Enzymatic Synthesis of Dipeptide Derivatives Containing Non-coded Amino Acids in Organic Solvents†

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A series of dipeptide derivatives containing non-coded amino acids, *N*-Boc-4-X-Phe-Ala-NHNHPh (X = Cl, Br, I, NO_2), were synthesized by using thermoase in organic solvents. The physical data were consistent with the same samples prepared by 3-(diethoxyphosphoryloxy)-1, 2, 3-benzotriazin-4(3*H*)-one (DEPBT). Influence of different substituted groups of the non-coded amino acids and different organic solvents on the enzymatic peptide synthesis was studied.

Keywords thermoase, DEPBT, non-coded amino acid, enzymatic synthesis, peptide

Introduction

Enzymatic peptide synthesis has drawn much attention because of its advantage of enzyme stereospecificity, mild reaction conditions, minimum side-chain protection and avoidance of racemization. But the strict substrate specificity of the enzymes limits their applications. However, recent progress showed that such specificity of enzymes can be modified by media engineering so that they can accept compounds other than *L*-amino acid as their substrates in organic solvents. Furthermore, enzymatic synthesis of peptide in organic solvents offers special advantages. The enzymes are more stable in organic solvents where the formation of peptide bonds is more favorable than the hydrolysis of peptide bonds. And most organic compounds are soluble in organic solvents.[1,2]

We have reported the enzymatic synthesis of sweet dipeptide derivatives,[3] *N*-protected Leu-enkephalin[4] and *N*-protected osteogenic growth peptide fragment (10—14).[5] Recently, we have focused our attention on extending the application of protease for amide bond formation in organic solvents. And the effects of different substrates on the yields of those enzymatic reactions were studied. For example, subtilisin was successfully used for the synthesis of *N*-protected amino acid-steriod conjugates in DMF. The results indicated that Boc was better as the amino protecting group for the acyl donor than Z or Fmoc, and it was found that electron-withdrawing groups such as trifluoroethyl and phenyl esters in the carboxyl component were preferable to electron-donating groups such as methyl and ethyl esters, as well as 17-hydrazo-estra-1, 3, 5(10)-trien-3-ol acted as a better nucleophilic substrate than 17-amino-estra-1, 3, 5(10)-trien-3-ol due to its favorable electronic and steric effects.[6] In addition, a series of *N*-protected peptide alcohols were synthesized by subtilisin in different organic solvents, where amino alcohols with unprotected hydroxyl groups were used as amino components, such as Throl and Alaol. The results showed that *N*-protected aromatic amino acid esters were more suitable as acyl donor for subtilisin.[7]

Non-coded amino acids play an important role as building blocks in the synthesis of analogues of biologically active peptides for structure-activity studies.[8] Traditionally, peptides containing non-coded amino acids were prepared by using such coupling reagents as BOP[9] and DCC[10] which would possibly cause side reactions and racemization. However, these disadvantages can be large-

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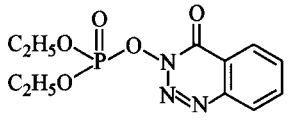
ly avoided by enzymatic method. For instance, Miyazawa *et al.*[11] reported the use of non-coded amino acids like halogenophenylalanines as acyl donor for the synthesis of a series of dipeptide derivatives catalyzed by immobilized α-chymotrypsin. And Ishikawa *et al.*[12] published their work of the synthesis of Z-(TMS-Ala)-Xaa-OMe (Xaa = Leu, Ile, Phe) by thermolysin.

Thermoase is crude powder mainly made up of thermolysin, a metal protease first separated from *Bcillus thermoproteolyticus* Rokko in Japan.[13] We have reported the enzymatic synthesis of *N*-Boc-Phe-Ala-NHNHPh, a peptide fragment of the analogue of delta sleeping-inducing peptide,[14] catalyzed by thermoase with high yields in organic solvents.[15] In this paper, dipeptide derivatives containing non-coded amino acids, *N*-Boc-4-X-Phe-Ala-NHNHPh (X = Cl, Br, I, NO_2), were synthesized by thermoase (Eq. 1), the physical data of which are consistent with those of the same samples prepared by 3-(diethoxyphosphoryloxy)-1, 2, 3-benzotriazin-4 (3*H*)-one (DEPBT), an efficient coupling reagent developed by our group for the synthesis of peptides (Eq. 2).[16,17]



DEPBT

N-Boc-4-X-Phe-OH + HCl·Ala-NHNHPh

$$\xrightarrow[\text{toluene, 5\% } H_2O,\ pH=8.5-9.0]{\text{thermoase, 37 °C}} \underset{\mathbf{1-5}}{\textit{N}\text{-Boc-4-X-Phe-Ala-NHNHPh}} \qquad (1)$$

1: X=H; **2**: X=Cl; **3**: X=Br; **4**: X=I; **5**: X=NO_2

N-Boc-4-X-Phe-OH + HCl·Ala-NHNHPh

$$\xrightarrow[\text{THF, pH=8.5}]{\text{DEPBT, r.t.}} \underset{\mathbf{1-5}}{\textit{N}\text{-Boc-4-X-Phe-Ala-NHNHPh}} \qquad (2)$$

1: X=H; **2**: X=Cl; **3**: X=Br; **4**: X=I; **5**: X=NO_2

Experimental

Apparatus

The melting points were determined on a Yanaco micromelting point apparatus and were uncorrected. Mass spectra were recorded on a VG-ZAB-HS spectrometer. ^{1}H NMR spectra were recorded on a Bruker ARX-400. Elemental analyses were performed on a Hezaeus CHN-Rapid elemental analyzer. Optical rotations were measured by a Perkin-Elmer 241 or 241 MC Polarimeter.

Reagents and materials

Thermoase (specific activity 22 units/mg protein) was purchased from DAINA KASEI K. K. company. *N*-Boc-4-X-Phe-OH (X = Cl, Br, I, NO_2) were purchased from BIOCHEM company. Methanol, phenyl hydrazine, THF and toluene were of analytical grade, and were dried and redistilled by standard techniques. HCl·Ala-NHNHPh was prepared by the standard technique.[18]

General procedure for the synthesis of N-Boc-4-X-Phe-Ala-NHNHPh by thermoase

To a solution of *N*-Boc-4-X-Phe-OH (0.4 mmol) in 5 mL of toluene, H_2O (250 μL, 5% *V/V*), HCl·Ala-NHNHPh (0.2 mmol), Et_3N (70 μL) and thermoase (30 mg) were added successively. The mixture was stirred at 37 ℃ and the reaction was monitored by TLC. When the starting materials disappeared, the solvent was removed *in vacuo* and the oily residue was dissolved in 100 mL of ethyl acetate. After filtrating Et_3N·HCl, the solution was successively washed with 5% Na_2CO_3, H_2O, 5% citric acid, H_2O and saturated NaCl solution, and dried over anhydrous Na_2SO_4 for 10 h. The solvent was then removed *in vacuo* to give the crude product, which was recrystallized from chloroform and petroleum.

General procedure for the synthesis of N-Boc-4-X-Phe-Ala-NHNHPh by DEPBT

To a solution of *N*-Boc-4-X-Phe-OH (0.3 mmol) and DEPBT (0.33 mmol) in 5 mL of THF, HCl·Ala-NHNHPh (0.45 mmol) was added. The pH of the solution was adjusted to 8—9 by Et_3N. The mixture was stirred at 20 ℃ and the reaction was monitored by TLC. When the starting materials disappeared, the product was purified as the same procedure described above.

Compound **1** White solid, m.p. 143—144 ℃, $[\alpha]_D^{20}$ − 17.9 (*c* 1.0, CH_3OH) (the physical data of this compound, m.p. 139—140 ℃ and $[\alpha]_D^{20}$ − 11.5 (*c* 1, MeOH), that we previously reported[15] should be revised to the physical data reported this time); ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.34—1.38 (m, 12H), 3.01—

5.13 (s, 1H), 6.73—6.78 (m, 3H), 6.85—6.89 (m, 1H), 7.14—7.28 (m, 8H), 8.39 (s, 1H); MS (FAB) m/z: 427 $(M+H)^+$. Anal. calcd for $C_{23}H_{30}N_4O_4$: C 64.77, H 7.09, N 13.14; found C 64.52, H 7.21, N 13.11.

Compound **2** White solid, m.p. 159—163 ℃, $[\alpha]_D^{20}$ - 8.8 (c 1.0, CH_3OH); ^1H NMR ($CDCl_3$, 400 MHz) δ: 0.83—0.87 (m, 1H), 1.25—1.26 (m, 1H), 1.32—1.40 (m, 12H), 2.94—3.06 (m, 2H), 4.38—4.40 (s, 1H), 4.56—4.59 (m, 1H), 5.12—5.13 (s, 1H), 6.77—6.79 (m, 2H), 6.87—6.90 (m, 2H), 7.05—7.07 (m, 2H), 7.18—7.27 (m, 4H), 8.46 (s, 1H); MS (FAB) m/z: 461 $(M+H)^+$. Anal. calcd for $C_{23}H_{29}ClN_4O_4$: C 59.93, H 6.34, N 12.15, Cl 7.69; found C 60.04, H 6.38, N 11.83, Cl 7.58.

Compound **3** Yellowish solid, m.p. 191—194 ℃, $[\alpha]_D^{20}$ - 5.6 (c 1.0, CH_3OH); ^1H NMR (DMSO-d_6, 400 MHz) δ: 0.83—1.32 (m, 12H), 2.64—2.70 (m, 1H), 2.93—2.97 (m, 1H), 4.14—4.20 (m, 1H), 4.35—4.40 (m, 1H), 6.67—6.71 (m, 3H), 6.97—6.99 (m, 1H), 7.10—7.14 (m, 2H), 7.18—7.29 (m, 2H), 7.44—7.46 (m, 2H), 7.75—7.76 (s, 1H), 8.20—8.22 (m, 1H), 9.77 (s, 1H); MS (FAB) m/z: 505 $(M+H)^+$. Anal. calcd for $C_{23}H_{29}BrN_4O_4$: C 54.66, H 5.78, N 11.09, Br 15.81; found C 54.88, H 5.90, N 10.85, Br 15.65.

Compound **4** White solid, m.p. 195—198 ℃, $[\alpha]_D^{20}$ - 2.5 (c 1.0, CH_3OH); ^1H NMR (DMSO-d_6, 400 MHz) δ: 1.19—1.32 (m, 12H), 2.62—2.68 (m, 1H), 2.92—2.95 (m, 1H), 4.14—4.18 (m, 1H), 4.34—4.38 (m, 1H), 6.69—6.70 (m, 3H), 6.95—6.97 (m, 1H), 7.08—7.22 (m, 4H), 7.60—7.62 (m, 2H), 7.76 (s, 1H), 8.18—8.20 (m, 1H), 9.76—9.79 (s, 1H); MS (FAB) m/z: 553 $(M+H)^+$. Anal. calcd for $C_{23}H_{29}IN_4O_4$: C 50.01, H 5.29, N 10.14; found C 50.23, H 5.26, N 9.81.

Compound **5** Yellowish solid, m.p. 151—153 ℃, $[\alpha]_D^{20}$ - 0.6 (c 1.0, CH_3OH); ^1H NMR (DMSO-d_6, 400 MHz) δ: 1.20—1.34 (m, 12H), 2.81—2.87 (m, 1H), 3.10—3.14 (m, 1H), 4.24—4.30 (m, 1H), 4.35—4.39 (m, 1H), 6.67—6.70 (m, 3H), 7.06—7.07 (m, 1H), 7.10—7.14 (m, 2H), 7.55—7.57 (m, 2H), 7.76 (s, 1H), 8.14—8.16 (m, 2H), 8.26—8.28 (m, 1H), 9.77—9.78 (s, 1H); MS (FAB) m/z: 472 $(M+H)^+$. Anal. calcd for $C_{23}H_{29}N_5O_6$: C 58.59, H 6.20, N 14.85; found C 58.29, H 6.08, N 14.39.

Results and discussion

From Tables 1 and 2, it can be obviously seen that the physical data of **1**—**5** synthesized by thermoase were consistent with those synthesized by DEPBT. And when the chemical method was used (Eq. 2 and Table 2), there were no obvious differences among the yields.

In the case of the enzymatic method (Eq. 1 and Table 1), it can be found that organic solvents are very important for this enzymatic reaction. Many organic solvents were tried for the peptide bond formation such as acetonitrile, toluene, tert-amyl alcohol, 1, 4-dioxane, chloroform, acetic ether, THF, DMF and DMSO, but only toluene could be successfully used in this reaction (Table 3). And the optimum reaction conditions were 37 ℃, 5% water content and the pH value of 8.5—9.0.

In toluene, thermoase can broaden its substrate selectivity and accept some non-coded amino acid derivatives like Boc-X-Phe-OH (X = Cl, Br, I, NO_2) as its substrates. In most cases, the yields were reasonably rather lower (30.2%, 25.9%, 28.2% for X = Cl, Br, I, respectively) than those of the synthesis using Boc-Phe-OH as the substrate (69.2%). This is consistent with Miyazawa's results when immobilized α-chymotrypsin was used as the catalyst,[11] since such selectivity broadening is limited. However, there was an exception. When X is NO_2, the yield is quite high (74.6%), even higher than that of the synthesis using Boc-Phe-OH as the substrate. Since NO_2 can only slightly activate the carboxyl group of the acyl donor, it is supposed that NO_2, with its two oxygen atoms, may form two hydrogen bonds with thermoase. In this way, it may be easier for Boc-4-NO_2-Phe-OH to bind to the hydrophobic pocket of thermoase's active site, and thus to facilitate this enzymatic reaction.

It is also found that α-chymotrypsin can catalyze this reaction, due to its highly efficient recognition of aromatic amino acids. Yet it usually requires esters of amino acids for acyl donors. By using thermoase, however, there was no need to prepare esters, and carboxylic acids could act as acyl donors for convenience. Furthermore, the effects of organic solvents on stereo-selectivity of various proteases in this reaction are being pursued and the results will be published elsewhere.

Table 1 Physical data of *N*-Boc-4-X-Phe-Ala-NHNHPh synthesized by thermoase

Compd	X	Yield (%)	M.p. (℃)	$[\alpha]_D^{20}$ (*c* 1.0, CH_3OH)
1	H	69.2	143—144	-17.9
2	Cl	30.2	159—163	-8.8
3	Br	25.9	191—194	-5.6
4	I	28.2	195—198	-2.5
5	NO_2	74.6	154—156	-0.6

Table 2 Physical data of *N*-Boc-4-X-Phe-Ala-NHNHPh synthesized by DEPBT as the coupling reagent

Compd	X	Yield (%)	M.p. (℃)	$[\alpha]_D^{20}$ (*c* 1.0, CH_3OH)
1	H	63.5	139—141	-19.9
2	Cl	66.2	162—165	-9.3
3	Br	64.7	189—191	-5.2
4	I	53.9	195—197	-2.0
5	NO_2	68.4	151—153	-1.4

Table 3 Enzymatic synthesis of *N*-Boc-Phe-Ala-NHNHPh catalyzed by thermoase in different organic solvents[a]

Entry	Organic solvent	Yield (%)	Entry	Organic solvent	Yield (%)
1	DMSO	0	7	AcOEt	0
2	1,4-Dioxane	0	8	*t*-Amyl alcohol	0
3	DMF	0	9	CH_2Cl_2	Trace
4	MeOH	0	10	CH_3Cl	0
5	CH_3CN	0	11	CCl_4	Trace
6	THF	0	12	Toluene	69.2

[a] Other reaction conditions: pH = 8.5—9.0, H_2O (5% *V/V*), 37 ℃.

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19 Abbreviations: the amino acid residues which are not indicated with configuration in this paper are of *L*-configuration. Standard abbreviations for amino acid and peptide derivatives 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, carbobenzyloxy; Fmoc, 9-fluorenylmethyloxycarbonyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; THF, tetrahydrofuran; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulphoxide; Et_3N, triethylamine; BOP, benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate.

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