incubated at 40°C. The reaction was followed by ^{31}P NMR. The kinetic data were obtained by the integral of the ^{31}P NMR peaks, and the disappearing rates of the reactants were considered as the hydrolysis rates of N-phosphoamino acids.

The ^{31}P NMR stack spectra of DIPPSer **20** hydrolysis reaction was showed in Figure 1. According to the results obtained by integrating of the appropriate peak areas gave the curve lnC-t and it was found to be a straight line (Figure 2). Therefore the hydrolysis reactions of N-phosphoamino acids was kinetically first order (equation 1):

$$\ln C_o/C = kt \tag{1}$$

Where C was the concentration of N-phosphoamino acids at time **t** in mol/l; *k* was the kinetic constant in sec^{-1} which could be obtained from the slope of the lnC-t line; t was the reacting time in sec.

The half life time $t_{1/2}$ of each N-phosphoamino acids could be obtained by the equation 2:

$$t_{1/2} = \ln 2/k \tag{2}$$

The *k* and $t_{1/2}$ values of N-phosphoamino acids were listed in Table I.

TABLE I Hydrolysis Rate Constants k and Half Life Time $t_{1/2}$ of N-Phosphoamino Acids

	compounds	k (10^{-6} sec^{-1})	$t_{1/2}$ *(hr)*
1	DIPP-Ala-Ala	0	
2	DIPP-$NHCH_2CH_2OH$	0	
3	DIPP-NHBu	0	
4	DIPP-β-Ala	0	
5	DIPPHPro	1.3	148
6	DIPPPro	3.3 ± 0.1	58.3
7	DIPPGly	3.3	58.3
8	$(DIPP)_2Lys$	9.4	20.5
9	DIPPTyr	10.6	18.2
10	DIPPPhe	10.8	17.8
11	DIPPTrp	11.1	17.3
12	DIPPMet	11.7	16.5
13	DIPPAla	12.5 ± 0.1	15.4
14	DIPPLeu	12.8	15.0
15	DIPPIle	12.9	14.9
16	DIPPVal	12.9	14.9
17	DIPPAsn	13.3	14.5
18	DIPPGln	13.3	14.5
19	DIPPGlu	15.1	12.8
20	DIPPSer	15.1 ± 0.7	12.8
21	DIPPAsp	17.4	11.1
22	DIPPThr	17.4 ± 0.4	11.1
23	DIPPCys	29.4	6.5
24	DIPPHis	32.5	5.9

*Initial pH value was 7.5 for each solution

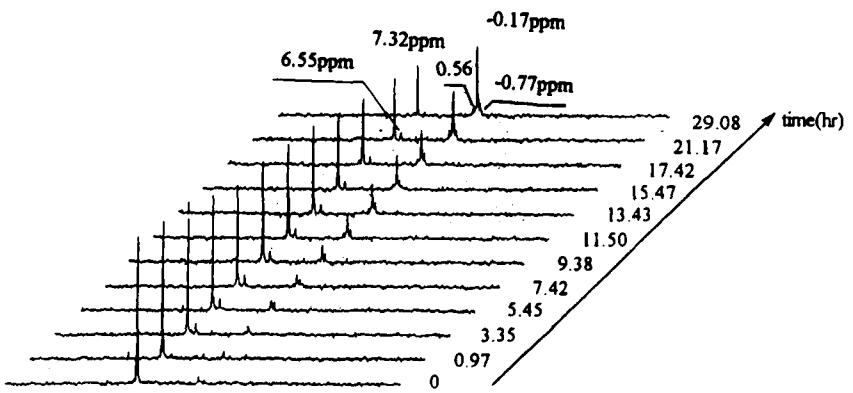


FIGURE 1 The stack ^{31}P NMR spectra of DIPPSer **20** hydrolysis reaction. DIPPSer **20** (7.32ppm), (HO)(iPrO)P(O)Ser **27** (6.55ppm), DIPPOH **25** (−0.77ppm), $(iPrO)P(O)(OH)_2$ **26** (−0.17ppm), H_3PO_4 **28** (0.56ppm).

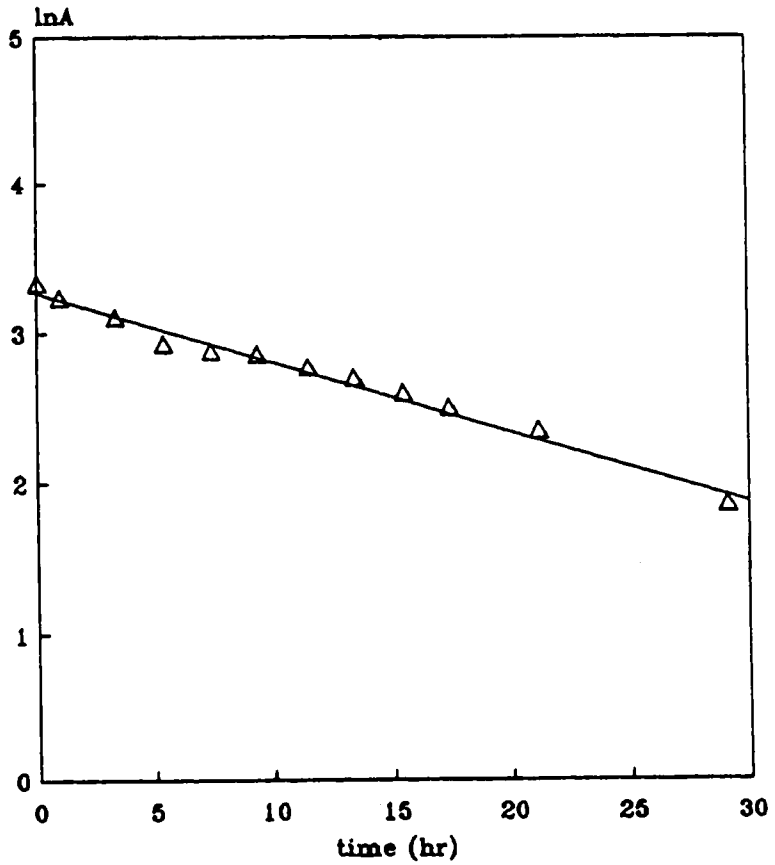


FIGURE 2 The kinetic curve lnA—t of DIPPSer **20** hydrolysis reaction (A was the integral value of DIPPSer **20**, ^{31}P NMR peak at 7.32ppm).

2. Effect of Carboxyl Group

The kinetic results showed that the N-phosphoamino acids were much more easily hydrolyzed than the simple phosphoamidates under the same conditions. For example, the hydrolysis rate constant for DIPPA1a **13** was $k = 25 \times 10^{-6}$ sec^{-1}, while for DIPP-$NHCH_2CH_2OH$ **2** and DIPP-NH-Bu **3** were $k \approx 0$. Comparison of their structures led to the conclusion that the only difference was that there wasn't intramolecular carboxyl groups in phosphoamidates **2** and **3**. This difference suggested that the carboxyl group may act as a neighboring group to catalyze the hydrolysis reaction. It was also found that the catalysis of carboxyl group was regioselective and stereoselective.

A. Regioselectivity

If the carboxyl group were acting only as the acid catalyst, it would be expected that phosphoamidates **2** and **3** should undergo hydrolysis reaction easily in the presence of acetic acid. However, with addition of acetic acid, the solution of compound DIPP-NHBu **3** (pH4) was kept at 40°C for 20hr, ^{31}P NMR spectra showed that there was still no obvious hydrolysis reaction occurring. Therefore, the carboxyl group should be an intramolecular catalysis group.

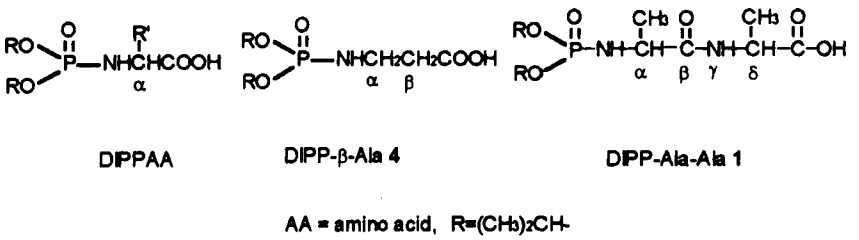
Furthermore, it seemed that not all intramolecular carboxyl groups could catalyze the reaction efficiently. For example, DIPPA1a **13** with a carboxyl group at α position could be hydrolyzed easily, while DIPP-β-A1a **4** and DIPP-A1a-A1a **1** with carboxyl group at β or δ position respectively (Scheme 1) were not be able to be hydrolyzed at all under the same mild conditions. It was interesting to find that although there were two phosphoryl groups in compound **8**, only the P_α-N bond could be cleavaged but not the P_ϵ-N bond (Figure 3). This observation strongly suggested that the intramolecular carboxyl group could only catalyze the hydrolysis of the P_α-N bond, in another word, only the molecular α carboxyl group could catalyze the hydrolysis reaction of N-phosphoamino acids.

B. Stereoselectivity

In compounds DIPPSer **20** and DIPPHPro **5**, containing both carboxyl and hydroxyl groups, the hydrolysis rate constant of DIPPSer **20** was 15.1×10^{-6} sec^{-1}, while for DIPPHPro **5** was 1.3×10^{-6} sec^{-1}. Comparison of their structures, DIPPSer **20** was a linear compound, its carboxyl group could easily catalyze the reaction, whereas for DIPPHPro **5** was a cyclic compound, the steric hindrance would inhibit the catalysis of the carboxyl group.

3. Effect of Side Chain Groups

From the kinetic data in Table I we find that the hydrolysis rates for different phosphoamino acids also varied greatly with their side chain groups. For ex-



SCHEME 1 The position of carboxyl group in different compounds.

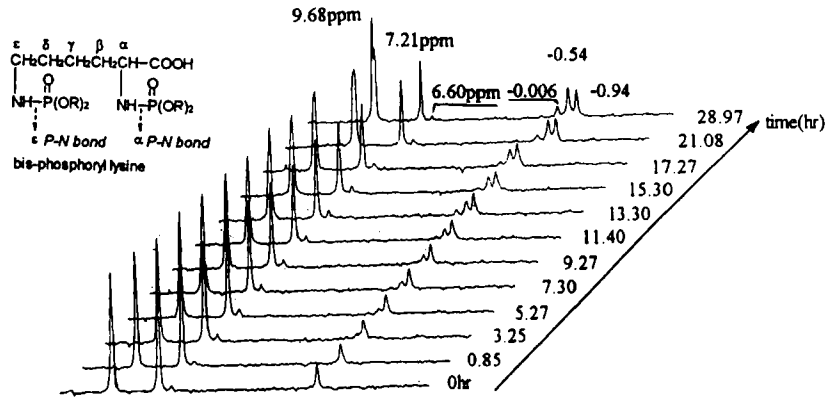


FIGURE 3 The structure of $(DIPP)_2$Lys **8** and its hydrolysis reaction stack ^{31}P NMR spectra.

ample, the hydrolysis rate constant of DIPPHis **24** with an imidazole side chain group was $k = 32.5 \times 10^{-6}$ sec^{-1}, while for DIPPGly **7** without any side chain group $k = 3.3 \times 10^{-6}$ sec^{-1}. According to their hydrolysis rates, the N-phosphoamino acids could be classified into three categories:

A. N-phosphoamino acids with polar side chain groups such as DIPPHis **24**, DIPPCys **23**, DIPPAsp **21**, DIPPGlu **19**, DIPPSer **20**, DIPPThr **22**, DIPPAsn **17**, DIPPGln **18**, had much greater rate constants. Among them the most reactive one was DIPPHis **24**. It was expected that these polar side chains might take part in the intramolecular catalysis function.
B. The DIPPPro **6** and DIPPGly **7** had the smallest rates (vide ante).
C. N-phosphoamino acids with aliphatic and aromatic nonpolar side chains such as DIPPMet **12**, DIPPTrp **11**, DIPPAla **13** et al., had the moderate rate constants compared with the above two categories. In DIPPMet **12**, the mercapto group was blocked by the methyl group. In $(DIPP)_2$Lys **8**, the ε amino group was also phosphorylated, these two side chains couldn't participate the catalysis. In DIPPTrp **11**, DIPPTyr **9** and DIPPPhe **10**, there were large aromatic group in side chain which might cause steric hindrance to reaction. For compounds **13**, **14**, **15** and **16**, their aliphatic nonpolar side chains were unable to participate the intramolecular catalysis. Therefore these compounds could not be hydrolyzed as fast as the compounds of category A.

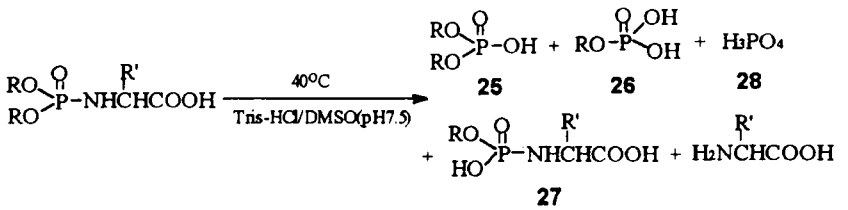
4. Identification of the Hydrolysis Products

0.1M DIPPSer **20** was dissolved in water and incubated at 40°C for 15 hr, the ^{31}P NMR spectrum showed that there were three hydrolysis products. The authentic samples were synthesized and the products were identified respectively

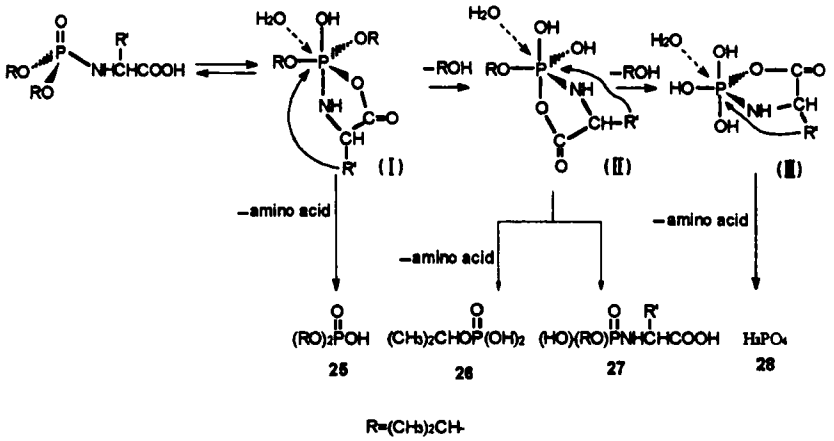
to be diisopropyl phosphate **25** (−0.77ppm), isopropyl phosphate **26** (−0.17ppm) and phosphoric acid **28** (0.56ppm). For the hydrolysis reaction of DIPPSer **20** in Tris-HCl buffer(pH7.5)/DMSO (1:1) mixed solution, the products were found to be compounds **25**, **26**, **28** and (HO)(iPrO)P(O)Ser **27** by the same method. The general hydrolysis reaction of N-phosphoamino acids is illustrated in Scheme 2.

5. Mechanism Study

To account for all the above facts, the coparticipation of carboxyl group, phosphoryl group and amino acids' side chain groups was proposed for the hydrolysis reaction of N-phosphoamino acids (Scheme 3). In this mechanism, the phosphoryl group was attacked by α-carboxyl group and the penta-coordinate transition state **I** was formed first, which then was activated by polar side chains and attacked by water molecule. There were two possible pathes to follow. The cleavage of P-O bond to form the transition state **II**, on the cleavage of the P-N bond to form the product dialkyl phosphate **25**. The same process could happen to the transition state **II** to give the products **26**, **27** and transition state **III** which finally to reach the phosphoric acid **28**. In compounds DIPP-NH-Bu **3**, DIPP-NH-CH_2OH **2**, the penta-coordinate transition state couldn't be formed without the catalysis of intramolecular carboxyl group. In compounds DIPP-β-Ala **4** and DIPP-Ala-Ala **1**, there are carboxyl groups which are too far away to form the five-membered ring transition states. In compounds DIPPPro **6** and DIPPHPro **5**, the steric hindrance prevented the carboxyl group from forming the penta-coordinate transition states. Therefore these compounds couldn't be hydrolyzed easily under mild conditions. In fact, their hydrolysis reaction occurs only under strongly acid condition such as concentrated hydrochloric acid similar to simple phosphoamidates.[12,13] The acid catalyzed hydrolysis of phosphoamidates has been investigated before,[14,15] and the protonization of the phosphoryl group was proposed to be important, which is different from our proposed mechanism.



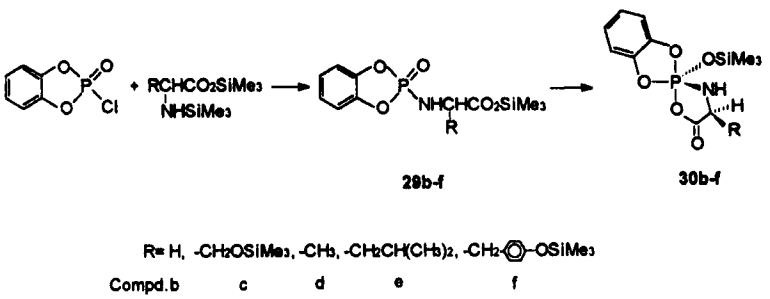
SCHEME 2 The hydrolysis reaction of N-phosphoamino acids.



SCHEME 3 Proposed mechanism for N-phosphoamino acid hydrolysis reaction.

The life time of the penta-coordinate phosphorus transition states were too short to be observed by ^{31}P NMR spectra. However, the transition state analogues compounds **30** were obtained in the following reaction (Scheme 4). The phosphoamino acids **29** with stabilizing trimethylsilyl groups $(Me)_3SiO$- were formed easily, then they were completely transferred into the penta-coordinate products **30** within 30 min. These compounds **30** were stable and their structures were identified (Table II).

The kinetic rates for the formation of the penta-coordinate phosphorus compounds **30** were also studied. Their rate constants were listed in Table III. It was interesting to find that the formation rates of these compounds **30** had the same order as the hydrolysis reaction rates of corresponding N-phosphoamino acids. It supported the contention that the proposed penta-coordinate transition states were reasonable for the mechanism of the N-phosphoamino acids' hydrolysis reaction, and that the formation of the transition state was the rate-controling step.



SCHEME 4 Synthesis of the penta–coordinate phosphorus transition state's analogue.

TABLE II The Structure Data of Penta-Coordinate Phosphorus Compounds **30**

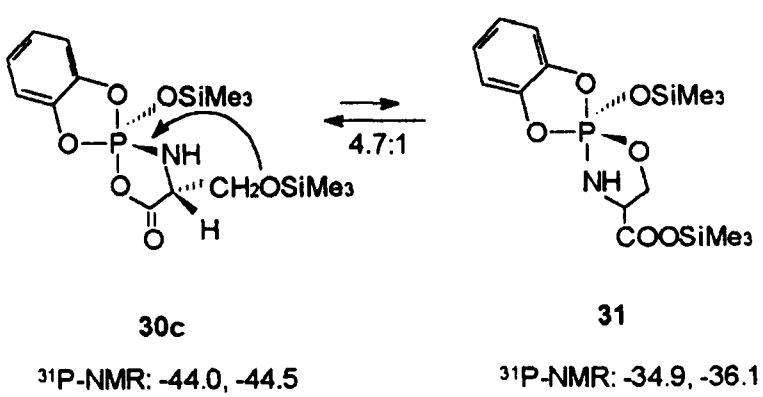
Comp.	*corrsp.aa.*	*HR EI-MS*		*NMR(ppm)*
		Cal.	*Ana.*	
30b*	Gly			^{31}P-NMR: −42.0
30c	Ser	403.1036	403.1033	^{13}C-NMR: 0.48, 1.95, 57.25, 55.69, 110.38, 119.92, 123.04, 141.85, 145.23, 167.25. ^{31}P-NMR: −44.0, −44.5.
30d	Ala	315.0692	315.0695	^{1}H-NMR: 0.40(s,9H), 1.38–1.42(m,3H), 1.63–1.66(d,1H), 3.75–4.05(m,1H), 6.73–6.98(m,4H). ^{31}P-NMR: −45.4, −46.0.
30e	Leu	357.1161	357.1165	^{1}H-NMR: 0.43(s,9H), 0.91–1.02(q,6H), 1.71–1.82(m,1H), 3.83–3.91(m,1H), 4.56(s,1H), 6.75–7.04(m,4H). ^{13}C-NMR: 0.82, 21.71, 23.01, 25.18, 42.86, 53.25, 109.86, 110.38, 111.16, 111.95, 120.27, 123.30, 169.50. ^{31}P-NMR: −45.1, −45.6.
30f	Tyr	479.1349	479.1353	^{1}H-NMR: 0.17(s,9H), 0.25–0.28(s,9H), 2.70–2.79(m,1H), 3.15–3.20(m,1H), 3.66–3.79(q,1H), 4.06(w,1H), 6.67–7.14(m,8H). ^{13}C-NMR: 0.39, 0.72, 38.85, 56.30, 109.71, 110.25, 111.01, 111.27, 120.33, 123.26, 128.35, 128.89, 130.30, 142.11, 144.89, 154.46, 168.44. ^{31}P-NMR: −45.4, −45.5.

*Only 75% of linear compound **29b** could be transferred into **30b** cyclic compound.

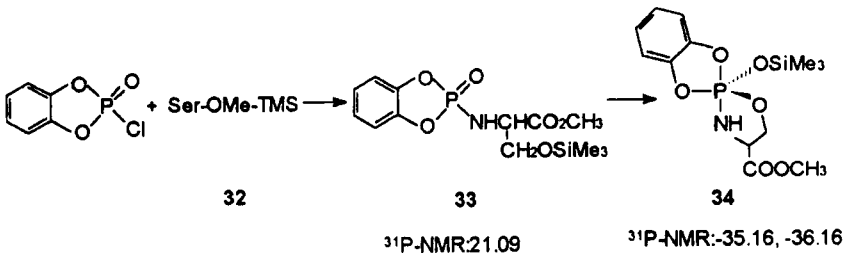
TABLE III The Formation Rate Constant k' and Half Time $t_{1/2}$ of Penta-Coordinate Compounds **30**

Starting Amino Acids	*^{31}PNMR of linear comp.* **29**	*^{31}PNMR of cyclic comp.* **30**	$k'(10^{-4}\ sec^{-1})$	$t_{1/2}$ *(min)*
β-Ala	22.6	non	\	\
Pro	17.8	non	\	\
HPro	18.1	non	\	\
Gly	21.1	−42.0		
Leu	21.3	−45.1, −45.6	3.6	32
Tyr	20.5	−45.4, −45.5	4.7	25
Phe	20.3	−45.4, −45.5	7.3	16
Ala	20.7	−45.4, −46.0	8.5	13
Met	20.9	−44.0, −44.5	13	9
Ser	21.3	−44.0, −44.5	15	7
Thr	22.7	−44.1, −44.9	20	5.8
Asp	21.0	−44.8, −45.4	16	7.2
His	21.8	−45.0, −45.2	38	3.0

When the penta-coordinate phosphoserine **30c** was formed, it was easily transformed into another penta-coordinate phosphorus compound **31** (Scheme 5) with an equilibrium ratio 4.7/1 after 12 hr. To identify the hydroxyl isomer compound **31**, standard compound **34** with similar structure was synthesized (Scheme 6), which had ^{31}P NMR shifts similar to compound **31**. It demonstrated that in DIPPSer **20**, the side chain group -CH_2OH could really attack the phosphorus atom and catalyze the hydrolysis reaction. The observed ratio 4.7/1 also demonstrated that the catalysis of carboxyl group was more efficient than that of the side chain groups.



SCHEME 5 Transfer of two penta-coordinate phosphoserine compounds.

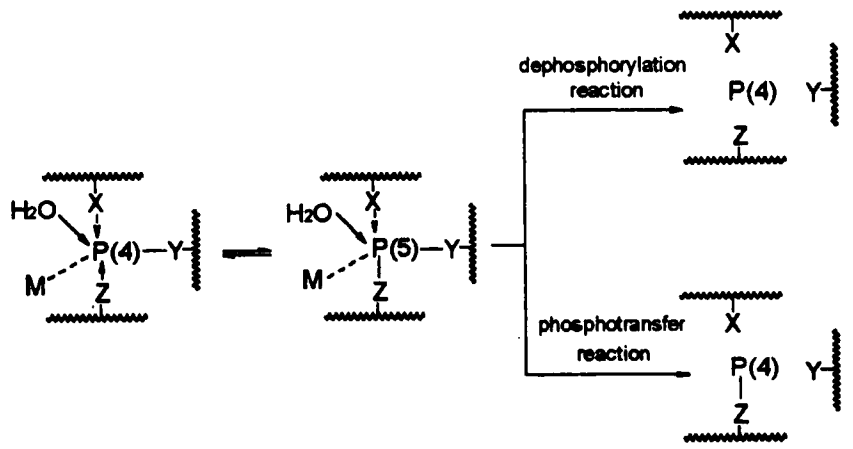


SCHEME 6 Synthesis of the analogue of compound **31**.

The reason for the retardation on hydrolysis of DIPPPro **6** and DIPPGly **7** might also be due to the inhibition on the formation of the transition state too. Indeed, it was found that the linear phosphoproline derivative **29** was unable to form a penta-coordinate phosphorus compound corresponding to **30**, while for phosphoglycine derivative **29b**, only 75% could be transferred into its penta-coordinate derivative **30b**. Therefore DIPPPro **6** could only be hydrolyzed like simple phosphoamidates and DIPPGly **7** could be hydrolyzed partially with the catalysis of carboxyl group. They were the least reactive ones toward hydrolysis reaction of N-phosphoamino acids.

DISCUSSION

Recently more and more N-phosphoproteins were discovered as new techniques were used.[16,17] They may also play very important role in regulating biochemical process like O-phosphoproteins.[18–20] In this paper, the N-phosphoamino acids' hydrolysis reaction could act as a model of N-phosphoproteins' dephosphorylation. It is expected that the dephosphorylation of N-phosphoproteins might occur through a transition state similar to the hydrolysis reaction of N-phosphoamino acids. In additional, the hydroxyl groups of Ser and Thr were found to be able to attack the phosphoryl group in N-phosphoamino acids. The P-O bond of hydroxyl group and P-N bond of amino group could exist together in the same penta-coordinate transition state (Scheme 3 and Scheme 5). The O-phosphoamino acids may share the same activated transition state with the N-phosphoamino acids. Therefore N-phosphoproteins and O-phosphoproteins were expected to have the same activated transition state in their dephosphorylation reaction. The proposed mechanism is illustrated in Scheme 7. First, the negative charge of $P\text{-}O^-$ bond in phosphoryl group was neutralized by metal ions or positive charged base residues in protein so that the phosphorus atom could be attacked by nucleophilic molecules or groups. For N-phosphoamino



SCHEME 7 Proposed mechanism for protein dephosphorylation and phosphotransfer reactions.

acids this step is not needed because their P-O$^-$ bonds are blocked by isopropyl groups. The second step is the formation of the activated penta-coordinate phosphorus transition state by the nucleophilic attack of water or protein's polar residues such as hydroxyl, carboxyl, amino, imidazole et al. In the third step, the transition state is promoted by polar residues to cleave either P-O or P-N bond followed by subsequent dephosphorylation or phosphotransfer reactions. In fact, it was found that the N-phosphoamino acids could also take place phosphotransfer reaction via the same mechanism as hydrolysis reaction.[8] Thus the dephosphorylation and phospotransfer of proteins were expected to follow similar mechanisms. If these nucleophilic residues came from the phosphoprotein, it would be autodephosphorylated or autophosphotransferred. If they came from another protein, the phosphoprotein would be dephosphorylated or phosphotransferred by protein kinase.

In biochemical systems, a similar mechanism was proposed in E. Coli bacterial chemotaxis. J. Fred Hess et al found that phosphohistidine of CheA protein was important in signal transduction systems for E. Coli bacterial chemotaxis.[21] Lukat et al found that the phosphoryl group could be transferred from histidine in CheA to aspartic acid in CheY. In their proposed mechanism, the coparticipation of amino group in Lys, carboxyl group in Asp and Mg^{2+} metal ion could activate the phosphoryl group to form a penta-coordinate transition state which could result in phosphotransfer and dephosphorylation of phosphohistidine.[22] Their proposed transition state was similar to our proposed transition state. However, we are looking foward to more biochemical examples of our proposed mechanism.

In conclusion, this study found that the hydrolysis reaction of N-phosphoamino acids was strongly dependent on the amino acids' side chains which may be related to the dephosphorylation and phosphotransfer mechanism of phosphoprotein.

EXPERIMENTAL SECTION

MATERIALS

Amino acids (Sino-American Biotechnology Co. China, Chromatography grade) were all L-allo. Compounds **1–24** were prepared as described before.[5,6]

METHODS

The ^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR spectra were taken on a BRUKER ACP200 spectrometer. Chemical shifts of ^{31}P-NMR are referenced to external 85% H_3PO_4. ^{1}H-NMR and ^{13}C-NMR are referenced to internal tetramethylsilane. EI HR-MS data were taken on a ZAR-HS GC-MS spectrometer.

1: Effect of Acetic acid on DIPP-NH-Bu **3**: 60 mg (1 mmol) acetic acid was added into 1 ml 0.5M compound **3** solution in water, and then the solution was kept at 40°C for 20 hr. ^{31}P-NMR spectra showed that there was no reaction occurred.

2: Kinetic study For each N-phosphoamino acids, a 0.1M solution in Tris-HCl buffer (0.1M, pH 7.5) and DMSO mixed solvent (1 : 1) was prepared respectively. The solution was kept at 40°C and the reaction was monitored by ^{31}P-NMR spectra. For quantitive determination of each compound, the NMR spectrometer parameters were RG = 100, RD = 10, NS = 16, CPD = 22H. The total hydrolysis rate constants for compounds **1–24** were measured by the disappearing rates of these compounds as calculated by equation $\ln C_o/C = kt$.

3: Preparation of compounds **30**: All operations were under protection of nitrogen. 0.4 ml 0.5M N,O-bis(trimethylsilyl)amino acid[23] in $CHCl_3$ and 0.2 ml 1M O,O-phenylene phosphochloridate[24] in CCl_4 were added into a 5 mm NMR tube. The tube was sealed immediately and then the reaction was monitored by ^{31}P-NMR. First, the tetra-coordinate phosphoamino acids **29** were formed, and then they were transformed into the penta-coordinate products **30** completely as determined by ^{31}P-NMR. The solvent was removed in vacuum and the colorless products **30** were obtained, yields near 99%.

4: Methyl O-trimethylsilylserine ester **32** was prepared by the method of Smith.[24] ^{1}H-NMR: 0.04(s, 9H), 1.70(s, 2H), 3.67(s, 3H), 3.47(t, 1H), 3.76(t, 2H). ^{13}C-NMR: −1.01, 51.52, 56.11, 64.51, 173.99.

5: Compound **34** was prepared by the similar method for **30**. ^{1}H-NMR: −0.01(s, 9H), 3.66(s, 3H), 3.57–4.21 (m, 3H), 6.57–6.89(m, 4H). ^{31}P-NMR: −35.16, −36.16. ^{13}C-NMR: 0.74, 51.8–52.9, 60.4–61.2, 54.9, 109.2, 109.8, 122.4, 123.4, 142.3, 145.6, 175.0. EI:($C_{13}H_{20}NO_6PSi$)345. HR EI-MS:345.0799(calculated), 345.0784(found).

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