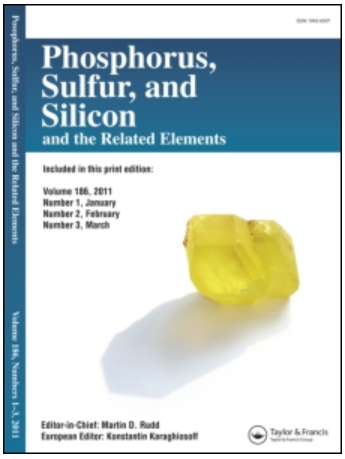

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PHOSPHORYL GROUP PARTICIPATION IN THE REACTIONS OF *N*-PHOSPHORYLDIPEPTIDE ACIDS

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The phosphoryl group participation is the key to the intramolecular cyclocondensation and esterification reactions of *N*-phosphoryldipeptide acids and their coupling reactions with amino acid esters.

Key words: Self-activation; coupling reaction; esterification; mixed anhydride intermediate; *N*-phosphoryltripeptide.

In recent years it has become evident that phosphorylated proteins play a central role in enzyme regulation and protein biosynthesis in biological systems.[1–3] The increasing interest in the biological function of phosphorylated proteins attracts considerable attention to their structures and particularly to the question of the effect of the phosphoryl residue on the protein.[4–6] But it is not so easy to understand the chemistry on these macromolecules, and a systematic investigation on the phosphorylated amino acids and small peptides might give some clue to the above question. Based on this point of view, in this laboratory a number of *N*-phosphoryl amino acids and peptides have been synthesized.[7–11] Previously, it was found that through self-activation the *N*-phosphoryl-*L*-Prolyl-*L*-proline **1** could be cyclocondensed to cyclodipeptide **2** when heated in toluene or 1-butanol (Scheme I).[8] In this paper we wish to report that due to the presence of a phosphoryl group the *N*-phosphoryldipeptide acids become active and can perform esterification and amide-formation reactions. Thus, in the absence of any activators or catalysts, they are able to esterify in alcohol to give *N*-phosphoryldipeptide acid esters and to couple with amino acid esters to form the corresponding *N*-phosphoryltripeptide acid esters. The coupling reaction can be catalyzed by *p*-toluenesulfonic acid. All these reactions might go through a mixed phosphoric-carboxylic anhydride intermediate, which seems to grant a clue to the important function of phosphorylated proteins in protein biosynthesis.

RESULTS AND DISCUSSION

1. Intra- and Intermolecular Condensation Reactions

As shown in Scheme I, when the *N*-phosphoryl-*L*-prolyl-*L*-proline **1** was heated in toluene or 1-butanol at 105–110°C, a cyclodipeptide **2** was formed.[8] But under

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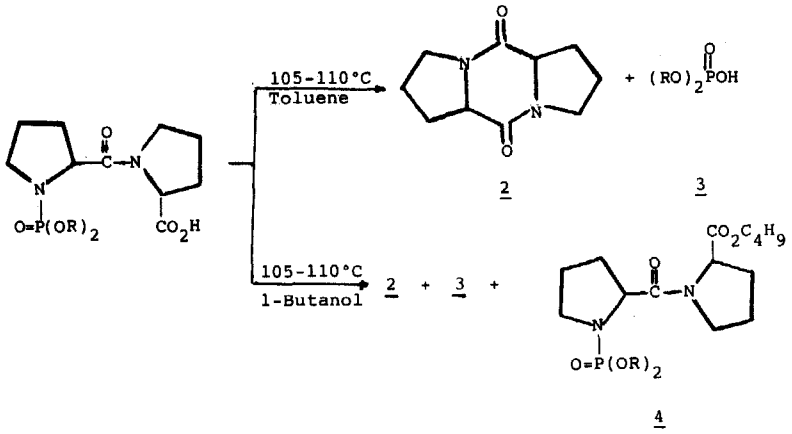


SCHEME I

Dbp-L-Pro-L-Pro-OH (1) —L-Tyr-OMe • HCl→ Dbp-L-Pro-L-Pro-L-Tyr-OMe (9)

Dbp-Gly-Gly-OH (5) —L-Tyr-OMe • HCl→ Dbp-Gly-Gly-L-Tyr-OMe (10)

Dbp-Gly-Gly-OH (5) —L-Pro-OMe • HCl→ Dbp-Gly-Gly-L-Pro-OMe (11)

Dbp-DL-Ala-DL-Ala-OH (6) —L-Tyr-OMe • HCl→ Dbp-DL-Ala-DL-Ala-L-Tyr-OMe (12)

Dbp-L-Ala-L-Tyr-OH (7) —Gly-OMe • HCl→ Dbp-L-Ala-L-Tyr-Glt-OEt (13)

Dipp-L-Leu-L-Asn-OH (8) —Gly-OEt • HCl→ Dipp-L-Leu-L-Asn-Gly-OEt (14)

Dipp-L-Leu-L-Asn-OH (8) —L-Phe-OMe • HCl→ Dipp-L-Leu-L-Asn-L-Phe-OMe (15)

*Reaction Conditions: Et_3N, xylenes, 120℃ , 16–18hrs or Et_3N, p-TsOH, 1,4-dioxane, reflux, 11–16 hrs. Dbp = $(n\text{-}C_4H_9O)_2P(O)$; Dipp = $(i\text{-}C_3H_7O)_2P(O)$.

SCHEME II

the same conditions, the similar compounds, *N*-phosphoryldipeptide acids **5–8**, which contained only the acyclic amino acid residues, did not give any cyclodipeptides. Instead, very complicated results were obtained.

However, when *N*-(*O,O*-dibutylphosphoryl)-glycyl-glycine **5** was mixed with one equivalent of tyrosine methyl ester and heated in xylenes at 120°C for 16 hours, *N*-(*O,O*-dibutylphosphoryl)-glycyl-glycyl-*L*-tyrosine methyl ester 10 could be obtained in 20.3% yield. Similarly, *N*-phosphoryltripeptide ester **11** was produced in 15.2% yield when the mixture of **5** and proline methyl ester was stirred in xylenes at 120°C for 14–15 hours. As indicated by FAB mass spectrometry, other *N*-phosphoryldipeptide acids **1** and **6–8** could also couple with amino acid esters to give the corresponding *N*-phosphoryltripeptide esters under similar conditions (Scheme II).

TABLE I
The FABHRMS of *N*-phosphoryltripeptide esters **9–15**

Product	MH^+	$(MH^+)/z$ Calcd.	Obs.
9	$C_{28}H_{45}N_3O_8P$	582.2941	582.2941
10	$C_{22}H_{37}H_3O_8P$	502.2319	502.2319
11	$C_{18}H_{35}N_3O_7P$	436.2203	436.2202
12	$C_{24}H_{41}N_3O_8P$	530.2621	530.2620
13	$C_{24}H_{41}N_3O_8P$	530.2631	530.2632
14	$C_{20}H_{40}N_4O_8P$	495.2584	495.2579
15	$C_{26}H_{44}N_4O_8P$	571.2897	571.2897

As shown by T.L.C. and FAB-MS, among the reactions (**1**), (**2**) and (**4**), only the reaction (**1**) took place when heated in 1,4-dioxane at 90°C for 5 hours. If these reactions were carried out in refluxing 1,4-dioxane for 4 hours, both of the reactions (**1**) and (**2**) were successful to give the corresponding *N*-phosphoryltripeptide esters whereas the reaction (**4**) remained unchanged. Only upon heating at 120°C in xylenes for over 4 hours did the reaction (**4**) take place to give the tripeptide ester **12**.

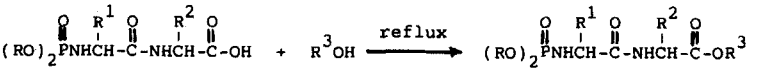
When 0.2 eq. of *p*-toluenesulfonic acid was added to the mixture of *N*-phosphoryldipeptide acid and amino acid ester, the coupling reactions could be carried out at lower temperature and the yields were higher. For instance, in the presence of 0.2 eq. of *p*-toluenesulfonic acid, products **10** and **11** could be obtained in 30.1% and 27.3% yields when **5** was refluxed in 1,4-dioxane with tyrosine methyl ester and proline methyl ester respectively for 14–16 hours.

However, when the mixture of the *N*-benzyloxycarbonyl-*L*-alanyl-*L*-proline and tyrosine methyl ester was treated similarly, no coupling reaction occurred either in the presence or in the absence of *p*-toluenesulfonic acid.

2. *The Self-Catalytic Esterification Reactions*

Previously we found that besides cyclodipeptide **2**, the esterification product **4** could be obtained in 29% yield when *N*-phosphoryl-*L*-prolyl-*L*-proline **1** was heated at 105–110°C in butanol (Scheme I). Even at the ambient temperature the esterification reaction could be observed when **1** was dissolved in ethanol for about 10 days.[8]

In order to understand the behavior of other *N*-phosphoryldipeptide acids in alcohol, in the absence of any catalysts or activators, *N*-phosphoryldipeptide acids **5–7** and **17–18** were refluxed in alcohol for 48 hours. After removal of the solvent the FAB high and low resolution mass spectrometry of the residues showed that the esterification products were formed (Scheme III and Table II).



SCHEME III

TABLE II

The FABMS and FABHRMS of the esterification products of *N*-phosphoryldipeptide acids **5–7** and **17–18**

N-Protected Dipeptide Acids				Esterification Products[b] $(MH^+)/z$	
Structure	M.W.[a]	Alcohol	MH^+	LR	HR: Obs.(Cal.)
5. Dbp-Gly-Gly-OH	324	i-PrOH	$C_{15}H_{32}N_2O_6P$	367(31.8)	
6. Dbp-Ala-Ala-OH	352	MeOH	$C_{15}H_{32}N_2O_6P$	367(20.4)	367.2052 (367.2047)
7. Dbp-Ala-Tyr-OH	444	EtOH	$C_{22}H_{38}N_2O_7P$	473(2.7)	473.2406 (473.2405)
17. Dipp-Ala-Ala-OH	324	EtOH	$C_{14}H_{30}N_2O_6P$	353(92.3)	353.1825 (353.1823)
18. Dbp-Pro-Asn-OH	421	MeOH	$C_{18}H_{35}N_3O_7P$	436(3.6)	
19. Boc-Ser-Lys-OH Bzl Z	423	i-PrOH		—	
16. Z-Ala-Pro-OH[c] DCHA	501	i-PrOH		—	

[a] M.W. = Molecular weight of *N*-phosphoryldipeptide acids
[b] LR = low resolution; HR = high resolution
[c] DCHA = dicyclohexylamine.

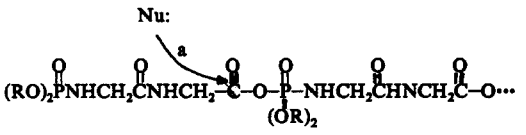
Regarding the other *N*-protected dipeptide acids, we have investigated the reactivities of compounds **16** and **19**, and have observed that no esterification reactions occurred under the same conditions as with the *N*-phosphoryldipeptide acids (Table II).

3. The Self-Activation Mechanism

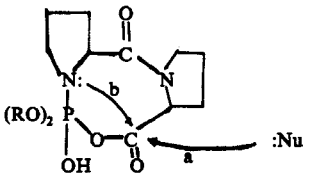
As stated above, the absence of any catalysts or activators, *N*-phosphoryldipeptide acids could perform esterification and some amide-formation reactions such as intramolecular cyclocondensation reactions and intermolecular condensation reactions with amino acid esters. It seems that all three reactions are attributable to the presence of a phoshoryl group since under the similar conditions none of the above reactions occurred for the other *N*-protected dipeptide acids. Thus, due to the presence of a phosphoryl group in the *N*-phosphoryldipeptide acids, they can activate themselves to carry out the above reactions.

For this self-activation, there are two possible mechanisms: inter- and intramolecular activation mechanisms via mixed phosphoric-carboxylic anhydride intermediates A and B. As shown in Scheme IV, the nucleophiles such as alcohols and amino acid esters could attack the carbonyl carbon through path a to give the corresponding *N*-phosphoryldi- and tripeptide acid esters. In addition, for the intermediate B, the nucleophilic attack of the nitrogen atom on the carbonyl carbon through path b leads to the formation of the intramolecular cyclocondensation product **2**.

In order to differentiate these two mechanisms, two three-component reactions were elaborated as follows. One is that the mixture of *Z*-protected dipeptide acid

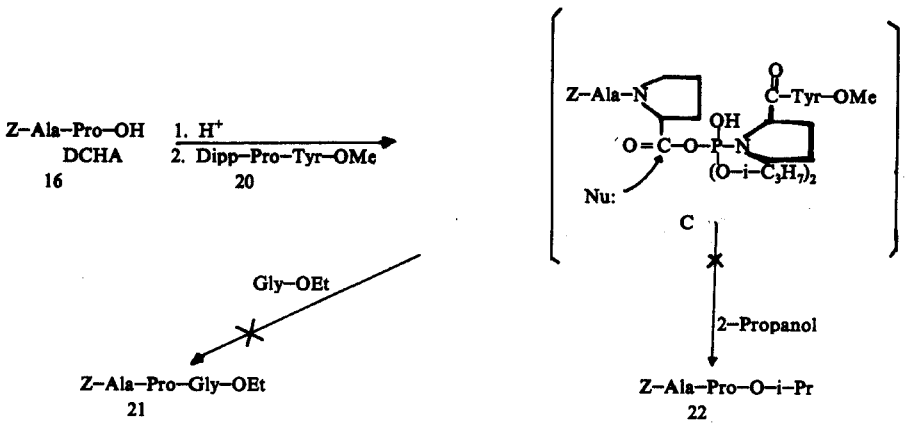


A



B

SCHEME IV



SCHEME V

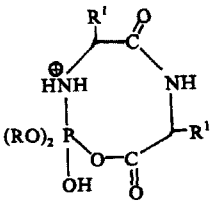
16, *N*-phosphoryldipeptide acid ester **20** and glycine ethyl ester was refluxed in 1,4-dioxane for 48 hours. The second was that the mixture of **16** and **20** was stirred in refluxing 2-propanol for 48 hours. If the above intermolecular mixed anhydride intermediate was possible, the Z-protected tripeptide acid ester **21** or *Z*-protected dipeptide acid ester **22** would be obtained via the nucleophilic attack of glycine ethyl ester or 2-propanol on the carbonyl carbon of the intermediate C. However, as indicated by FAB-MS, there was no tripeptide acid ester **21** formed either in the absence or in the presence of *p*-toluenesulfonic acid in the first experiment. Also, no esterification reaction was observed in the second one (Scheme V).

From the above interesting results, it seems that the reactions do not go through the intermolecular mixed anhydride intermediate A. Accordingly, the *N*-phosphoryldipeptide acids might activate themselves via intramolecular mixed anhydride intermediate B. For the dipeptide acid **1**, owing to the relatively rigid conformation attributable to the proline residue, the intermediate B is easy to form, which makes

it more easy to perform the esterification and amide-formation reactions in contrast to the *N*-phosphoryldipeptide acids **5–8** and **17–18** containing only acyclic amino acid residues. The difference between *N*-phosphoryldipeptide acids **1** and **5–8** is that there was no cyclodipeptide formed for **5–8**, which might be due to the stronger P—N bond in the intermediate D as compared to the intermediate B in which the nitrogen atom of the P—N bond is at the bridge position. In addition, apparently the intermolecular condensation reaction of *N*-phosphoryldipeptide acids with amino acid esters were catalyzed by *p*-toluenesulfonic acid, which might be explained by the protonation on the oxygen or nitrogen atom of the intramolecular mixed anhydride intermediate. It is obvious that the attack of the nucleophiles on the carbonyl carbon is easier after protonation (Scheme VI).

In this paper the *N*-phosphoryldipeptide acids consist of simple amino acid residues and the distance between phosphoryl group and carboxyl group are five atoms, hence, their reactivities are more inert and the reaction temperature is rather high. However, the *N*-phosphoryl amino acids or peptide acids containing side chain functional groups are very active, they can perform similar reactions under much milder conditions.[10,11] On the other hand, in the real enzyme, a three-dimensional conformation might bring the phosphoryl and carboxyl groups close enough to introduce the important phosphoric-carboxylic mixed anhydride intermediate for initiating the biochemical process under mild conditions. Anyway, the phosphoryl group participation is the key to the chemistry of phosphorylated enzymes.

In conclusion, due to the existence of a phosphoryl group, *N*-phosphoryldipeptide acids were able to perform the amide-formation reactions and esterification reactions via self-activation which might go through an intramolecular mixed phosphoric-carboxylic anhydride intermediate as proposed by F. Lipmann.[12] The phosphoryl group interaction with neighboring functional groups such as carboxyl, hydroxyl, imidazolyl, amino, arginyl, etc. might be the clue to the basic unit of biochemistry. These results indicate that the phosphoryl group participation reaction is essential in the biochemistry of the phosphorylated enzymes, which has been ignored by the modern bioorganic chemists.



D

SCHEME VI

EXPERIMENTAL

Methods. All *N*-phosphopeptide acids used here were synthesized by the new methods developed recently and confirmed by IR, NMR, FABMS and elemental analyses.[13] ^1H and ^{13}C NMR spectra were taken in $CDCl_3$ on a JEOL FX-100 spectrometer with chemical shifts reported in ppm downfield from

internal tetramethylsilane. ^{31}P NMR spectra were recorded in $CDCl_3$ (vs. 85% H_3PO_4 as external standard) on a JEOL FX-100 spectrometer. Positive-ion FAB-MS data and FAB high-resolution mass spectral (FABHRMS) data were obtained on a double-focussing mass spectrometer (VG-ZAB-HS) and VG 11-250 data system. IR spectra were measured as KBr plates or film on NaCl on a Shimadzu 430 spectrometer. Optical rotations were measured with WZZ polarimeter made by Shanghai Optical Company, China. Column chromatography was performed on 10–40 μ silica gel under the reduced pressure. Elemental analyses were performed by the Analytical Laboratory, Institute of Chemistry, Academia Sinica, Beijing, China.

Reaction of N-(O,O-dibutylphosphoryl)-glycyl-glycine **5** *with tyrosine methyl ester.* To a stirred solution of tyrosine methyl ester hydrochloride (1.43 g, 6.2 mmol) in 10 mL of methanol was added triethylamine (1.7 mL, 12.4 mmol). After complete neutralization the solvent and excess triethylamine were removed with a rotary evaporator. And then to the resulting mixture was added *N*-(*O,O*-dibutylphosphoryl)-glycyl-glycine **5** (2.0 g, 6.2 mmol) and 5 mL of xylenes. The mixture was stirred at 120°C for 16 hrs. After removal of the solvent by distillation in vacuo, 20 mL of ethyl acetate was added, and the mixture was washed successively with saturated $NaHCO_3$ solution (3 × 10 mL), 10% citric acid (3 × 10 mL) and saturated NaCl solution (2 × 10 mL), dried over anhydrous $MgSO_4$. Removal of solvent and flash column chromatography on silica gel eluting sequentially with neat EtOAc and EtOAc/EtOH (15:1) afforded *N*-(dibutyloxyphosphinyl)-glycyl-glycyl-*L*-tyrosine methyl ester **10** (0.63 g, 20.3%) as white crystals, mp 29–30°C. $[\alpha]_D$ −26.8° (c, 0.84, $CHCl_3$). ^1H NMR 1.0 (t, 6H, $2CH_3$), 1.2–1.9 (m, 10H, $2CH_2CH_2$, CH_2), 2.8–3.2 (m, 1H, CHN), 3.4–3.8 (m, 4H, J = 7.4 Hz, $2CH_2N$), 3.83 (s, 3H, CH_3O), 3.9–4.3 (m, 4H, J = 7.8 Hz, $2CH_2O$), 4.85 (s, brd, 1H, OH), 6.7–7.1 (m, $4H_{arom}$), 7.2–7.5 (3s, brd, 3H, 3NH). ^{31}P NMR δ = 8.30 ppm. ^{13}C NMR 172.0 (O=C—O); 171.1 (J = 6.4, O=C—N); 169.0 (O=C—N); 156.0, 130.2, 126.6, 115.6 (C_{arom}); 66.9 (J = 5.9, $C_3H_7CH_2O$); 32.4, 18.7, 13.6 ($CH_3CH_2CH_2$); 36.9 (CH_2-Ar); 53.4 (CH_3O); 52.4, 44.4, 42.8 (CHN, $2CH_2N$). IR 1660, 1740 cm^{-1}. Anal. Calcd. for $C_{22}H_{36}N_3O_8P$ (mol. weight .501) C, 52.69; H, 7.19; N, 8.38. Found: C, 52.35; H, 7.21; N, 8.21.

Reaction of N-(dibutyloxyphosphinyl)-glycyl-glycine **5** *with proline methyl ester.* General Procedure. A solution of proline methyl ester hydrochloride (1.28 g, 7.72 mmol) in methanol (10 mL) was neutralized with sodium hydroxide (0.31 g, 7.72 mmol) and concentrated to dryness in vacuo, and then to the mixture was added *N*-(dibutyloxyphosphinyl)-glycyl-glycine **5** (2.50 g, 7.72 mmol), and 10 ml of xylenes. The mixture was heated with stirring at 120°C for 14–15 hrs. After removal of the solvent by evaporation in vacuo, 20 mL of ethyl acetate was added. The resultant mixture was washed successively with saturated aqueous $NaHCO_3$ (3 × 20 mL), 10% citric acid (3 × 20 mL) and saturated aqueous NaCl solution and dried ($MgSO_4$). The solvent was evaporated under reduced pressure to give an oily residue, which was purified by flash column chromatography, eluted with EtOAc/MeOH (15:1) to give *N*-(dibutyloxyphosphinyl)-glycyl-glycyl-*L*-proline methyl ester **11** (0.51 g, 15.2%) as a colorless sticky oil. $[\alpha]_D$ −27.2° (c 0.75, $CHCl_3$). ^1H NMR 0.9 (t, 6H, $2CH_3$), 1.1–1.8 (m, 8H, $2CH_2CH_2$), 1.9–2.4 (m, 4H, CH_2CH_2), 3.4–3.7 (m, 4H, $2CH_2N$), 3.8 (s, 3H, CH_3O), 3.9–4.4 (m, 7H, J = 7.8 Hz, $2CH_2O$, CHN, CH_2N), 7.5 (s, brd, 2H, 2NH). ^{31}P NMR δ = 8.82 ppm. ^{13}C NMR 171.2 (O=C—O); 169.8 (J = 5.8 Hz, O=C—N); 166.0 (O=C—N); 65.2 (J = 5.8 Hz, $C_3H_7CH_2O$); 31.4 (5.8 Hz, $CH_3CH_2CH_2CH_2O$); 17.7, 12.5 ($CH_3CH_2CH_2CH_2O$); 28.0, 23.6 (CH_2CH_2); 45.0, 43.5, 51.0, 57.9 (6.3) ($3CH_2N$, CHN); 53.6 (CH_3O). IR 1650, 1745 cm^{-1}. Anal. Calcd. for $C_{18}H_{34}O_7P$ (mol. weight .435), C, 49.66; H, 7.82; N, 9.66. Found: C, 49.61; H, 7.97; N, 9.70.

Similarly, the coupling reactions of other *N*-phosphoryldipeptide acids **1** and **6–8** with amino acid esters were also observed via FAB high resolution mass spectrometry (Table I).

In the presence of 0.2 equivalents of p-TsOH, the coupling reactions of *N*-phosphoryldipeptide acids **1** and **5–8** with amino acid esters occurred at lower temperature, and the yields were higher. For instance, in the presence of 0.2 eq. of p-toluene-sulfonic acid, *N*-phosphoryltripeptide esters **11** and **12** could be obtained in 30.1% and 27.3% when **5** was refluxed with tyrosine methyl ester and proline methyl ester respectively in 1,4-dioxane for 14–16 hrs.

Esterification reactions of N-phosphoryldipeptide acids **5–7** *and* **15–18.** General Procedure. A solution of *N*-phosphoryldipeptide acid (0.2 g) in 10 mL of alcohol was refluxed with stirring for 48 hrs. After removal of the solvent with a rotary evaporator, the FABMS and FABHRMS of the residue were recorded (Table II), which showed the esterification products for *N*-phosphoryldipeptide acids **5–7** and **15–16**. However, no esterification products were observed for the Boc or Z protected dipeptide acids **17** and **18.**

The investigation on the intermolecular activation. To a solution of Z-Ala-Pro-OH · DCHA (0.21 g, 0.42 mmol) in methanol (5 mL) was added dropwise diluted sulphuric acid with shaking until pH = 3. The mixture was evaporated in vacuo to dryness, and then the residue was added to the mixture in xylene (10 mL), of Dipp-L-Pro-L-Tyr-OMe (0.19 g, 0.42 mmol) and glycine ethyl ester hydrochloride

(0.12 g, 0.84 mmol) which was neutralized with Et_3N (0.15 ml, 1.1 mmol) in advance. The resultant mixture was heated with stirring at 120°C for 20–36 hrs. After removal of the solvent, the FABMS of the residue showed that no coupling reaction occurred. Even in the presence of *p*-TsOH (0.02 g, 0.11 mmol), no tripeptide ethyl ester **21** was observed. Similarly, no esterification reaction was observed when Z-Ala-Pro-OH · DCHA (0.33 g, 0.66 mmol) was heated at reflux with Dipp-L-Pro-L-Tyr-OMe (0.30 g, 0.66 mmol) in 2-propanol (2 mL) for 15 hrs (Z-Ala-Pro-OH · DCHA was neutralized with diluted H_2SO_4 prior to being used).

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