BIOORGANIC CHEMISTRY 14, 182–193 (1986)

Thermolysin as a Catalyst in Enzymatic Synthesis of Asparagine-Containing Peptides

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Received July 10, 1985

The following di- and tripeptides were synthesized to study the potential utility of thermolysin as a catalyst in reactions of incorporation of N_α-acyl-L-asparagine into esters of amino acids and peptides: Boc-Asn-Ile-OBzl, Z-Asn-Ile-OBzl, Moz-Asn-Ile-OBzl, Boc-Asn-Leu-OBzl, Z-Asn-Leu-OBzl, Moz-Asn-Leu-OBzl, Boc-Asn-Phe-OBzl, Z-Asn-Phe-OBzl, Moz-Asn-Phe-OBzl, Z-Asn-Val-OBzl, Moz-Asn-Val-OBzl, Moz-Asn-Ile-Gly-OBzl, Moz-Asn-Ile-Ala-OBzl, Moz-Asn-Ile-Leu-OBzl, and Moz-Asn-Ile-Phe-OBzl. All of these peptides were obtained in pure form in good yield and characterized by thin-layer chromatography, melting point, elemental analysis, amino acid analysis, and proton magnetic resonance. The use of benzyloxycarbonyl (Z) and *p*-methoxybenzyloxycarbonyl (Moz) as protecting groups for asparagine gave excellent yields of the dipeptides. Relative to the dipeptides, the synthesis of the tripeptides was found to require lower enzyme concentrations and reaction times. Since the yields of the tripeptides failed to exhibit significant differences, it was not possible to establish the existence of a secondary specificity of thermolysin for the residue P_2'. A methodological study was also performed to determine the optimum conditions for synthesis of Boc-Asn-Ile-OBzl. This study consisted of an analysis of the influence of pH, enzyme concentration, volume and concentration of the solution of sodium acetate, relative proportions of carboxyl and amine components, temperature, and addition of organic solvent to the reaction medium.

INTRODUCTION

Incorporation of asparagine as a carboxyl component in peptide synthesis is problematic because any process used to active asparagine leads to dehydration of the amide in the side chain with subsequent formation of the corresponding nitrile as a secondary product (*1, 2*) (Scheme 1). This compound, incorporated into the peptide chain, contaminates the synthesized peptide and is difficult to separate.

To avoid this problem, couplings involving asparagine as a carboxyl component have been performed mainly by the active ester method (*2*). In this method, the active ester obtained is separated from the undesirable subproduct by successive recrystallizations and utilized in pure form in the coupling reactions. Alternative methods such as coupling by oxido-reduction (*3*) and addition of 1-hydroxybenzotriazole together with *N,N'*-dicyclohexylcarbodiimide (*4*) have been reported. However, none of these methods totally eliminate the occurrence of dehydration

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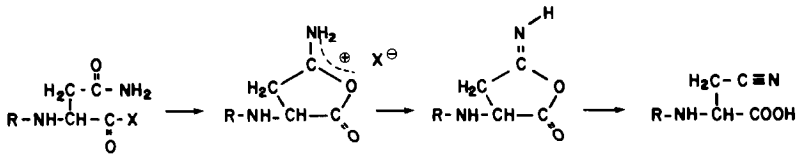

SCHEME 1. R = protecting group. X = electron-withdrawing group.

(*2*). Protection of the amide function with the 4,4′-dimethoxybenzhydryl group as described by König and Geiger (*5*) has proved to be quite effective at preventing nitrile formation, but does not always provide high coupling yields and requires an additional step in the preparation of the protected amino acid (*2*). An alternative form of protection of the amide group is total transformation of carboxyamide into nitrile, which is then utilized in the couplings (*6*). The resulting β-cyanoalanyl peptide can be rehydrated in the presence of sodium carbonate/hydrogen peroxide (*6*) or hydrogen bromide/glacial acetic acid (*7*) or during the final deprotection with hydrogen fluoride (*2*). One of the disadvantages of this type of protection, however, is that rehydration is not always quantitative (*6*).

In the present paper, we report the enzymatic synthesis of di- and tripeptides having N_α-acyl-L-asparagine as the carboxyl component. Thermolysin was chosen as the enzyme because it has no amidase or esterase activity (*8*) and has been used successfully as a catalyst in peptide synthesis by several investigators (*9–14*).

RESULTS AND DISCUSSION

1. Optimum Conditions for Boc-Asn-Ile-OBzl Synthesis

Optimum conditions for Boc-Asn-Ile-OBzl synthesis were established after a methodological study in which we analyzed the influence of pH, reaction time, enzyme concentration, sodium acetate solution concentration, relative proportions of carboxyl and amine components, temperature, and organic solvent on the coupling yield.

The effect of pH on Boc-Asn-Ile-OBzl synthesis (Fig. 1) indicated an optimum pH of 6.0. For comparison, Oka and Morihara (*12*) detected a greater extent of synthesis at pH close to 7.0 for the Z-Phe-OH and H-Leu-NH_2 coupling, and Isowa and Ichikawa (*15*) obtained high peptide yields using buffer at pH 8.0. These data suggest that the optimum pH for synthesis also depends on the substrates used.

The effect of reaction time (Fig. 2) indicated that, under the conditions employed, the reaction reached apparent equilibrium within 24 h; no significant difference in yield was observed after an additional 24 and 48 h.

The results obtained for the effect of enzyme concentration indicate that the extent of Boc-Asn-Ile-OBzl synthesis increases with increasing enzyme concen-

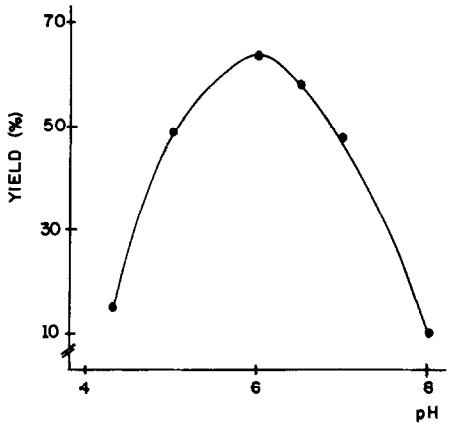

FIG. 1. Effect of pH on the yields of Boc-Asn-Ile-OBzl. The reaction mixtures contained 1.0×10^{-3} mol Boc-Asn-OH, 1.0×10^{-3} mol H-Ile-OBzl · Tos, 1 ml 1 M NaOH, 2.0×10^{-7} mol thermolysin, and 20 ml 0.2 M acetate buffer (pH 4.3–6.5) or 0.2 M Tris buffer (pH 7.0–8.0). The buffers contained 5.0×10^{-2} M calcium acetate. The reaction was performed by shaking at 38°C for 72 h.

tration up to 1.0×10^{-5} M (Fig. 3), the yield being practically unchanged above this concentration.

As shown in Fig. 4, the extent of Boc-Asn-Ile-OBzl synthesis also depends on the concentration of the sodium acetate solution. The decrease in yield observed above 0.5 M may be explained by the decrease in the solubility of the reagents in the reaction medium.

The effect of changing the relative proportions of the carboxyl (C) and amine

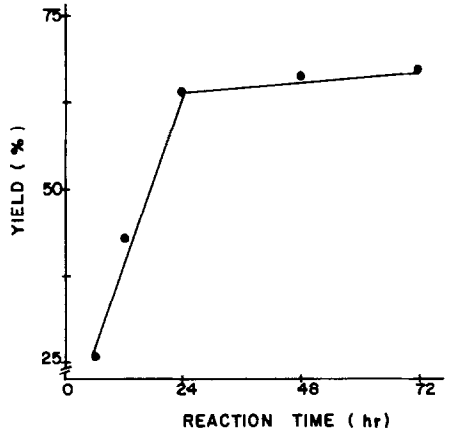

FIG. 2. Effect of reaction time on the synthesis of Boc-Asn-Ile-OBzl. The reaction mixtures contained 1.0×10^{-3} mol Boc-Asn-OH, 1.0×10^{-3} mol H-Ile-OBzl · Tos, 1 ml 1 M NaOH, 4.0×10^{-7} mol thermolysin, and 20 ml 0.2 M sodium acetate solution, pH 6, containing 5.0×10^{-2} M calcium acetate. The reaction was performed by shaking at 38°C.

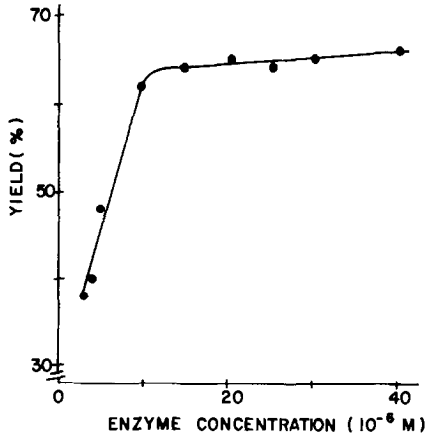

FIG. 3. The effect of enzyme concentration on the synthesis of Boc-Asn-Ile-OBzl. The reaction mixtures contained 1.0×10^{-3} mol Boc-Asn-OH, 1.0×10^{-3} mol H-Ile-OBzl · Tos, 1 ml 1 M NaOH, and 20 ml 0.2 M sodium acetate solution, pH 6, containing 5.0×10^{-2} M calcium acetate. The reaction mixtures were shaken for 48 h at 38°C.

(A) components (Table 1) showed that a higher percentage of synthesis (83% yield) can be obtained using a C:A ratio of 2:1; proportions of 1.0:1.5 and 1.0:2.0 were less effective. This may be attributed to the different solubilities of Boc-Asn-OH and H-Ile-OBzl · Tos in the sodium acetate solution. Boc-Asn-OH is quite soluble while H-Ile-OBzl is not. Consequently, an increase in the amount of Boc-Asn-OH shifts the reaction equilibrium toward the formation of a peptide bond; increasing the amount of H-Ile-OBzl is ineffective since it fails to dissolve further in the reaction medium.

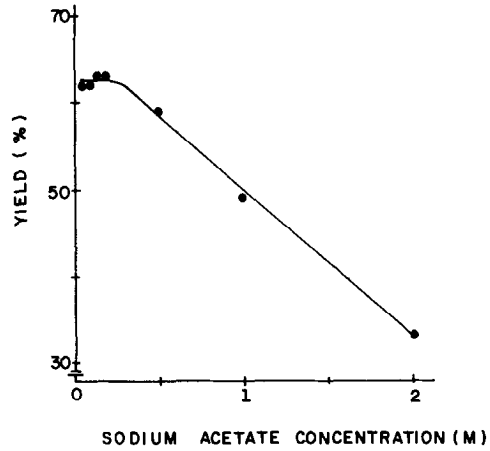

FIG. 4. Effect of sodium acetate concentration on the synthesis of Boc-Asn-Ile-OBzl. The reaction mixtures contained 1.0×10^{-3} mol Boc-Asn-OH, 1.0×10^{-3} mol H-Ile-OBzl · Tos, 1 ml 1 M NaOH, 2.0×10^{-7} mol thermolysin, and 20 ml sodium acetate solution, pH 6, containing 5.0×10^{-2} M calcium acetate. The reaction was performed for 48 h at 38°C.

TABLE 1

EFFECT OF RELATIVE PROPORTION OF THE CARBOXYL AND AMINE COMPONENTS ON THE SYNTHESIS OF Boc-Asn-Ile-OBzl[a]

Boc-Asn-OH : H-Ile-OBzl · Tos	Boc-Asn-OH (10^{-3} mol)	H-Ile-OBzl-Tos (10^{-3} mol)	Yield (%)
1.0 : 1.0	1.0	1.0	66
1.0 : 1.5	1.0	1.5	67
1.0 : 2.0	1.0	2.0	61
1.5 : 1.0	1.5	1.0	75
2.0 : 1.0	2.0	1.0	83
2.0 : 1.0[b]	2.0	1.0	53

[a] The reaction mixture contained 4.0×10^{-7} mol thermolysin, 1 ml 1 M NaOH, and 20 ml 0.2 M sodium acetate solution, pH 6, containing 5×10^{-2} M calcium acetate. The amounts of starting material are given in the table. The reaction was performed by shaking at 38°C.

[b] Synthesis was carried out in the presence of 2.0×10^{-7} mol thermolysin.

Since the Boc group is thermolabile, the effect of temperature on the extent of synthesis was studied only at 38 and 48°C,using the following experimental conditions: 1.0×10^{-3} mol Boc-Asn-OH; 1.0×10^{-3} mol H-Ile-OBzl · Tos; 4.0×10^{-7} mol thermolysin; 1 ml 1 M NaOH; 20 ml 0.2 M sodium acetate, pH 6, containing 5.0×10^{-2} M calcium acetate. For the two temperatures tested, the peptide was obtained in 36 and 53% yields, respectively, after 9 h and in 51 and 56% yields after 13 h of reaction. The expected yield at 48°C for the 13-h reaction was higher than that observed in this experiment.

The effect of temperature on the thermolysin-catalyzed synthesis of Z-Asn-Ile-OBzl was studied over a wider temperature range, i.e., at 28, 38, 48, 55, and 68°C. The choice of this protecting group for the N_α-amine group was based on the fact that it is more thermostable than Boc. The results showed that the yields obtained after 3 h of incubation were significantly higher than those obtained after 1 h (Table 2). This suggests that thermolysin maintains its activity, at least partially, after 1 h of incubation at temperatures above 50°C. From a synthetic point of view, these results indicate that, in addition to better thermostability, the Z group is a more adequate protecting group for L-asparagine than Boc, since the yields are higher in a shorter reaction time.

The synthesis of Boc-Asn-Ile-OBzl was carried out in the presence of organic solvents in an attempt to improve the yields obtained in sodium acetate solution alone. The minimum amount of the aqueous organic solvent mixture required for total dissolution of the starting materials was used. The experimental conditions and yields are given in Table 3. The best results were obtained in the presence of methanol, with a reaction mixture containing relative C : A proportions of 2 : 1. At relative C : A proportions of 1 : 2, the yield in the presence of ethyl acetate (73%) was higher than in its absence (61%). The presence of methanol failed to produce a similar effect at this latter ratio (47% yield).

TABLE 2

EFFECT OF TEMPERATURE ON THE SYNTHESIS OF Z-Asn-Ile-OBzl[a]

Reaction time (h)	Yield (%)				
	28°C	38°C	48°C	58°C	68°C
1	33	53	66	74	75
3	—	73	80	83	81

[a] The reaction mixture contained 1.0×10^{-3} mol Z-Asn-OH, 1×10^{-3} mol H-Ile-OBzl · Tos, 1 ml 1 M NaOH, 2.0×10^{-7} mol thermolysin, and 20 ml 0.2 M sodium acetate solution, pH 6, containing 5.0×10^{-2} M calcium acetate.

2. Synthesis of Various Asparagine-Containing Di- and Tripeptides

Comparative data for couplings of Boc-Asn-OH, Z-Asn-OH, and Moz-Asn-OH with H-Ile-OBzl · Tos, H-Leu-OBzl · Tos, H-Phe-OBzl · Tos, and H-Val-OBzl · Tos, carried out under different experimental conditions, are shown in Table 4. All peptides protected with the Z and Moz groups gave higher yields than those protected with Boc. Two possible factors may contribute to these results: the lower solubility of the products formed in the reaction medium, and the favored interaction of the protecting group with the S_2 subsite of thermolysin. The latter hypothesis is based on Fruton's data (*11*), which demonstrate the stereospecific preference of thermolysin for hydrophobic amino acids at position P_2. The author showed that the K_{cat}/K_M (min^{-1} mM^{-1}) value for Z-Phe-Leu-NHPh synthesis is 73 ± 5, whereas for Boc-Phe-Leu-NHPh it is 49 ± 3.

The coupling of Boc-Asn-OH with H-Val-OBzl · Tos was also performed under several experimental conditions, but the amount of dipeptide obtained was always negligible, preventing its isolation (data not shown). In contrast, Moz-Asn-Val-OBzl was obtained in excellent yields (Table 4).

In the synthesis of Boc-Asn-Leu-OBzl, Boc-Asn-Phe-OBzl, and Z-Asn-Val-OBzl, most of the amine components were found to be insoluble in the 0.2 M sodium acetate solution used. Since the reaction yields are related to the solubility of the reagents and products, we studied the effect of addition of organic solvents to the reaction mixture. The yields obtained in the presence of organic solvents (ethyl acetate, dimethylformamide, ethanol, and methanol) were consistently below 50% (Table 3). Isowa *et al.* (*16*) observed an extensive synthesis of Z-Ala-Phe-OBzl in papain-catalyzed reactions when the proportions of organic solvent : water were within the range of 3:8 to 8:3. Since the proportions of organic solvent : water were within this range in our reaction more expressive synthesis yields would be expected. More detailed studies of the effect of these solvents on the extent of synthesis of these peptides are currently in progress in our laboratory.

TABLE 3

SYNTHESIS OF ASPARAGINE-CONTAINING DIPEPTIDE IN THE PRESENCE OF ORGANIC SOLVENTS[a]

Peptide	Yield (%)	*N*-Acyl-Asn (10^{-3} mol)	Carboxyl component (10^{-3} mol)	pH	0.2 M AcONa, pH 6.0, or 0.2 M Tris–HCl, pH 8.0 (ml)	Organic solvent (ml)				Thermolysin (10^{-7} mol)	Reaction time (h)
						MeOH	EtOH	DMF	EtAc		
Boc-Asn-Ile-OBzl	47	1	2	6	18.0	2.0	—	—	—	4.0	48
Boc-Asn-Ile-OBzl	73	1	2	6	16.0	—	—	—	0.8	4.0	48
Boc-Asn-Ile-OBzl	79	2	1	6	10.0	4.0	—	—	—	4.0	48
Boc-Asn-Leu-OBzl	10	2	1	6	8.0	—	5.0	—	—	4.0	48
Boc-Asn-Leu-OBzl	9	2	1	6	14.0	—	4.0	2.0	—	4.0	72
Boc-Asn-Leu-OBzl	10	2	1	6	10.0	—	3.0	3.0	—	4.0	48
Boc-Asn-Phe-OBzl	25	2	1	6	10.0	—	—	2.0	—	4.0	48
Boc-Asn-Phe-OBzl	6	2	1	6	8.0	—	5.0	—	—	4.0	48
Z-Asn-Val-OBzl	44	2	1	8	7.0	2.0	—	1.0	—	4.0	72
Z-Asn-Val-OBzl	49	2	1	8	10.5	2.0	—	1.0	—	4.0	72
Z-Asn-Val-OBzl	25	2	1	6	10.5	2.0	—	1.5	—	4.0	72

[a] The solution contained 5.0×10^{-2} M calcium acetate. All couplings were performed at 38°C, with shaking.

TABLE 4

CONDITIONS OF SYNTHESIS AND YIELDS OF DIPEPTIDES OBTAINED BY THE ENZYMATIC METHOD

Peptide	Yield (%)	*N*-Acyl-Asn (10^{-3} mol)	Amine component (10^{-3} mol)	pH	0.2 M AcONa, pH 6, or 0.2 M Tris–HCl, pH 8 (ml)[a]	Thermolysin (10^{-7} mol)	Reaction time (h)
Boc-Asn-Ile-OBzl	70	1	1	6	5	2.0	48
Boc-Asn-Ile-OBzl	62	1	1	6	20	2.0	48
Boc-Asn-Ile-OBzl	62	1	2	6	20	4.0	48
Boc-Asn-Ile-OBzl	83	2	1	6	20	4.0	48
Boc-Asn-Ile-OBzl	10	1	1	8	20	2.0	72
Boc-Asn-Ile-OBzl	79	2	1	6	10	4.0	48
Z-Asn-Ile-OBzl	87	1	1	6	20	4.0	48
Z-Asn-Ile-OBzl	96	2	1	6	20	4.0	48
Moz-Asn-Ile-OBzl	87	1	1	6	20	2.0	48
Moz-Asn-Ile-OBzl	95	2	1	6	20	4.0	48
Boc-Asn-Leu-OBzl	25	1	1	6	30	2.0	48
Z-Asn-Leu-OBzl	79	1	1	6	20	4.0	48
Moz-Asn-Leu-OBzl	80	1	1	6	20	4.0	48
Moz-Asn-Leu-OBzl	94	2	1	6	20	4.0	48
Boc-Asn-Phe-OBzl	57	1	1	6	30	2.0	48
Boc-Asn-Phe-OBzl	60	1	1	6	30	2.0	72
Boc-Asn-Phe-OBzl	25	2	1	6	10	4.0	48
Z-Asn-Phe-OBzl	78	1	1	6	35	4.0	72
Moz-Asn-Phe-OBzl	79	1	1	6	30	2.0	48
Moz-Asn-Phe-OBzl	95	1	1	6	35	4.0	72
Moz-Asn-Val-OBzl	80	1	1	6	20	4.0	48
Moz-Asn-Val-OBzl	91	2	1	6	20	4.0	48

[a] The solutions contained 5.0×10^{-2} M calcium acetate. All couplings were performed at 38°C, with shaking.

To determine the effect of residue P_2' on thermolysin-catalyzed synthesis, the following tripeptides were synthesized: Moz-Asn-Ile-Gly-OBzl, Moz-Asn-Ile-Ala-OBzl, Moz-Asn-Ile-Leu-OBzl, and Moz-Asn-Ile-Phe-OBzl. The results in Table 5 show that the yields of these tripeptides exceeded 85% in 5 h at an enzyme concentration of 4.0×10^{-6} M. Under the same conditions, Boc-Asn-Ile-OBzl was obtained in 40% yield (Fig. 3). These data demonstrate that the rate of tripeptide formation is higher than the rate of dipeptide formation for a given enzyme concentration. It was not possible to establish the secondary specificity of thermolysin for residue P_2' since the yields obtained in the synthesis of the tripeptides did not exhibit significant differences.

The results presented in this paper suggest that, once established, the enzymatic method of coupling N_α-acyl-L-asparagine to amino acid residues and peptides may prove useful for obtaining peptide chains containing this amino acid.

EXPERIMENTAL PROCEDURE

Enzyme

Thermolysin (EC 3.4.24.2) was purchased from the Protein Research Foundation, Osaka, and used without purification (activity, 10,000 PU mg^{-1}).

TABLE 5

CONDITIONS OF SYNTHESIS AND YIELDS OF ASPARAGINE-CONTAINING TRIPEPTIDES

Peptide	Yield (%)	Carboxyl : amine (10^{-3} mol)	0.2 M AcONa, pH 6.0 (ml)[a]	Thermolysin (10^{-8} mol)	Reaction time (h)
Moz-Asn-Ile-Gly-OBzl	88	1 : 1	20.0	8.5	5
Moz-Asn-Ile-Ala-OBzl	95	1 : 1	20.0	20.0	6
Moz-Asn-Ile-Leu-OBzl	88	1 : 1	20.0	8.5	5
Moz-Asn-Ile-Phe-OBzl	89	1 : 1	20.0	8.5	5

[a] The solution contained 5.0×10^{-2} M calcium acetate. All couplings were performed at 37°C, with shaking.

L-Amino Acid and Peptide Derivatives

The following compounds were synthesized in our laboratory according to techniques described in the literature: Boc-Asn-OH, Boc-Ile-OH, and Moz-Asn-OH (*17*); H-Ile-OBzl · Tos, H-Leu-OBzl · Tos, H-Val-OBzl · Tos, H-Phe-OBzl · Tos, H-Gly-OBzl · Tos, and H-Ala-OBzl · Tos (*18*); Boc-Asn-Ile-OBzl (*19*); Boc-Ile-Gly-OBzl, Boc-Ile-Leu-OBzl, Boc-Ile-Ala-OBzl, and Boc-Ile-Phe-OBzl (*4*). The α-amine groups of the dipeptides used as substrates in the synthesis of tripeptides were deprotected with trifluoroacetic acid.

Analytical Procedures

Thin-layer chromatography (TLC) was carried out on 0.25-mm-thick silica gel G plates, prepared in our laboratory, using the following solvent systems for development: (A) chloroform : methanol (95 : 5); (B) chloroform : methanol : acetic acid (95 : 5 : 3); and (C) chloroform : methanol : acetic acid (85 : 10 : 5). Proton magnetic resonance spectra (^{1}H NMR) were obtained with a Varian Model T-60 spectrometer. Amino acid analyses were performed using a Beckman 120C automatic amino acid analyzer after hydrolysis of protected peptides for 72 h with twice-distilled 6 M hydrochloric acid. Elemental analyses were performed with a Perkin–Elmer Model 740 CHN-type microanalyzer. The melting points are uncorrected.

General Method Used for the Formation of Peptide Bonds

The enzymatic synthesis of di- and tripeptides containing asparagine was carried out in homogeneous reaction systems: carboxyl and amine components were fully or partially dissolved in buffer or in sodium acetate solution containing 5.0×10^{-2} M calcium acetate, and stoichiometric amounts of a sodium hydroxide solution were added to neutralize the amine components. In those reactions in which the carboxyl and amine components were only slightly soluble in the buffer or saline solution, organic solvents such as ethanol, methanol, and dimethylformamide were added. Thermolysin was added and the resulting mixture was incubated for variable times and temperatures, with shaking. The precipitated prod-

ucts were filtered and washed with 1 M hydrochloric acid, water, and 5% aqueous sodium bicarbonate solution (w/v) and dried under vacuum over phosphorus pentoxide. These products were chromatographically homogeneous in solvent systems A, B, and C. Reaction yields were determined gravimetrically and refer to crude products. The products were recrystallized using the following solvent systems: methanol/water, Boc-Asn-Ile-OBzl and Boc-Asn-Phe-OBzl; ethyl acetate/petroleum ether, Z-Asn-Ile-OBzl, Boc-Asn-Leu-OBzl, Z-Asn-Leu-OBzl, and Z-Asn-Val-OBzl; chloroform/petroleum ether, Moz-Asn-Ile-OBzl, Moz-Asn-Leu-OBzl, and Moz-Asn-Val-OBzl; acetone, Z-Asn-Phe-OBzl; acetone/petroleum ether, Moz-Asn-Ile-Leu-OBzl; chloroform/ethanol/petroleum ether, Moz-Asn-Phe-OBzl, Moz-Asn-Ile-Gly-OBzl, Moz-Asn-Ile-Ala-OBzl, and Moz-Asn-Ile-Phe-OBzl.

The analytical data for the recrystallized peptides obtained enzymatically are listed below.

Boc-Asn-Ile-OBzl. R_{fA} 0.35, R_{fB} 0.60; R_{fC} 0.86; mp 126–127°C. ^{1}H NMR (DMSO-d_6) 0.63–1.13 (m, 6H), 1.42 (s, 9H), 1.45–2.03 (m, 2H), 2.42 (d, 2H), 3.95–4.65 (m, 1H), 5.17 (s, 2H), 6.88 (s, 1, 2H), 7.38 (s, 5H, phenyl), 7.95 (d, 1H). *Anal.* Calcd for $C_{22}H_{33}N_3O_6$: C, 60.67; N, 9.65; H, 7.64. Found: C, 60.76, N, 9.60; H, 7.64. Amino acid analysis: Asp 1.01, Ile 0.98.

Z-Asn-Ile-OBzl. R_{fA} 0.39, R_{fB} 0.64, R_{fC} 0.86; mp 160–163°C; ^{1}H NMR (DMSO-d_6), 0.67–0.83 (m, 6H), 0.83–1.47 (m, 2H), 2.43 (d, 2H), 3.90–4.70 (m, 1H), 5.00 (s, 2H), 5.10 (s, 2H), 6.87 (s, 1, 2H), 7.32 (s, 5H, phenyl), 7.93 (d, 1H). *Anal.* Calcd for $C_{25}H_{31}N_3O_6$: C, 63.95; N, 8.95; H, 6.65. Found: C, 64.30; N, 8.85; H, 6.72. Amino acid analysis: Asp 1.03, Ile 0.97.

Moz-Asn-Ile-OBzl. R_{fA} 0.41, R_{fB} 0.60, R_{fC} 0.85; mp 127–129°C; ^{1}H NMR (DMSO-d_6), 0.50–0.97 (m, 6H), 0.97–1.53 (m, 2H), 2.37 (d, 2H), 3.67 (s, 3H), 4.07–4.57 (m, 1H), 4.87 (s, 2H), 5.07 (s, 2H), 6.83 (d, 2H, arom. ring), 7.22 (d, 2H, arom. ring), 7.33 (s, 5H, phenyl), 8.00 (d, 1H). *Anal.* Calcd for $C_{26}H_{33}N_3O_7$: C, 62.50; N, 8.41; H, 6.66. Found: C, 62.17; N, 8.14; H, 6.60. Amino acid analysis: Asp 1.03, Ile 0.97.

Boc-Asn-Leu-OBzl. R_{fA} 0.36, R_{fB} 0.59, R_{fC} 0.83; mp 140–143°C; ^{1}H NMR (DMSO-d_6), 0.70–1.13 (m, 6H), 1.38 (s, 9H), 1.38–1.83 (m, 2H), 2.42 (d, 2H), 4.10–4.60 (m, 1H), 5.15 (s, 2H), 6.93 (s, 1, 2H), 7.40 (s, 5H, phenyl), 8.17 (d, 1H). *Anal.* Calcd for $C_{22}H_{33}N_3O_6$: C, 60.67; N, 9.65; H, 7.64. Found: C, 60.86; N, 9.50; H, 7.66. Amino acid analysis: Asp 1.03, Leu 0.97.

Z-Asn-Leu-OBzl. R_{fA} 0.38, R_{fB} 0.57, R_{fC} 0.81; mp 139–151°C; ^{1}H NMR (DMSO-d_6), 0.63–1.07 (m, 6H), 1.30–1.80 (m, 3H); 2.42 (d, 2H), 4.07–4.63 (m, 2H), 4.98 (s, 2H), 5.07 (s, 2H), 6.80 (s, 1, 2H), 7.27 (s, 10H, phenyl), 8.13 (d, 1H). *Anal.* Calcd for $C_{25}H_{31}N_3O_6$: C, 63.95; N, 8.95; H, 6.65. Found: C, 63.07; N, 8.69; H, 6.56. Amino acid analysis: Asp 1.00, Leu 1.00.

Moz-Asn-Leu-OBzl. R_{fA} 0.34, R_{fB} 0.66, R_{fC} 0.85; mp 161–163°C; ^{1}H NMR (DMSO-d_6), 0.63–1.07 (s, 1, 6H), 1.22–1.78 (m, 3H), 2.33 (d, 2H), 3.63 (s, 3H), 4.10–4.47 (m, 2H), 4.85 (s, 2H), 5.03 (s, 2H), 6.78 (d, 2H, arom. ring), 7.18 (d, 2H, arom. ring), 7.23 (s, 5H, phenyl), 8.13 (d, 1H). *Anal.* Calcd for $C_{26}H_{33}N_3O_7$: C, 62.50; N, 8.41; H, 6.66. Found: C, 62.71; N, 8.29; H, 6.63. Amino acid analysis: Asp 1.07, Leu 0.92.

Boc-Asn-Phe-OBzl. R_{fA} 0.35, R_{fB} 0.58, R_{fC} 0.81; mp 148–150°C; ^{1}H NMR (DMSO-d_6), 1.27 (s, 9H), 2.28 (d, 2H), 2.92 (d, 2H), 3.95–4.63 (m, 2H), 4.97 (s, 2H), 6.75 (s, 1, 2H), 7.10 (s, 5H, phenyl), 7.20 (s, 5H, phenyl), 8.03 (d, 1H). *Anal.* Calcd for $C_{25}H_{31}N_3O_6$: C, 63.95; N, 8.95; H, 6.65. Found: C, 63.51; N, 8.85; H, 6.60. Amino acid analysis: Asp 0.99, Phe 1.01.

Z-Asn-Phe-OBzl. R_{fA} 0.39, R_{fB} 0.72, R_{fC} 0.85; mp 165–168°C; ^{1}H NMR (DMSO-d_6), 2.35 (d, 2H), 2.97 (d, 2H), 4.12–4.70 (m, 2H), 4.92 (s, 2H), 5.02 (s, 2H), 6.78 (s, 1, 2H), 7.10 (s, 5H, phenyl), 7.25 (s, 10H, phenyl), 8.17 (d, 1H). *Anal.* Calcd for $C_{28}H_{29}N_3O_6$: C, 66.79; N, 8.34; H, 5.80. Found: C, 66.17; N, 8.19; H, 5.83. Amino acid analysis: Asp 0.97, Phe 1.02.

Moz-Asn-Phe-OBzl. R_{fA} 0.29, R_{fB} 0.55, R_{fC} 0.82; mp 191–192°C; ^{1}H NMR (DMSO-d_6), 2.38 (d, 2H), 3.00 (d, 2H), 3.70 (s, 3H), 4.05–4.68 (m, 2H), 4.92 (s, 2H), 5.05 (s, 2H), 6.85 (d, 2H, arom. ring, J = 8.0 Hz), 7.15 (s, 5H, phenyl), 7.27 (s, 5H, phenyl), 8.22 (d, 1H). *Anal.* Calcd for $C_{29}H_{31}N_3O_7$: C, 65.28; N, 7.88; H, 5.86. Found: C, 65.68; N, 7.80; H, 5.98. Amino acid analysis: Asp 0.97, Phe 1.02.

Z-Asn-Val-OBzl. R_{fA} 0.36, R_{fB} 0.65, R_{fC} 0.78; mp 162–164°C; ^{1}H NMR (DMSO-d_6), 0.78 (d, 6H, J = 6.9 Hz), 1.73–2.10 (m, 1H), 2.38 (d, 2H), 3.97–4.42 (m, 2H), 4.93 (s, 2H), 5.03 (s, 2H), 6.78 (s, 1, 2H), 7.22 (s, 10H, phenyl), 7.93 (d, 1, 1H). *Anal.* Calcd for $C_{24}H_{29}N_3O_6$: C, 63.28; N, 9.22; H, 6.42. Found: C, 63.11; N, 9.08; H, 6.33. Amino acid analysis: Asp 1.01, Val 0.98.

Moz-Asn-Val-OBzl. R_{fA} 0.32, R_{fB} 0.63, R_{fC} 0.84; mp 155–157°C; ^{1}H NMR (DMSO-d_6), 0.65 (d, 6H, J = 6.9 Hz), 1.62–2.10 (m, 1H), 2.28 (d, 2H), 3.55 (s, 3H), 3.88–4.48 (m, 2H), 4.77 (s, 2H), 4.97 (s, 2H), 6.68 (d, 2H, arom. ring, J = 8.0 Hz), 7.08 (d, 2H, arom. ring, J = 8.0 Hz), 7.18 (s, 5H, phenyl), 7.87 (d, 1, 1H). *Anal.* Calcd for $C_{25}H_{31}N_3O_7$; C, 61.80; N, 8.65; H, 6.43. Found: C, 61.01; N, 8.44; H. 6.25. Amino acid analysis: Asp 1.03, Val 0.97.

Moz-Asn-Ile-Gly-OBzl. R_{fA} 0.67, R_{fB} 0.75, R_{fC} 0.89; mp 225–230°C; ^{1}H NMR (DMSO-d_6), 0.55–1.02 (m, 6H), 1.42–1.95 (m, 3H), 2.42 (d, 2H), 3.68 (s, 3H), 3.85 (d, 2H, J = 6.0 Hz), 3.98–4.60 (m, 2H), 4.90 (s, 2H), 5.08 (s, 2H), 6.82 (d, 2H, arom. ring, J = 8.0 Hz), 7.22 (d, 2H, arom. ring, J = 8.0 Hz), 7.32 (s, 5H, phenyl), 8.28 (d, 1H). *Anal.* Calcd for $C_{28}H_{36}N_4O_8$: C, 60.42; N, 10.07; H, 6.51. Found: C, 60.29; N, 9.92; H, 6.54. Amino acid analysis: Asp 1.03, Ile 0.95, Gly 1.01.

Moz-Asn-Ile-Ala-OBzl. R_{fA} 0.60, R_{fB} 0.77, R_{fC} 0.90; mp 219–224°C; ^{1}H NMR (DMSO-d_6), 0.53–0.97 (m, 6H), 1.23 (d, 3H, J = 7.0 Hz), 1.37–1.97 (m, 2H), 2.37 (d, 2H), 3.68 (s, 3H), 3.93–4.53 (m, 2H), 4.88 (s, 2H), 5.03 (s, 2H), 6.78 (d, 2H, arom. ring, J = 8.0 Hz), 7.13 (d, 2H, arom. ring, J = 8.0 Hz), 7.27 (s, 5H, phenyl), 8.33 (d, 1H). *Anal.* Calcd for $C_{29}H_{38}N_4O_8$: C, 61.04; N, 9.82; H, 6.71. Found: C, 60.08; N, 9.58; H, 6.59. Amino acid analysis: Asp 1.01, Ile 1.03, Ala 0.96.

Moz-Asn-Ile-Leu-OBzl. R_{fA} 0.36, R_{fB} 0.71, R_{fC} 0.87; mp 214–216°C; ^{1}H NMR (DMSO-d_6), 0.53–1.03 (m, 9H), 1.40–1.72 (m, 2H), 2.40 (d, 2H), 3.70 (s, 3H), 3.90–4.60 (m, 2H), 4.92 (s, 2H), 5.07 (s, 2H), 6.85 (d, 2H, arom. ring, J = 8.0 Hz), 7.22 (d, 2H, arom. ring, J = 8.0 Hz), 7.30 (s, 5H, phenyl), 8.28 (d, 1H). *Anal.* Calcd for $C_{32}H_{44}N_4O_8$: C, 62.73; N, 9.14; H, 7.23. Found: C, 59.08; N, 8.56; H, 6.90. Amino acid analysis: Asp 1.00, Ile 1.03, Leu 0.98.

Moz-Asn-Ile-Phe-OBzl. R_{fA} 0.72, R_{fB} 0.87, R_{fC} 0.94; mp 194–197°C; ^{1}H NMR (DMSO-d_6), 0.52–0.93 (m, 6H), 1.22–1.95 (m, 3H), 2.38 (d, 2H), 2.98 (d, 2H), 3.68

(s, 3H), 3.93–4.77 (m, 2H), 4.92 (s, 2H), 4.98 (s, 2H), 6.85 (d, 2H, arom. ring, J = 8.0 Hz), 7.15 (5H, phenyl), 7.23 (s, 5H, phenyl), 8.40 (d, 1H). *Anal.* Calcd for $C_{35}H_{42}N_4O_8$: C, 64.70; N, 8.62; H, 6.52. Found: C, 63.29; N, 8.37; H, 6.37. Amino acid analysis: Asp 1.01, Ile 0.96, Phe 1.03.

ACKNOWLEDGMENTS

This work was supported by grants from CNPq, FAPESP, and FINEP. We thank Dr. Frank Quina for critically reading the manuscript.

REFERENCES

1. Bodansky, M. (1984) Principles of Peptide Synthesis, pp. 158–201, Springer-Verlag, Berlin.
2. Mojsov, S., Mitchell, A. R., and Merrifield, R. B. (1980) *J. Org. Chem.* **45,** 555.
3. Mukaiyama, T., Ueki, M., Maruyama, H., and Matsueda, R. (1968) *J. Amer. Chem. Soc.* **90,** 4490.
4. König, W., and Geiger, R. (1970) *Chem. Ber.* **103,** 788.
5. König, W., and Geiger, R. (1970) *Chem. Ber.* **103,** 2041.
6. Liberek, B. *Chem. Ind.* (1961) **26,** 987.
7. Zaoral, M., and Rudinger, J. (1959) *Collect. Czech. Chem. Commun.* **24,** 1993.
8. Morihara, K., and Tsuzuki, H. (1966) *Biochim. Biophys. Acta* **118,** 215.
9. Isowa, Y., Ichikawa, T., and Ohmori, M. (1978) *Bull. Chem. Soc. Japan* **51,** 271.
10. Kullmann, W. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 2840.
11. Fruton, J. S. (1983) *Trans. N.Y. Acad. Sci.* **41,** 49.
12. Oka, T., and Morihara, K. (1980) *J. Biochem.* **88,** 807.
13. Isowa, Y., Ohmori, M., Ichikawa, T., and Mori, K. (1979) *Tetrahedron Lett.* **28,** 2611.
14. Petkov, D. D., and Stoineva, I. B. (1984) *Tetrahedron Lett.* **25,** 3751.
15. Isowa, Y., and Ichikawa T. (1979) *Bull. Chem. Soc. Japan* **52,** 796.
16. Isowa, Y., Kakutani, M., and Yaguchi, M. (1982) Peptide Chemistry 1981 (Shioiri, T., ed.), pp. 25–30, Protein Research Foundation, Osaka.
17. Nagasawa, T., Kuroiwa, K., Narita, K., and Isowa, Y. (1973) *Bull. Chem. Soc. Japan* **46,** 1269.
18. Cipera, J. D., and Nicholls, R. V. V. (1955) *Chem. Ind. (London)*, 16.
19. Bodansky, M. (1955) *Nature (London)* **175,** 685.