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β-Carboxyl Catalytic Effect of N-Phosphoryl Aspartic Acid

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The β-carboxylic group in N-dialkylphosphorylated aspartic acid has an activating effect that gives rise to peptides, esters, and ester exchange at the phosphoryl group. In contrast, the γ-carboxylic group of N-alkylphosphorylated glutamic acid has a much smaller effect. Some of the self-activating products were isolated and many model compounds were synthesized to study the novel activating effect of the β-carboxylic group. Mixed anhydride intermediates derived from α-carboxylphosphoryl and β-carboxylphosphoryl groups are proposed for the self-activation mechanism. © 1992 Academic Press, Inc.

INTRODUCTION

Acidic amino acid residues are biologically interesting in several respects. It has been established that the active center of many enzymes is a serine or threonine flanked by both basic and acidic amino acid residues, and the activation of these enzymes is regulated through phosphorylation–dephosphorylation. For example, the active center of the alkaline phosphatase from the bacterium prodigiosum is Asp–Ser–Ala and that of the thrombase from beef is Gly–Asp–Ser–Gly (*1*). It is widely known that there are 20 natural amino acids in living organisms and slight variations in the side chain can cause tremendous deviation in the chemical properties. What are the different effects of the β- and γ-carboxylic groups on the life processes? In order to understand the chemistry of this biological problem, a single amino acid residue was phosphorylated and the novel properties of the N-dialkylphosphorylated amino acids were studied. Our research group has synthesized 18 N-diisopropylphosphorylated (DIPP) amino acids (*2, 3*) and the interesting properties of DIPP-Ser, DIPP-Thr, N-dibutylphosphoryl-Ala, etc., were studied (*4–7*). In this paper, we report the synthesis and the novel properties of N-phosphorylated aspartic acid.

Peptide formation, esterification, and ester exchange at the phosphoryl group were the fundamental chemical reactions of the amino acid during life processes. Without peptide formation, no enzymes would form and no life process would work. Esterification of the amino acids is the first step in protein biosynthesis. Without esterification, the amino acids could not be carried by tRNA and no

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protein could be synthesized. Ester exchange at the phosphoryl group is the key to the self-catalysis of the RNA, and this has been hypothesized to relate to the foundation of biocatalysis (*8–10*).

RESULTS AND DISCUSSION

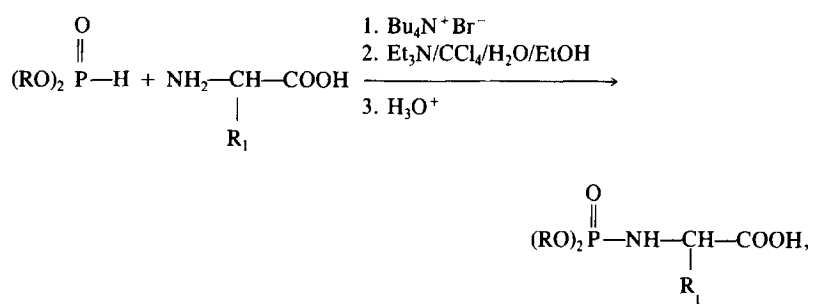
Synthesis

At first, aspartic acid was phosphorylated in a mixed EtOH/H_2O system and then extracted by ethyl acetate, as described in the literature (*2*). However, there was none of the expected product. It was found that the phase transfer catalyst, such as *n*-tetrabutylammonium bromide was essential to the synthesis of *N*-dialkylphosphoryl aspartic acids. Due to the polar effect of the extra carboxylic side chain, the ethyl acetate used previously (*2*) was too weak to extract the *N*-dialkylphosphoryl aspartic acids from the mixture. Therefore, pure 1-butanol or a mixed solvent of *tert*-butanol and ethyl acetate should be applied for an efficient extraction. In doing so, the pure *N*-dialkylphosphoryl aspartic acids (e.g., *N*-diisopropylphosphoryl aspartic acid, DIPP-Asp, *N*-dibutylphosphoryl aspartic acid, DBP-Asp, and *N*-diethylphosphoryl aspartic acid, DEP-Asp) were isolated in 60–70% yield (Scheme 1).

Similarly, the analogues *N*-dibutylphosphoryl glutamic acid (DBP-Glu) and *N*-diethylphosphoryl glutamic acid (DEP-Glu) were synthesized in 60–75% yield. The purity and structure of these novel compounds were determined by microanalysis, fast atom bombardment mass spectrometry (FAB-MS), and ^1H NMR, ^{13}C NMR, and ^{31}P NMR spectrometry (Tables 1 and 2).

Novel Properties of N-Diisopropylphosphoryl Aspartic Acid (DIPP-Asp)

Compared with the other non-functional-group amino acids, the DIPP-Asp was very unstable. For example, as the fresh pure sample was incubated in 1-butanol at 40°C for 8 h, some solid substances precipitated out. Its ir spectrum shows



SCHEME I

TABLE 1

The ^{1}H NMR and FAB-MS Data for Some *N*-Dialkylphosphoryl Amino Acids

Entry compound	^{1}H NMR (ppm)	FAB-MS $(MH)^+$
5, DIPP-(α-OBz)-Asp ($CDCl_3$)	1.1–1.5 (d, 12H), 2.7–3.1 (m, 2H) 3.6–4.0 (m, 2H), 4.3–4.8 (m, 2H) 5.0–5.2 (s, 2H), 7.2–7.4 (s, 5H) 9.5–9.7 (br, s, 1H)	388
6, DIPP-(α-OBz)-Glu ($CDCl_3$)	1.0–1.4 (d, 12H), 1.9–2.4 (m, 4H) 3.3–4.0 (m, 2H), 4.1–4.5 (m, 2H) 5.0–5.3 (m, 3H), 7.3–7.4 (s, 5H)	402
11, DIPP-Gln ($CDCl_3$)	0.9–1.2 (d, 12H), 1.7–2.1 (m, 4H) 3.2–3.6 (m, 1H), 4.1–4.5 (m, 3H) 6.4–6.7 and 6.9–7.2 (br, s, 3H)	311
14, DBP-Asp (d_6-DMSO)	0.7–1.1 (t, 6H), 1.2–2.0 (m, 8H) 2.8–3.1 (m, 2H), 3.6–4.3 (m, 6H) 10.7–11.3 (br, s, 2H)	326
15, DEP-Asp (d_6-DMSO)	1.0–1.6 (t, 6H), 2.5–2.7 (d, 2H) 2.8–3.2 (m, 1H), 3.7–4.2 (m, 5H) 7.7–8.1 (br, s, 2H)	270
16, DBP-Glu (d_6-DMSO)	0.7–1.0 (t, 6H), 1.1–2.0 (m, 10H) 2.1–2.5 (m, 2H), 2.8–3.1 (m, 1H) 3.4–4.0 (m, 5H), 5.6–6.0 (br, s, 2H)	340
17, DEP-Glu (d_6-DMSO)	1.0–1.3 (t, 6H), 1.8–2.0 (m, 2H) 2.2–2.5 (t, 2H), 2.7–3.2 (m, 2H) 3.7–4.1 (m, 4H), 4.7–6.6 (br, s, 2H)	284
13, DIPP-Asp ($CDCl_3$)	1.1–1.5 (d, 12H), 2.6–2.9 (m, 2H) 3.8–4.2 (m, 2H), 4.3–4.9 (m, 2H) 10.5–11.0 (br, s, 2H)	298

the amide group absorption around 1605–1640 cm^{-1} and the carboxylic group absorption around 1660–1760 cm^{-1}. The FAB mass spectra of the solution portion indicated that in addition to the signal at $(MH)^+/z$ 298 for DIPP-Asp, there were several new signals at $(MH)^+/z$ 312, 326, 354, 368, 382, 410, 424, 438, 413 which could be respondes to the ester exchange at the phosphoryl products *N*-(iPrO) (BuO)-P(O)-Asp, and *N*-$(BuO)_2$-P(O)-Asp; the self-catalysis for the esterification products DIPP-Asp · OBu, *N*-(iPrO) (BuO)-P(O)-Asp · OBu, *N*-$(BuO)_2$-P(O)-Asp · OBu, DIPP-Asp · $(OBu)_2$, *N*-(iPrO) (BuO)-P(O)-Asp · $(OBu)_2$, and *N*-$(BuO)_2$-P(O)-Asp · $(OBu)_2$; and the self-activating for the peptide formation product DIPP-Asp–Asp, respectively (Table 3). These compounds showed overlapping ^{31}P NMR signals at 5.3, 6.4, and 7.5 ppm. Incubation of the fresh pure DIPP-Asp in benzyl

TABLE 2

^{13}C NMR and ^{31}P NMR Chemical Shifts (ppm) and Coupling Constants (Hertz in Parentheses) of *N*-Dialkylphosphoryl Amino Acids

	^{13}C NMR					
	$(RO)_2P(=O)-NH-C_\alpha(-C_\beta-C_\gamma)-COOH$					
Entry compound	COOH	C-α	C-β	C-γ	C of R	^{31}P NMR
5	172.8(7.3)	52.0	39.6(2.9)	174.6 68.6 136.8 129.7 129.3	23.6 71.9	5.6
6	172.8(2.9)	53.6	28.6(7.3)	29.5 173.6 69.7 135.9 128.3 127.8	23.9 63.8	5.8
11	174.4(2.9)	53.7	29.4(5.9)	31.0 173.6	23.6 69.7	6.0
14	174.7(7.3)	50.4	38.2(5.9)	174.2	11.8 17.2 18.6 62.9	7.8
15	174.2	51.8	32.1	172.5	16.2 63.6	7.7
16	175.1(4.4)	53.4	29.4(8.8)	31.0 176.5	13.4 18.3 18.6 61.6	7.5
17	173.8(7.3)	53.4	28.7(5.9)	29.9 174.2	16.0 63.4	7.5
13	174.2(5.8)	51.9	32.0	172.4	23.5 69.0	5.1

alcohol at 40°C for 8 h, would result in the peptide formation product DIPP-Asp–Asp ($(MH)^+ = 413$), ester exchange at the phosphoryl product *N*-(iPrO)(BzlO)-P(O)-Asp ($(MH)^+ = 346$), and the esterification product DIPP-Asp · (OBzl) ($(MH)^+ = 388$).

Isolation of the Peptide and Esterification Products

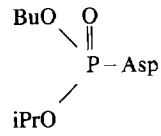
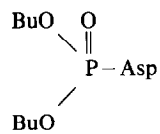
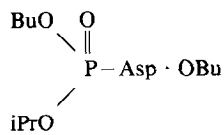
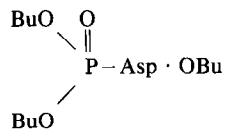
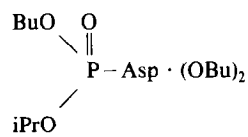
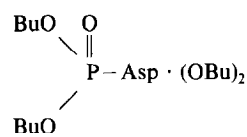
In order to identify and isolate the final products, a series of experiments were designed to obtain each product group.

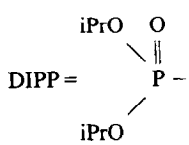
First, the fresh pure sample was incubated in $CHCl_3$, the ester exchange and esterification products were inhibited so that only the peptide was yielded (Scheme 2).

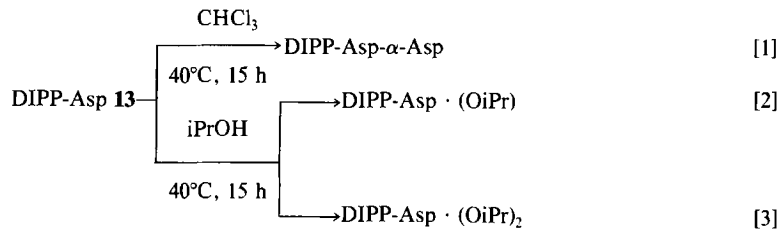
Second, the fresh pure sample was incubated in isopropyl alcohol, since the ester exchange at the phosphoryl will give itself. The mono- and diesterification products were obtained (Scheme 2).

TABLE 3

The FAB-MS Data for DIPP-Asp[a] after Incubation in 1-Butanol

Solid portion		Liquid portion		
$(MH)^+$	Possible compound	$(MH)^+$	Possible compound	Notes
364	Asp–Asp–Asp	298	DIPP-Asp	Original compound
		312		Ester exchange at phosphoryl products
		326		
		368		
		382		Esterification and ester exchange at phosphoryl products
		424		
		438		
		354	DIPP-Asp · OBu	Esterification products
		410	DIPP-Asp · $(OBu)_2$	
		413	DIPP-Asp-Asp	Peptide formation

[a] DIPP = 



SCHEME II

All of the products isolated were determined by ^{31}P NMR, ^{1}H NMR, ^{13}C NMR, ir, FAB-MS, and FAB-HRMS (Tables 4, 5, and 6).

Mechanism Study

In order to study the self-activating mechanism of DIPP-Asp, we extended our experiment to a series of amino acids with free or blocked α, β, or γ-carboxylic groups (compounds **4–13**), and the results are given in Table 7. It can be seen that compounds **4–6**, which do not possess a free α-COOH group (without or blocked by a benzyl group), were unchanged under these conditions, but compounds **7–11**, which possess a free α-COOH group without free β- or γ-COOH, gave small amounts of products with monoester exchange at the phosphoryl. These ester exchange products must have occurred through an intramolecular mixed α-COOH and phosphoryl anhydride intermediate. However, the compound DIPP-Asp 13 with both free α- and β-COOH under the same conditions produced much more complicated products which could be explained as showed in Scheme 3. In addition to the five-member mixed α-COOH and phosphoryl anhydride, there could be an extra catalytic effect of the six-member mixed anhydride derived from the β-COOH and phosphoryl groups. In this intermediate, the phosphoryl group and the carboxyl group activated each other, while the analogue DIPP-Glu, **12,** which has a γ-COOH side chain, yielded a much simpler product distribution. DIPP-Glu gave only trace products of ester exchange at the phosphoryl group. Neither the esterification products nor the peptide was observed. This can be rationalized by the fact that the γ-COOH in DIPP-Glu could only form a much less favorable

TABLE 4

The Infrared ^{31}P NMR, and FAB-MS of the Isolated Products

Product	ir(cm^{-1})	^{31}P NMR (ppm)	FAB-MS $(MH)^+$
1	1715(s, br) 1650(s)	5.1	413
2	1745(s, br)	5.5	340
3	1750(s, br)	6.8	381

TABLE 5

The FAB-HRMS of the Product Isolated

Product	Molecular formula	$(MH)^+/z$ Calculated mass	$(MH)^+/z$ Observed mass
1	$C_{14}H_{25}O_{10}N_2P$	413.1325	413.1303
2	$C_{13}H_{26}O_7NP$	340.1525	340.1490
3	$C_{16}H_{32}O_7NP$		

intramolecular seven-member mixed carboxylic phosphoryl anhydride and plays a very minor role in self-activation. Therefore, it is believed that the six-member mixed anhydride derived from the β-COOH and phosphoryl groups, might play a vital role in the self-catalysis mechanism in the aspartic acid containing the phosphoenzyme.

It is worthwhile to note that the peptide formed in the system can be accounted for as follows. The cleavage of a P—N bond, which is 30 kcal/mol weaker than a P—O bond, would release an amino group that could form an amide bond with a second molecule.

In conclusion, the slight difference between N-phosphorylated aspartic acid and N-phosphorylated glutamic acid can induce a great divergence in many reactions

TABLE 6

^1H NMR and ^{13}C NMR of the Products Isolated (in $CDCl_3$)

Product	^1H NMR (ppm)	^{13}C NMR (ppm) J(Hz) COOH	COOR	CONH	C_α	C_β	C of R
1	1.12–1.50(d, 12H) 2.07–2.87(m, 6H) 3.97–4.39(m, 2H) 4.39–4.81(m, 2H) 8.64(br, 3H)	1.70.4(7.8) 169.8 166.8		172.9	50.6 58.2	38.3 38.3	23.5 69.0
2	1.05–1.45(d, 18H) 1.95–2.05(m, 2H) 2.65–3.10(m, 1H) 4.00–4.75(m, 3H) 4.95–5.15(m, 1H) 9.05–9.10(m, 1H)	173.5	170.6(7.8)		50.9	38.5	23.6 71.9
3	1.15–1.25(m, 24H) 2.05–2.10(m, 2H) 3.20–3.35(m, 1H) 4.05–4.20(m, 4H) 6.50–6.60(d, 1H)		171.2 170.7		58.7	39.3	23.9 63.8

TABLE 7

FAB-MS and ^{31}P NMR of the Incubated Reaction Mixture

Entry compound	Temperature (°C)	Time (h)	FAB-MS (%) M + 1	(M + 1) +14	(M + 1) +28	^{31}P NMR[a]
4, DIPP-β-Ala[b]	40	0	100	0	0	7.9
		8	100	0	0	7.9
5, DIPP-(α-OBz)-Asp	40	0	100	0	0	5.6
		6	100	0	0	5.6
6, DIPP-(α-OBz)-Glu	50	0	100	0	0	5.8
		4	100	0	0	5.8
7, DIPP-Ala[b]	40	0	100	0	0	5.5
		8	88	12	0	5.5 (96%) 5.6 (4%)
8, DIPP-(β-OBz)-Asp[b]	40	0	100	0	0	5.2
		6	84.1	15.9	0	5.2(81%) 6.3(19%)
9, DIPP-Asn[b]	40	0	100	0	0	5.8
		6	97.1	2.9	0	5.8
10, DIPP-(γ-OBz)-Glu	50	0	100	0	0	5.5
		4	66	34	0	5.3(11%) 5.5(44%) 6.5(16%) 7.0(29%)
11, DIPP-Gln	40	0	100	0	0	6.0
		6	94	6	0	6.1(br)
12, DIPP-Glu	40	0	100	0	0	5.4
		6	94	4.2	2.8	6.7(9%) 6.5(7%) 5.4(84%)
13, DIPP-Asp[c]	40	0	100	0	0	5.2
		8	13.1	15.2	13.1	7.5(3%) 6.4(25%) 5.3(71%)

[a] Using 85% H_3PO_4 as external reference.

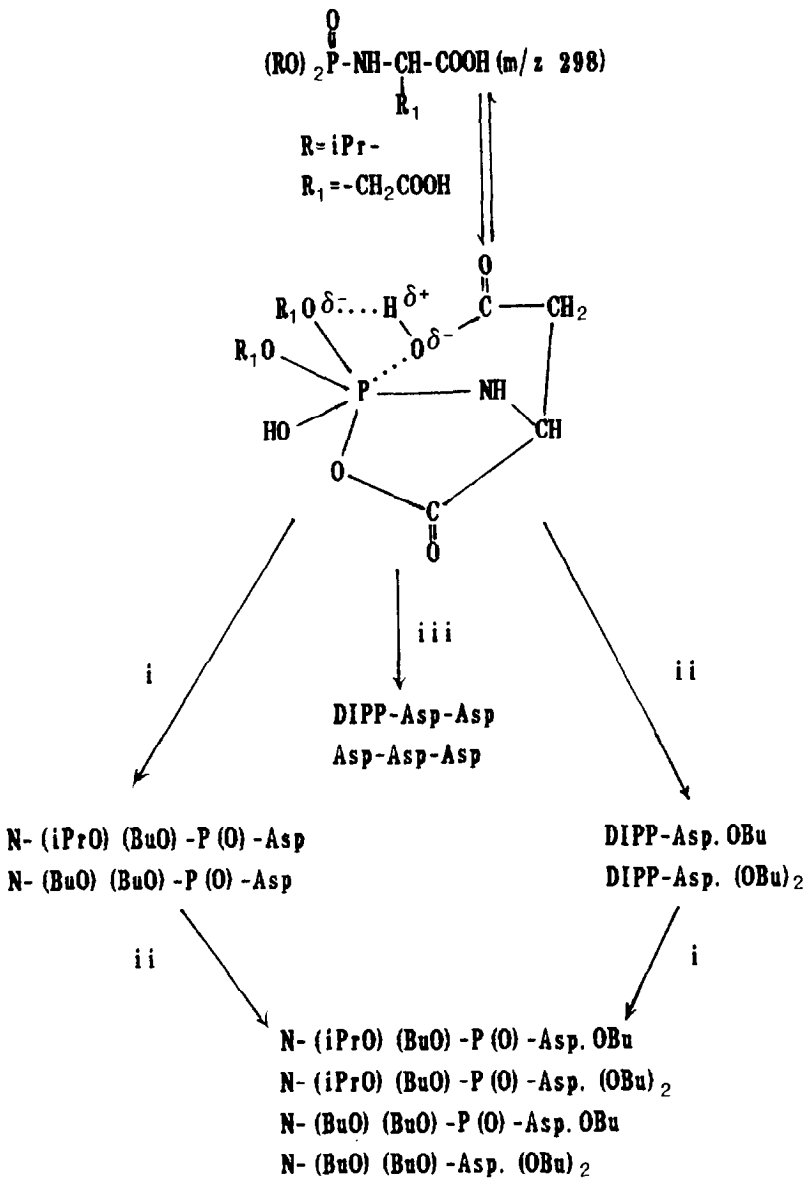
[b] Spectra data can be found in Ref. (*2*)

[c] (M + 1) +56 33.4%; (M + 1) + 56 + 14 13.1%; (M + 1) + 56 + 28 5.8%; (M + 1) + 112 2.5%; (M + 1) + 112 + 14 1.8%; (M + 1) + 112 + 28 1.3%; (M + 1) = 413 0.7%.

that are common to bioprocesses. A free β-COOH group is important for the self-activation of the amino acid. It implies that the phosphoenzyme's active center containing basic as well as acidic amino acid residues (*11*) might involve a five- or six-member ring as a rigid and precise transition state. The mixed anhydride intermediate would be a plausible intermediate.

EXPERIMENTAL

The ^{13}C NMR, ^{31}P NMR, and ^{1}H NMR spectra were taken on a JEOL FX-100, FT-80, Bruker AM-300, EM-360L 60-MHz spectrometer. The ^{31}P NMR shifts used 85% phosphoric acid as the external reference. ^{13}C NMR spectra used chloroform-*d* at 76.9 ppm or DMSO-d_6 at 39.5 ppm as the internal reference. TMS was used



i: ester exchange at the phosphoryl
ii: self catalysis for esterification
iii: self catalysis for peptide formation

SCHEME III

as the internal standard for the ^1H NMR spectra. Positive-ion FAB-MS data were obtained on a KYKY Zhp-5 double-focusing mass spectrometer from the Scientific Instrument Factory (Beijing, China) equipped with a standard KYKY fast atom gun. Infrared spectra were determined with a Carl Zeiss Jena Specord 75IR instrument. The melting points were uncorrected.

Synthesis of DIPP-Asp, DBP-Asp, DEP-Asp, DBP-Glu, and DEP-Glu

Ten millimoles of amino acid and 0.5 mmol of tetrabutylammonium bromide were dissolved in 5 ml of H_2O, 5 ml of C_2H_5OH, and 10 ml of NEt_3, and the solution was cooled to 0°C. To the above mixture, 10 mmol of dialkylphosphite in 10 ml CCl_4 was added dropwise and the mixture was stirred at 20°C for 12 h. Then, ethyl acetate and ether were used to extract the reaction mixture to remove the by-products of this reaction; this was acidified to pH 3 with dilute HCl. The solution was extracted with 1-butanol or with a mixed solvent of *tert*-butanol and ethyl acetate (1 : 1.5) (4 × 20 ml), the extract was dried ($MgSO_4$), and the solvent was evaporated to give *N*-dialkylphosphoryl amino acids.

DIPP-Asp, ***13***. Infrared: 1705, 1715 cm^{-1}; mp, 87–88°C. *Anal.* Calcd for $C_{10}H_{20}O_7NP$: C, 40.40; H, 6.73; N, 4.71. Found: C, 40.10; H, 6.80; N, 4.45. High-resolution FAB-MS: 298.1030.

Synthesis of Compounds ***5, 6, 8, 11***

Asp-(α-OBz), Asp-(β-OBz), and Glu-(α-OBz) were prepared according to Refs. (*12, 13*).

Compounds **5, 6, 8, 11** were prepared according to Ref. (*2*).

DIPP-(α-OBz)-Asp, ***5***. Infrared: 1700, 1720 cm^{-1}; mp, 70–71°C. *Anal.* Calcd for $C_{17}H_{26}O_7NP$: C, 52.71; H, 6.72; N, 3.62. Found: C, 52.48; H, 6.87; N, 3.69.

DIPP-(α-OBz)-Glu, ***6***. Infrared: 1710, 1730 cm^{-1}; mp, 57–58°C. *Anal.* Calcd for $C_{13}H_{28}O_7NP$: C, 53.86; H, 6.98; N, 3.49. Found: C, 53.90; H,7.19; N, 3.49.

DIPP-(β-OBz)-Asp, ***8***. The spectral data and physical properties can be found in Ref. (*2*).

DIPP-Gln, ***11***. Infrared: 1690, 1730 cm^{-1}; mp, 111–112°C. *Anal.* Calcd for $C_{11}H_{23}O_6N_2P$: C, 42.58; H, 7.42; N, 9.03. Found: C, 42.28; H, 7.63; N, 8.64.

Incubation of the Sample

To about 3.0 g of fresh pure sample, 20 ml of the solvent (either chloroform or isopropyl alcohol) was added. The mixture was incubated at 40°C for a certain period of time.

Isolation of Products ***1, 2, and 3***

The liquid portion and the solid portion were separated carefully. The solvent in the liquid portion was removed by distillation *in vacuo*. Vacuum liquid chromatography with gradient elution (the apparatus and technique are described clearly in the literature (*14, 15*)) was used to isolate products **1**, **2**, and **3**. Parameters related to the column: diameter, 4.5 cm; height, 7.0 cm; fritted dish, ASTM, 10–20

μm; absorbent, silica gel, 10–40 μm; vacuum, water aspirator, 20–60 mm Hg; solvent mixtures, petroleum ether/chloroform to ethyl acetate gradient. The rate of polarity change is dependent on the thin-layer chromatography analysis of each fraction collected.

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