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Enzymatic Syntheses of N-Protected Leu-enkephalin and Some Oligopeptides in Organic Solvents[#]

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Abstract: Enzymatic syntheses of bioacitive pentapeptide, N-protected Leu-enkephalin and some other oligopeptides in organic solvents were studied. The stereoselectivity of the enzymatic reaction was examined by using racemic substrates. Different enzymes, solvent systems and protecting groups were compared. The importance of the essential water was addressed. The side chain of tyrosine was not protected during all the enzymatic reactions. P-DL-AlaOY (P=Z or Boc, Y=H or Me) and P-DL-TyrOEt were coupled with GlyNHNHPh by papain and α-chymotrypsin in mixed solvent or organic solvent to obtain the expected optically pure products P-L-AlaGlyNHNHPh and P-L-TyrGlyNHNHPh respectively in good yield. Two sweetener precursors, ZAspXaaOR (XaaOR=PheOMe or AlaOcHex) were synthesized by thermolysin in *tert*-amyl alcohol and some reaction conditions were optimized to get the best yield. Full enzymatic synthesis of N-protected Leu-enkephalin ZTyrGlyGlyPheLeuOH was investigated using α-chymotrypsin and thermolysin as catalysts in dichloromethane and *tert*-amyl alcohol.

INTRODUCTION

Enzymatic syntheses of peptides are stereospecific without racemization. The side chain functional groups of amino acids do not need to be protected. Most studies on enzymatic synthesis in the past were carried out in aqueous buffers or in the mixture of aqueous and organic solvents. Thermodynamically, the enzymes and the product of the reaction are more stable in organic solvent where the formation of peptide bonds is more favorable than the hydrolysis of the product. Moreover, most organic compounds are soluble in organic solvents. In addition, in organic media undesirable side reaction such as hydrolysis of acid anhydrides caused by water can be prevented[1-3]. In the present study, we focused our research on the enzymatic syntheses

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Abbreviations: The amino acid residues which were not indicated configuration in this paper are of L-configuration. Standard abbreviations for amino acid derivatives and peptides are according to the suggestions of the IUPAC-IUB Commission on Biochemical Nomenclature (1984) *Eur. J. Biochem.* 138, 9-37. Other abbreviations: Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl; cHex, cyclohexyl; NHNHPh, phenyl hydrazine; DCM, dichloromethane; DMF, N,N-dimethylformamide; TFA, trifluoroacetic acid; DCC, N,N'-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DCHA, dicyclohexylamine; DEPBT, 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one; ATEE, N-acetyl-tyrosine ethyl ester.

[#] To the memory of Professor Yu Wang.

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of peptides in organic solvents. Although the enzymatic reaction is carried out in organic solvents, a small amount of water namely essential water is required for the reaction. Different enzymes, solvents, amount of essential water and pH etc. were studied and compared. The side chain of tyrosine was not protected during all the enzymatic reactions. P-DL-AlaOY (P=Z or Boc, Y=H or Me) and P-DL-TyrOEt were coupled with GlyNHNHPh by papain and α-chymotrypsin respectively in aqueous-organic mixed solvent or organic solvent to obtain the expected optically pure products P-L-AlaGlyNHNHPh and P-L-TyrGlyNHNHPh[4]. The precursors of two sweeteners, N-protected aspartame ZAspPheOMe and a new sweetener candidate ZAspAlaOcHex[5-6] were synthesized as model peptides by thermolysin in *tert*-amyl alcohol and the reaction conditions were studied systematically[7]. N-protected Leu-enkephalin phenyl hydrazine, ZTyrGlyGlyPheLeuNHNHPh was synthesized successfully by thermolysin in *tert*-amyl alcohol, and the phenyl hydrazine was easily removed by ferric chloride. The two fragments, ZTyrGlyGlyOEt and BocPheLeuNHNHPh were synthesized by α-chymotrypsin and thermolysin in dichloromethane and *tert*-amyl alcohol respectively. The preliminary results were briefly reported[8]. The physical constants of all peptides synthesized by enzymes were identical with those of peptides synthesized by chemical method.

RESULTS AND DISCUSSION

N-Protected racemic amino acids (or their esters) as carboxyl components for enzymatic peptide synthesis

Owing to the stereospecificity of proteases, with racemic amino acids (or their esters) as carboxyl components the optically pure protected oligopeptides were synthesized (Table 1).

At first, we used an organic-aqueous mixed solvent to carry out the enzymatic peptide synthesis. With papain as catalyst, Z-DL-AlaOH or Boc-DL-AlaOH was coupled with GlyNHNHPh in methanol-water mixed solvent at 38 ℃, pH 5.0, to give the expected optically pure product Z-L-AlaGlyNHNHPh or Boc-L-AlaGlyNHNHPh respectively. When carboxyl component was changed into Z-DL-AlaOCH$_3$ and pH was increased to 8.2, higher yield of Z-L-AlaGlyNHNHPh was obtained. The results indicated that ester substrate was more suitable than free acid substrate for papain. In the reaction of α-chymotrypsin as catalyst, the desired Boc-L-TyrGlyNHNHPh was got from racemic Boc-DL-TyrOEt and GlyNHNHPh in DMF-water mixed solvent at 38 ℃ and pH 10.0.

Our study also demonstrated that peptide synthesis catalyzed by α-chymotrypsin could be successfully performed in organic solvent. In dichloromethane containing a little essential water(0.25% V/V), Z-DL-TyrOEt or Z-L-TyrOEt reacted with GlyNHNHPh at room temperature and observed pH 10 to provide expected optically pure product Z-L-TyrGlyNHNHPh. Some tri- or tetra-peptide derivatives such as Z-L-GlyTyrGlyNHNHPh, Z-L-AlaTyrGlyNHNHPh and Z-L-AlaTyrGlyGlyOEt were also synthesized by this method in dichloromethane under same reaction conditions.

Table 1. Physical Constants of Peptides Synthesized by Enzymatic Method

No. Enzyme	C-Component	N-Component	Solvent	Product	yield[a] %
1. Papain	Z-DL-AlaOH	GlyNHNHPh	MeOH-H_2O	Z-L-AlaGlyNHNHPh	60
2. Papain	Z-DL-AlaOCH$_3$	GlyNHNHPh	MeOH-H_2O	Z-L-AlaGlyNHNHPh	80
3. Papain	Boc-DL-AlaOH	GlyNHNHPh	MeOH-H_2O	Boc-L-AlaGlyNHNHPh	60
4. α-Chymotrypsin	Boc-DL-TyrOEt	GlyNHNHPh	DMF-H_2O	Boc-L-TyrGlyNHNHPh	60
5. α-Chymotrypsin	Z-DL-TyrOEt	GlyNHNHPh	CH_2Cl_2[b]	Z-L-TyrGlyNHNHPh	68
6. α-Chymotrypsin	Z-L-TyrOEt	GlyNHNHPh	CH_2Cl_2[b]	Z-L-TyrGlyNHNHPh	83
7. α-Chymotrypsin	Z-L-GlyTyrOEt	GlyNHNHPh	CH_2Cl_2[b]	Z-L-GlyTyrGlyNHNHPh	86
8. α-Chymotrypsin	Z-L-AlaTyrOEt	GlyNHNHPh	CH_2Cl_2[b]	Z-L-AlaTyrGlyNHNHPh	85
9. α-Chymotrypsin	Z-L-AlaTyrOEt	GlyGlyOEt	CH_2Cl_2[b]	Z-L-AlaTyrGlyGlyOEt	40
10. α-Chymotrypsin	Z-L-TyrOEt	GlyGlyOEt	CH_2Cl_2[b]	Z-L-TyrGlyGlyOEt	70
11. α-Chymotrypsin	Z-L-TyrOEt	GlyGlyOEt	CH_2Cl_2[c]	Z-L-TyrGlyGlyOEt	0

a). The yields were based on the L-configuration of amino acid. b). CH_2Cl_2 contained 0.25%(V/V) essential water. c). CH_2Cl_2 contained no essential water.

Of these reactions, C-terminal was protected by a hydrophobic blocking group, phenyl hydrazine which not only is useful for the enzymatic reaction but also can readily be removed by $FeCl_3$ in dioxane-water system to afford N-protected C-terminal free peptide. All of these peptide derivatives synthesized by enzymatic method were identical with those peptides synthesized by chemical method using DEPBT [3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one] as coupling reagent which was developed by our group[9].

Synthesis of sweetener precursors by thermolysin in tert-amyl alcohol

The precursor of aspartame ZAspPheOMe, and the precursor of a new sweetener candidate, ZAspAlaOcHex were synthesized by thermolysin in *tert*-amyl alcohol.

The reaction was carried out in *tert*-amyl alcohol stirring at 40 ℃ for 3 days with ZAspOH as carboxyl component and PheOMe or AlaOcHex as amino component. Observed pH, water content of *tert*-amyl alcohol and excessive percent of Et_3N were studied systematically, since these reaction conditions have a great influence on the activity of the enzyme and product yield. The results showed the optimum observed pH was about 8 for synthesis of ZAspPheOMe and for ZAspAlaOcHex the pH was about 9, at the same time the corresponding excess Et_3N in these two reactions was 1.6%(V/V); and the optimum water content of *tert*-amyl

alcohol was 6~8%(V/V) containing 10 mM $CaAc_2$ in both model reactions. Under the optimum conditions, with the molar ratio of the carboxyl component to amino component keeping 1:3, the product yield of ZAspPheOMe and ZAspAlaOcHex were 83% and 44% respectively.

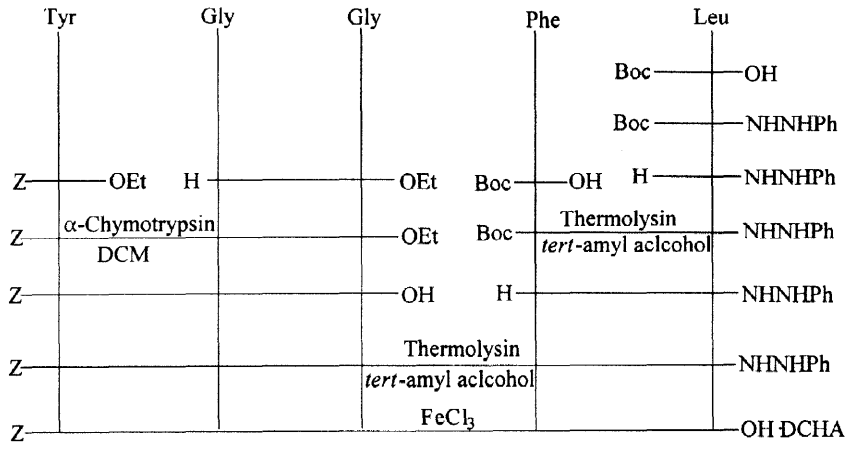
Moreover, using racemic amino acid derivative as amino component the expected optically pure sweetener precursors were obtained. The product was identical to that under the same conditions only using L-configuration amino component as substrate. The isolated yield was based on L-configuration in racemic substrate. The physical constants of protected sweetener peptides synthesized by thermolysin were listed in Table 2. These peptides were also compared with those peptides synthesized by chemical method and the results showed they were identical correspondingly. The detailed results will be published elsewhere.

Table 2. Physical Constants of Protected Sweeteners Synthesized by Thermolysin in *tert*-Amyl Alcohol

No.	Product[a]	Carboxyl component	Amino component	Molar ratio[b]	Yield %	m.p. (℃)	$[\alpha]^{25}_D$ (c, MeOH)
1	ZAspPheOMe	ZAspOH	PheOMe	1:3	83	120-122	- 14.8(0.5)
2	ZAspPheOMe	ZAspOH	PheOMe	1:1	41	120-122	- 14.8(0.5)
3	ZAspPheOMe	ZAspOH	DL-PheOMe	1:2	40[c]	118-121	- 15.1(0.5)
4	ZAspAlaOcHex	ZAspOH	AlaOcHex	1:3	44	119-121	- 31.6(0.5)
5	ZAspAlaOcHex	ZAspOH	AlaOcHex	1:2	20	119-121	- 31.6(0.5)
6	ZAspAlaOcHex	ZAspOH	DL-AlaOcHex	1:4	24[c]	117-120	- 29.5(0.5)

a). Carboxyl component was 100mM in every reaction. Water content(V/V) in No. 1~3 was 8%(10 mM $CaAc_2$); in No. 4~6 was 6%(10 mM $CaAc_2$). The observed pH in No.1~3 was about 8; in No. 4~6 was about 9. b). Molar ratio means the mole of carboxyl component/amino component in the enzymatic reaction. c). The yield was based on the L-configuration of amino acid.

Full enzymatic synthesis of N-protected Leu-enkephalin



Scheme 1. The Strategy for Enzymatic Synthesis of N-Protected Leu-enkephalin

Nakanishi[10] reported that ZTyrGlyGly^{3}Phe^{4}LeuOEt was synthesized by immobilized thermolysin in several solvent systems such as saturated ethyl acetate, the maximum yield was 68%, while the yield was low in the reactions by free thermolysin. Vulfson[11] synthesized N-P-TyrGlyGly^{3}Phe^{4}LeuNH$_2$ (P=Ac, CF_3CO, Z) by immobilized proteinase K in acetonitrile-ethanol-formamide. Kullman[12-13] studied the synthesis of N-protected enkephalin phenyl hydrazine by thermolysin or papain in alcohol-water mixed solvent, the desired peptide was not formed. Alternatively, the undesired tetrapeptide BocTyr(Bzl)Gly^{2}Phe^{4}LeuNHNHPh or dipeptide BocTyr^1(Bzl)Phe^{4}NHNHPh was obtained. We synthesized N-Protected Leu-enkephalin phenyl hydrazine by free thermolysin with modest yield (42%) in *tert*-amyl alcohol for the first time, and both fragments ZTyrGlyGlyOEt and BocPheLeuNHNHPh were also synthesized by α-chymotrypsin and thermolysin in dichloromethane and *tert*-amyl alcohol in good yields (71% and 67% respectively), indicating that the enzymatic synthesis in organic solvent has its advantage over that in water-organic mixed system. The enzymatic synthesis strategy for Z-protected Leu-enkephalin was shown in Scheme 1.

Firstly, ZTyrOEt was coupled with GlyGlyOEt by α-chymotrypsin in dichloromethane containing 0.15% water (V/V), at observed pH 10 to obtain ZTyrGlyGlyOEt in 71% yield. The water content of dichloromethane was investigated systematically from 0% to 1%(V/V). The experiments showed the optimized water content was 0.15%(V/V). No product formed without essential water in the reaction, indicating that small amount of water was necessary for the enzymatic reaction. When 0.25%(V/V) water content was chosen for the reaction, the isolated yield was obtained in 61%~70%. The result suggested that 0.15~0.25% water (V/V) was enough for the coupling reaction (Table 1).

Then, BocPheOH was condensed with LeuNHNHPh using thermolysin as a catalyst in *tert*-amyl alcohol at observed pH 9 and with 8% (V/V) water content (containing 10 mM $CaAc_2$) to provide BocPheLeuNHNHPh in 67% yield. The water content of *tert*-amyl alcohol was also optimized in this reaction and 6-8%(V/V) was found the best water content for this reaction. This result was almost the same as that in the synthesis of ZAspPheOMe or ZAspAlaOcHex.

About synthesis of pentapeptide, at the beginning we tried to synthesize the N-protected enkephalin methyl ester by the condensation of ZTyrGlyGlyOEt with PheLeuOMe catalyzed by subtilisin in *tert*-amyl alcohol at observed pH 10. However, MS-FAB suggested the major product isolated was diketopiperazine derivative of amino component (PheLeuOMe). When thermolysin was instead of subtilisin to catalyze the coupling reaction between ZTyrGlyGlyOH and PheLeuOMe at observed pH 9, the desired N-protected Leu-enkephalin methyl ester was not obtained, either. The most product was still diketopiperazine derivative determined by MS. In order to prevent the formation of diketopiperazine byproduct in the reaction process, PheLeuNHNHPh was selected as nucleophile for the reaction. Then the expected N-protected Leu-enkephalin was obtained successfully. The procedure was described as following. After ZTyrGlyGlyOEt was saponifed in pyridine with 1M NaOH, ZTyrGlyGlyOH reacted with PheLeuNHNHPh catalyzed by thermolysin in *tert*-amyl

alcohol containing 10mM $CaAc_2$ 6%(V/V), observed pH 9.0, 40 ℃ stirring for 4 days to obtain ZTyrGlyGlyPheLeuNHNHPh in 42% yield. The phenyl hydrazine group in the C-terminal was readily removed by $FeCl_3$ in dioxane-water at last to obtain the N-protected Leu-enkephalin ZTyrGlyGlyPheLeuOH in 73% yield. In addition, the Z-protected Leu-enkephalin phenyl hydrazine and its two fragments were synthesized by chemical method using DCC or DEPBT as coupling reagent. These peptide derivatives from different synthetic method were compared and the physical constants of them were found to be identical respectively.

CONCLUSIONS

Condensation of N-protected DL-amino acids (or their esters) with GlyNHNHPh or N-Protected AspOH with DL-PheOMe (or DL-AlaOcHex) in the presence of enzyme (papain, α-chymotrypsin or thermolysin) at optimum pH successfully formed the expected optically pure protected oligopeptides. The reactions can be carried out in an aqueous-organic mixed solvent or organic solvent containing essential water.

The amino acid ester as acyl donor was better than the corresponding acid for papain. C-terminal of amino component, phenyl hydrazine was a better substrate than the corresponding ester for thermolysin-catalyzed reaction.

The amount of essential water needed depends on different enzymes. Synthesis of ZTyrGlyGlyOEt catalyzed by α-chymotrypsin in dichloromethane, 0.15~0.25% water (V/V) was enough for the coupling reaction. The optimized water content was 6-8%(V/V) for the syntheses of ZAspPheOMe and ZAspAlaOcHex catalyzed by thermolysin in *tert*-amyl alcohol. For synthesis of BocPheLeuNHNHPh using thermolysin as a catalyst in *tert*-amyl alcohol, the optimized water content was also 6-8%. Usually for hydrophobic solvent the amount of essential water was needed less than relatively polar organic solvent. There was almost no product formation when there was no essential water in organic solvents.

The optimum pH value of enzymatic reaction in organic media depends on the substrate and enzyme. For example, in the synthesis of Z-L-AlaGlyNHNHPh catalyzed by papain, Z-DL-AlaOH as a substrate, pH was 5.0, while Z-DL-$AlaOCH_3$ as a substrate, pH increased to 8.2. In the syntheses of sweetener precursors by thermolysin, various amount of Et_3N was added to the reaction in *tert*-amyl alcohol to make the observed pH was changed differently, the results showed that the good yield could be obtained at observed pH 8 or 9, namely an excess of 1.6% Et_3N was good for peptide bond formation by thermolysin in *tert*-amyl alcohol.

N-Protected Leu-enkephalin phenyl hydrazine has been synthesized previously by enzymes in mixture solvents through different synthetic routines. However, the peptide bond between Gly^3-Phe^4 could not be formed by fragment condensation under the same conditions. In the current study, we synthesized ZTyrGlyGlyOEt and BocPheLeuNHNHPh by α-chymotrypsin and thermolysin in dichloromethane and *tert*-amyl alcohol in good yields (71% and 67% respectively). After saponification of ZTyrGlyGlyOEt and

deprotection of the Boc group, the two fragments were then coupled by thermolysin in *tert*-amyl alcohol for the first time in modest yield (42%).

EXPERIMENTAL

α-Chymotrypsin (EC 3.4.21.1, specific activity 9295ATEE/mg protein determined using ATEE as substrate[14]), papain [EC 3.4.22.2, specific activity 30.5 U/mg protein measured using hemoglobin as substrate (Anson method)[15]] were purchased from Shanghai Dong-Fong Biochemical Technology Company. Thermolysin (EC 3.4.24.4 from bacillus thermoproteolyticus rokko, specific activity 38U/mg protein determined according to literature[16] with casein as substrate) was obtained from Sigma Chemical Company. The above values of the protease specific activity of these enzymes referred to those determined by ourselves before use. All other chemicals and solvents were purchased from the local chemical factories, they were analytical grade and the solvents used for enzymatic reaction were dried and redistilled by standard techniques. Melting points: Yanaco micro melting point apparatus given without correction; Mass spectra: VG-ZAB-HS spectrometer; Elemental analysis: Carlo Erba 1106 or Hezaeus CHN-Rapid elemental analyzer; Optical rotation: Perkin-Elmer 241 or 241 MC polarimeter; Amino acid analysis: Beckman 121MB amino acid analyzer. All reactions were controlled by TLC on silica gel prepared by ourselves, the developing solvent systems were chloroform-methanol (9:1), chloroform-methanol-acetic acid (25:5:1), cyclohexane-acetone-acetic acid (25:25:1).

Z-L-AlaGlyNHNHPh (Z-DL-AlaOH as an acyl donor)

To a mixture of 400 mg (1.8 mmol) Z-DL-AlaOH and 725 mg (3.6 mmol) HCl · GlyNHNHPh, 4.8 ml 2 M NaAc-HAc buffer, 6 ml water and 4 ml methanol were added. The pH of the solution was adjusted to 5.0 by 2 M NaOH. 80 μl 2-mercaptoethanol and 1g crude papain powder were added under stirring. The reaction was detected by TLC. After stirring at 38 ℃ for 3 days, the resulting precipitate was filtered, washed successively with 0.1M HCl, saturated NaCl and 0.2M $NaHCO_3$ to give a white solid. Crystallization from methanol-ethyl ether provided a pure product 183-200 mg, 55-60% yield (the yield was based on its L-configuration), mp 182-183 ℃, $[\alpha]^{20}_D$ +3.9 (c 0.5, DMF), MS(FAB): 371$(M+H)^+$. Analysis for calculated $C_{19}H_{22}N_4O_4$(%): C 61.61, H 5.99, N 15.13; found(%): C 61.37, H 5.94, N 15.01. Amino acid analysis: Ala:Gly = 1:0.99. Compared with the same dipeptide prepared by chemical method. Yield 80%, mp 183-184 ℃, $[\alpha]^{20}_D$ +4.5 (c 0.5 DMF).

Z-L-AlaGlyNHNHPh (Z-DL-AlaOCH$_3$ as an acyl donor)

To a mixture of 112 mg (0.5 mmol) Z-DL-$AlaOCH_3$ and 200 mg (1 mmol) HCl · GlyNHNHPh, 1.2 ml 0.2 M Na_2CO_3-$NaHCO_3$ buffer, 1.5 ml water and 1 ml methanol were added. The pH of the solution was

adjusted to 8.2 by 2 M NaOH. 20 μl 2-mercaptoethanol and 250 mg crude papain powder were then added. The reaction was detected by TLC. Stirring at 38 ℃ for 3 days, the solid from the solution was washed with 0.1 M HCl, saturated NaCl. After crystallization from methanol, a white crystal (74 mg) was obtained in 80% yield (the yield was based on its L-configuration), mp 183-184 ℃, $[\alpha]^{20}_D$ +4.5 (c 0.5 DMF).

Boc-L-AlaGlyNHNHPh (Boc-DL-AlaOH as an acyl donor)

To a mixture of 95 mg (0.5mmol) Boc-DL-AlaOH and 200 mg (1 mmol) HCl · GlyNHNHPh, 1.2 ml 2 M NaAc-HAc buffer was added to make the pH adjust to 5.0, then 1.5 ml water and 1 ml methanol were added. After bubbling nitrogen for a few minutes, 20 μl 2-mercaptoethanol and 250 mg crude papain powder were added. The reaction was detected by TLC. Stirring at room temperature for 2 days, the collected precipitate was washed successively with 0.1 M HCl, water and 0.5 M $NaHCO_3$ and saturated NaCl. Crystallization from methanol gave the pure dipeptide 50 mg, 60 % yield (the yield was based on its L-configuration), mp 213-215 ℃, $[\alpha]^{20}_D$ -8.4 (c 0.5 DMF), MS(FAB): 337$(M+H)^+$. Compared with that from chemical synthesis. Yield 62%, mp 213-215 ℃, $[\alpha]^{20}_D$ -7.6 (c 0.5 DMF). [Lit[17]: mp 218-219 ℃, $[\alpha]^{25}_D$ -5.6 (c 0.5 MeOH)].

Boc-L-TyrGlyNHNHPh (Boc-DL-TyrOEt as an acyl donor)

155 mg (0.5 mmol) Boc-DL-TyrOEt and 200 mg (1 mmol) HCl · GlyNHNHPh were dissolved in 3.5 ml DMF, under stirring 3 ml 0.2 M Na_2CO_3 was added to make the pH adjust to 10.0, then 50 mg α-chymotrypsin was added and the solution was stirred at room temperature for 2 days (the reaction was detected by TLC). The obtained solid was crystallized from methanol to give a pure dipeptide 64 mg, 60% yield (the yield was based on its L-configuration), mp 193-195 ℃, $[\alpha]^{20}_D$ -11.5 (c 0.5 DMF), MS(FAB): 429$(M+H)^+$. Compared with product synthesized by chemical method. Yield 83%, mp 193-196 ℃, $[\alpha]^{20}_D$ -10.9(c 0.5 DMF). [Lit[12]: mp 194-196 ℃, $[\alpha]^{23}_D$ -10.9 (c 0.6 DMF)].

Z-L-TyrGlyNHNHPh (Z-DL-TyrOEt as an acyl donor)

To a mixture of 1.02 g (3 mmol) Z-DL-TyrOEt and 600 mg (3mmol) HCl · GlyNHNHPh, 30 ml anhydrous CH_2Cl_2, 0.44 ml Et_3N, 30 mg α-chymotrypsin and 75 μl water were added. The observed pH of the solution was adjusted to 10.0 (indicator paper). The reaction was followed by TLC. Stirring at room temperature for 2 days, the precipitate from the solution was crystallized from methanol to obtain the dipeptide 471 mg, 68% yield (the yield was based on its L-configuration), mp 191-193 ℃, $[\alpha]^{20}_D$ -19.2 (c 0.5 DMF), MS(FAB): 463$(M+H)^+$. Compared with the product obtained from chemical synthesis. Yield 60%, mp 189-190 ℃, $[\alpha]^{20}_D$ -19.2 (c 0.5 DMF).

ZTyrGlyNHNHPh (Z-L-TyrOEt as an acyl donor)

The procedure was same as that of Z-DL-TyrOEt as substrate. The yield was 83%, mp 192-193 ℃, $[\alpha]^{20}_D$ -18.8 (c 0.5 DMF).

ZGlyTyrGlyNHNHPh (ZGlyTyrOEt as an acyl donor)

400 mg (1.03 mmol) ZGlyTyrOMe and 210 mg (1.04 mmol) HCl · GlyNHNHPh were suspended in 10 ml CH_2Cl_2, 25 μl water and 10 mg α-chymotrypsin were then added and the observed pH of the solution was adjusted to 10.0 (indicator paper) with Et_3N. The reaction was detected by TLC. Stirring at room temperature for 3 days, the resulting precipitate from the solution was collected and recrystillized from methanol-ethyl ether to give a white crystal 459 mg, 86% yield, mp 222-225 ℃(dec), $[\alpha]^{25}_D$ -5.1 (c 0.5 DMF), MS(FAB): 520$(M+H)^+$. Compared with the product from chemical method. Yield 81%, mp 222-223(dec), $[\alpha]^{25}_D$ -3.4 (c 0.5 DMF). Analysis calculated for $C_{27}H_{29}O_6N_5$ (%): C 62.42, H 5.63, N 13.48; found: C 62.04, H 5.62, N 13.23.

ZAlaTyrGlyNHNHPh (ZAlaTyrOEt as an acyl donor)

The synthetic procedure was same as that for synthesis of ZGlyTyrGlyNHNHPh. Yield 85%, mp 216-218 ℃, $[\alpha]^{25}_D$ -20.1 (c 0.5 DMF), MS(FAB): 534$(M+H)^+$. Analysis calculated for $C_{28}H_{31}O_6N_5$ (%): C 63.03, H 5.86, N 13.13; found: C 62.43, H 5.84, N 12.91. Compared with the product from chemical method. mp 213-216, $[\alpha]^{25}_D$ -19.4 (c 0.5 DMF).

ZAlaTyrGlyGlyOEt (ZAlaTyrOEt as an acyl donor)

The synthetic procedure was same as that for synthesis of ZGlyTyrGlyNHNHPh. Yield 40%, mp 118-120 ℃, $[\alpha]^{25}_D$ -27.6 (c 0.5 MeOH), MS(FAB): 529$(M+H)^+$. Analysis calculated for $C_{26}H_{32}O_8N_4$ (%): C59.08, H 6.10, N 10.60; found: C 58.82, H 6.32, N 10.09. Compared with the product from chemical method. Yield 36%, mp 125-127 ℃, $[\alpha]^{25}_D$ -28.6 (c 0.5 MeOH).

Chemical Syntheses for above Compounds were according to literature[9] described from corresponding N-protected amino acid and HCl · GlyNHNHPh or HCl · GlyGlyOEt with DEPBT as coupling reagent.

ZAspPheOMe (molar ratio of carboxyl component/amino component being 1:3)

134 mg (0.5 mmol) ZAspOH was dissolved in 5 ml *tert*-amyl alcohol in the presence of 360 μl triethylamine, then 330 mg (1.5 mmol) HCl · PheOMe was added. The basicity of the solution was about 8.0 which determined by dropping a small amount of the mixture on a moist special pH-indicator paper. After 5 mg thermolysin and 0.4 ml 10 mM calcium acetate were added, the solution was stirred at 40 ℃ for 3 days. The reaction was detected by TLC. At the end of the reaction, the solvent was evaporated under reduced pressure and the oily residue was acidified by the mixed 1M HCl (8ml) and a small volume ethyl acetate. When the water layer was extracted by ethyl acetate(15ml × 3), the combined organic layers were washed with 1M HCl (4ml × 3) and saturated NaCl (4ml × 3), then dried over anhydrous Na_2SO_4. After removal of solvent, the crude product was recrystallized from methanol-water to give pure ZAspPheOMe 177 mg, 83% yield, mp 120-122 ℃, $[\alpha]^{25}_D$ -14.8 (c 0.5 MeOH), MS(FAB): 429$(M+H)^+$. Compared with product synthesized

by chemical method. mp 123-125 ℃, $[\alpha]^{15}_D$ -16.7(c 0.5 MeOH). [Lit[18]: mp 121-123 ℃, $[\alpha]^{25}_D$ -16.0 (c 1 MeOH)]. Under other conditions such as molar ratio of carboxyl component to amino component being 1:1 (for entry 2 in table 2) and DL-PheOMe as nucleophile (for entry 3 in table 2), the preparation of this compound was also same as this procedure.

ZAspAlaOcHex (molar ratio of carboxyl component/amino component being 1:3)

134 mg (0.5 mmol) ZAspOH was dissolved in 5 ml *tert*-amyl alcohol in the presence of 360 μl triethylamine, then 520 mg (1.5 mmol) TsOH · AlaOcHex was added. The basicity of the solution was about 9.0 determined by a moist special pH-indicator paper. After 5 mg thermolysin and 0.3 ml 10 mM calcium acetate were added, the solution was stirred at 40 ℃ for 3 days. The reaction was detected by TLC. The following isolation and purification procedure was same as that of ZAspPheOMe to give pure ZAspAlaOcHex 93 mg, 44% yield, mp 119-121 ℃, $[\alpha]^{25}_D$ -31.6 (c 0.5 MeOH), MS(FAB): 421$(M+H)^+$. Compared with product synthesized by chemical method. mp 120-122 ℃, $[\alpha]^{15}_D$ -29.8(c 0.5 MeOH). Analysis calculated for $C_{21}H_{28}O_7N_2$ (%): C 59.99, H 6.71, N 6.66; found: C59.79, H 6.79, N 6.36. The synthesis of this compound under other reaction conditions such as molar ratio of carboxyl component to amino component being 1:2 (for entry 5 in table 2) and DL-AlaOcHex as nucleophile (for entry 6 in table 2) in the presence of thermolysin was similarly as this procedure.

Chemical Synthesis of ZAspPheOMe was according to the reference[19] from Z-aspartic anhydride and HCl · PheOMe, and it was purified by recrystallization from methanol-water three times. ZAspAlaOcHex was prepared similarly as literature[5] described from Z-aspartic anhydride and TsOH · AlaOcHex and purified like ZAspPheOMe.

ZTyrGlyGlyOEt (water content of dichloromethane being 0.25% (V/V))

170 mg (0.5 mmol) ZTyrOEt and 100 mg (0.5 mmol) HCl · GlyGlyOEt were suspended in 5 ml dichloromethane. When 140 μl triethylamine was added, the solution was shaken to make the substrate dissolve completely. The observed pH was about 10.0 determined by a moist special pH-indicator paper. 12.5 μl water and 5 mg α-chymotrypsin were then added. The reaction was detected by TLC. After stirring at room temperature for 3 days. The white precipitate from the solution was filtered and washed with 1M HCl and water. Crystallization from acetone or methanol gave the pure product 139-160 mg, 61-70% yield, mp 164-165 ℃, $[\alpha]^{20}_D$ +3.1 (c 2 HAc), MS(FAB): 458$(M+H)^+$. Compared with product synthesized by chemical method. Yield 64%, mp 164-165 ℃, $[\alpha]^{20}_D$ +2.9(c 2 HAc). [Lit[11]: mp 169-171 ℃, $[\alpha]^{20}_D$ +8.1(c 1 MeOH)].

BocPheLeuNHNHPh (water content of tert-amyl alcohol being 8%(V/V))

80 mg (0.3 mmol) BocPheOH and 100 mg (0.3 mmol) TFA · LeuNHNHPh were suspended in 3 ml *tert*-amyl alcohol. 100 μl triethylamine was added and the solution was shaken to make the substrates dissolve.

The observed pH of the solution was about 9.0 (indicator paper). 240μl 10 mM calcium acetate and 3 mg thermolysin were then added. This reaction was performed at 40 ℃ for 3 days under stirring. The reaction was detected by TLC. At the end of the reaction, the solution was diluted with 50 ml ethyl acetate and washed successively with 5% citric acid, 5% $NaHCO_3$ and saturated NaCl. The organic layer was dried over anhydrous $MgSO_4$. Evaporation of the solvent under reduced pressure gave the crude product. It was recrystallized from ethanol-water to obtain pure BocPheLeuNHNHPh 95 mg, 67% yield, mp 192-194 ℃, $[\alpha]^{28}_D$ -24.4 (c 0.5 DMF). Compared with product synthesized by chemical method. Yield 84%, mp 193-195 ℃, $[\alpha]^{28}_D$ -24.8(c 0.5 DMF). [Lit[13]: mp 186-188 ℃].

ZTyrGlyGlyPheLeuNHNHPh

86 mg (0.2 mmol) ZTyrGlyGlyOH and 290 mg (0.6 mmol) TFA · PheLeuNHNHPh were suspended in 2 ml *tert*-amyl alcohol. 132 μl triethylamine and 120μl 10 mM calcium acetate were then added and the solution was shaken to dissolve the substrates. The observed pH of the solution was about 9.0 (indicator paper) and then 6 mg thermolysin was added. This solution was stirred at 40 ℃ for 4 days. The reaction was detected by TLC, at the end of the reaction, the solution was diluted with 100 ml ethyl acetate and washed with 1M HCl, 5% $NaHCO_3$ and saturated NaCl. The organic layer was dried over anhydrous $MgSO_4$ and evaporated *in vacuo* to give the crude product. Recrystallization from acetone or ethanol gave pure desired pentapeptide derivative 65 mg, 42% yield, mp 210-213 ℃, $[\alpha]^{28}_D$ -27.4 (c 0.5 DMF), MS(FAB): 780$(M+H)^+$. Analysis calculated for $C_{42}H_{49}O_8N_7$ (%): C 64.68, H 6.33, N 12.57; found: C 64.67, H 6.48, N 12.42. Amino acid analysis: Gly 2.22, Tyr 0.81, Leu 1.06, Phe 1.08. Compared with product synthesized by chemical method. Yield 76%, mp 209-211 ℃, $[\alpha]^{28}_D$ -27.6(c 0.5 DMF).

Chemical Synthesis ZTyrGlyGlyOEt and ZTyrGlyGlyPheLeuNHNHPh were prepared using DEPBT as coupling reagent according to literature[9]. BocPheLeuNHNHPh and BocLeuNHNHPh were synthesized by usual DCC/HOBt coupling method, and these two compounds were treated by TFA-DCM (1:1 V/V) or TFA to give trifluoroacetate salt according to the standard deacylation procedure in Boc chemistry. The saponification of ZTyrGlyGlyOEt was carried out in pyridine with 1 M NaOH (see Literature[20]). The deblocking of phenyl hydrazine of ZTyrGlyGlyPheLeuNHNHPh (84 mg, 0.11 mmol) using $FeCl_3$ (1.4 M) in dioxane-water was similarly as the reference[12] described, then the oily residue was dissolved in small volume of methanol and proper amount of dicyclohexylamine was added. After stirring for several minutes, this solution precipitated with ether or petroleum ether to get a white solid of ZTyrGlyGlyPheLeuOH · DCHA 70 mg in 73% yield, mp 125-128 ℃, $[\alpha]^{28}_D$ -26.8 (c 0.5 $MeCONMe_2$), MS(FAB): 690$(M\text{-}DCHA+H)^+$, 712$(M\text{-}DCHA+Na)^+$, 871$(M)^+$. [Lit[21]: mp 128-131 ℃, $[\alpha]^{25}_D$ -28.3 (c 0.5 $MeCONMe_2$)].

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