The Enzymatic Synthesis of Protected Valine-5 Angiotensin II Amide-1

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An example of the use of proteolytic enzymes to facilitate the peptide synthesis by fragment condensation is provided for the preparation of protected valine-5 angiotensin II amide-1 using *t*-butoxycarbonylpeptides as a carboxyl component and a peptide ethyl ester as an amine component. The proteolytic enzymes used are papain, nagarse (subtilisin BPN') and microbial metalloenzyme isolated from *St. caespitosus*. Papain-catalyzed condensation reaction afforded the corresponding oligopeptide in the ester form, whereas nagarse and microbial metalloenzyme-catalyzed condensation reactions afforded the products in the carboxyl free form. Assignment of the product was made on the basis of a comparison of physical properties with those of the peptide prepared by the solution method.

Previous work in this series has shown that typical proteolytic enzymes, *e.g.*, papain, thermolysin, nagarse (subtilisin BPN') and pepsin, may be capable of catalyzing peptide bond formation between acylamino acids or acyldipeptides and amino acid or dipeptide esters.[1] The first instance of such a reaction was the amide bond formation between an acylamino acid and amino acid anilide in the presence of papain reported by Bergmann and Fraenkel-Conrat,[2] who also examined the effect of the peptide bond formation by chymotrypsin using an amino acid anilide as an amine component.[3,4] These observations indicate that the proteolytic enzymes catalyze the formation of small peptides such as di, tri, and tetrapeptides. However, since the enzymes essentially catalyze the hydrolysis of peptides, it is of interest to examine them with respect to their ability to catalyze the formation of oligopeptides without undesirable reactions.

This investigation was undertaken to provide a fundamental information on the enzymatic synthesis of oligopeptides using papain,[5] nagarse (subtilisin BPN')[6] and microbial metalloenzyme[7] as catalysts. For this purpose, the preparation of protected valine-5 angiotensin II amide-1 *via* the enzymatic fragment condensation was studied. This peptide hormon consists of eight amino acid residues having a L-configuration. Its constructional formula is as follows.

H-Asn[1]-Arg[2]-Val[3]-Tyr[4]-Val[5]-His[6]-Pro[7]-Phe[8]-OH

The octapeptide was first synthesized by Schwyzer and co-workers.[8] Several angiotensin peptides have since been prepared by means of solution or solid phase method.

It was demonstrated that the angiotensin undergoes cleavage at a tyrosyl residue on the carboxyl side by the action of chymotrypsin or chymotrypsin-like endopeptidases.[9,10] The peptide bond chosen for the fragment condensation was therefore Tyr[4]–Val[5] linkage in this synthetic procedure. The scheme for the preparation of N-terminal dipeptide and tetrapeptide used for carboxyl component is given in Fig. 1 and that of C-terminal tetrapeptide for amino component in Fig. 2.

In order to facilitate isolation the side-chain functional groups of Arg, Tyr, and His were protected by nitro or benzyl group.[11] As an amino protecting group for Arg(NO_2), His(BZL), and Pro, 2,4,6-trimethylbenzyloxycarbonyl (TMZ) group, which was readily

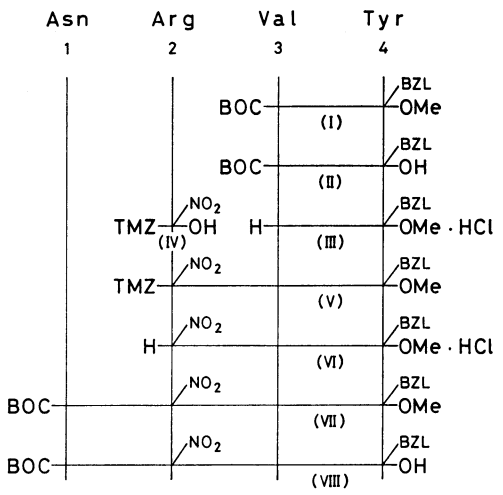

Fig. 1. Preparation of peptide substrates, II and VIII, as carboxyl component; prefix L for amino acids is omitted; BOC, *t*-butoxycarbonyl; TMZ, 2,4,6-trimethylbenzyloxycarbonyl; BZL, benzyl; Me, methyl.

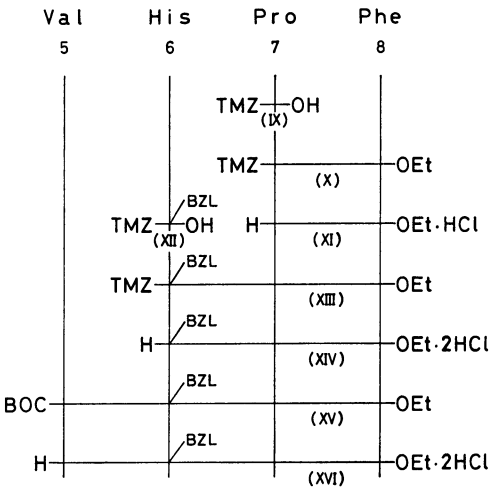

Fig. 2. Preparation of peptide substrate, XVI, as amine component; Et, ethyl.

introduced by the reaction with 2,4,6-trimethylbenzyl 2,4,5-trichlorophenyl carbonate by a modification of the *t*-butoxycarbonylation[12] and smoothly removed by acidolysis, was employed because of the fine crystallization of their peptide derivatives. In a comparison of

VI and XI with samples obtained from the corresponding BOC-derivatives, no detectable amount of optical inpurity was observed.

The reaction sequence for the preparation of the valine-5 angiotensin 3—8 fragment and the protected valine-5 angiotensin II amide-1 as authentic samples is shown in Fig. 3.

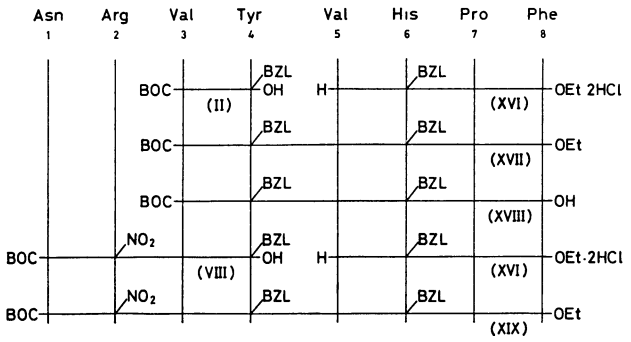

Fig. 3. Preparation of authentic samples, XVII, XVIII, and XIX, by solution method.

In all cases, the coupling reactions were carried out by means of HOBt/DCCI method,[13] the tertiary base being *N*-methylmorpholine, and the solvent *N,N*-dimethylformamide. The resulting products were isolated in the usual way. When necessary, the *t*-butoxycarbonyl or 2,4,6-trimethylbenzyloxycarbonyl group was removed by treatment with hydrogen chloride in ethyl acetate. Saponification of the peptide esters was carried out in methanol or *N,N*-dimethylformamide solution in the presence of sodium hydroxide at room temperature.

The enzymatic condensation reactions were carried out by incubating the substrates in buffer solutions of the pH comparable to those of the hydrolytic reactions, either in the absence or presence of methanol; the McIlvaine buffer of pH 5.5, 7.5 and Veronal buffer of pH 7.0 were employed for the papain, nagarse and microbial metalloenzyme-catalyzed reactions, respectively. When hydrochloride was used as an amine component, the required quantity of 1 M sodium hydroxide was added. Details of the composition of the reaction mixture and of the reaction conditions are given in Experimental.

All the products obtained in these reactions were identified by direct comparison of their melting point, optical rotation and R_f value of thin layer chromatography with those of samples prepared by the solution method (Fig. 3).

Results and Discussion

1) Syntheses of the Protected Valine-5 Angiotensin II 3-8 Fragment by Papain, Nagarse and Microbial Metalloenzyme-catalyzed Reaction. The possibility of the ploteolytic enzymes to catalyze the formation of oligopeptides was examined in the condensation of BOC–Val–Tyr(BZL)–OH (II) with H–Val–His(BZL)–Pro–Phe–OEt·2HCl (XVI). The results are summarized in Fig. 4.

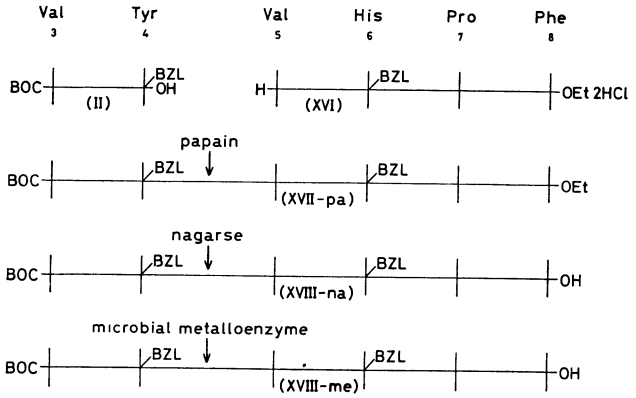

Fig. 4. Enzymatic condensation of BOC–Val–Tyr(BZL)–OH (II) with H–Val–His(BZL)–Pro–Phe–OEt·2HCl (XVI); suffix -pa, -na, and -me indicate the products obtained respectively by papain, nagarse, and microbial metalloenzyme-catalyzed reaction.

The papain-catalyzed reaction in McIlvaine buffer of pH 5.5 containing methanol (1/1, v/v) afforded the corresponding hexapeptide ethyl ester (XVII-pa) as the major product. The presence of all the amino acid residues constituting the hexapeptide and of the ethyl ester were determined by its NMR spectrum (see Experimental). Further structural elucidation was achieved by a comparison of physical properties with those of the peptide ester prepared by the HOBt/DCCI method (Table 1).

Table 1. Comparison of physical properties of XVII-pa with those of XVII prepared by solution method

		Type of condensation: Papain-catalyzed condensation (XVII-pa)	HOBt/DCCI (XVII)
Mp(°C)		167—173	164—173
$[\alpha]_D$ (c 1.0)	DMF	−36.0°	−35.3°
	MeOH	−58.5°	−59.2°
R_f (TLC)	system I	0.79	0.79
	system II	0.74	0.75

The observation that the protected hexapeptide ethyl ester (XVII-pa) is mainly obtained by the enzymatic reaction seems to be incompatible with the fact that papain possesses an esterase activity.[5,14] This is of considerable importance from a practical point of view.

The nagarse-catalyzed condensation reaction in McIlvaine buffer of pH 7.5 gave the corresponding hexapeptide in the carboxyl free form (XVIII-na). The melting point, optical rotation and R_f values of thin layer chromatography agree with those of XVIII obtained from the synthetic XVII by saponification (Table 2).

The result is in line with the fact that the serine proteinases catalyze the hydrolysis of ethyl or methyl ester linkage to a great extent.[15,16]

The microbial metalloenzyme-catalyzed reaction in Veronal buffer of pH 7.0 afforded the corresponding

Table 2. Comparison of physical properties of XVIII-na with those of XVIII prepared by saponification of the synthetic XVII

		Type of condensation	
		Nagarse-catalyzed condensation (XVIII-na)	Solution method (XVIII)
MP (°C)[a]		165—169	172—179
$[\alpha]_D$ (c 1.0)	DMF	−27.4°	−28.7°
R_f (TLC)	system I	0.41	0.41
	system II	0.64	0.63

a) Discrepancy in the melting points may be due to the water of crystallization; XVIII-na, $2H_2O$; synthetic XVIII, H_2O.

hexapeptide acid (XVIII-me). In spite of the fact that the microbial metalloenzymes are inactive against the ester substrates,[17,18] no measurable amount of the hexapeptide ester was detected in the condensation product. This might be attributed to an impurity in the microbial metalloenzyme employed.[7]

The investigations into the peptide bond forming ability of the proteolytic enzymes show that the protected hexapeptides can be enzymatically prepared by the fragment condensation of II with XVI without loss of amino acid residues. However, except in the case of the papain-catalyzed reaction, the hexapeptide was obtained in a carboxyl free form even when the condensation reactions were carried out in the presence of methanol.

2) Synthesis of the Protected Valine-5 Angiotensin II Amide-1 by the Papain-catalyzed Reaction. Since the papain-catalyzed reaction afforded the product in the form of the ethyl ester as described in the synthesis of hexapeptide, preparation of the protected valine-5 angiotensin II amide-1 was attempted with use of papain. The product obtained by the papain-catalyzed condensation of fragment 1—4 (VIII) with 5—8 (XVI) in a mixture of McIlvaine buffer of pH 5.5 and methanol (1/1, v/v) was the corresponding octapeptide ester (XIX-pa) containing two of the water of crystallization. Its structure was determined by means of elemental and NMR analysis.

BOC-Asn-Arg(NO_2)-Val-Tyr(BZL)-OH (VIII) + H-Val-His(BZL)-Pro-Phe-OEt (XVI)

→ (papain; in buffer/methanol=1/1 (v/v))

BOC-Asn-Arg(NO_2)-Val-Tyr(BZL)-Val-His(BZL)-Pro-Phe-OEt

Protected valine-5 angiotensin II amide-1 (XIX-pa)

The significant physical properties of XIX-pa were the same as those of XIX prepared by the solution method (Table 3). A small amount of the octapeptide acid was concomitantly obtained when the reaction was carried out in the absence of methanol. It seems to be reasonable to assume that the papain-catalyzed esterolysis is repressed by the addition of methanol to the reaction mixture.

This finding indicates that papain also catalyzes the octapeptide formation *via* fragment condensation without loss of amino acid residues. Furthermore, it is of interest to note that by the addition of methanol to the reaction mixture, the papain-catalyzed reaction affords the product in the form of ethyl ester.

Table 3. Comparison of physical properties of XIX-pa with those of XIX prepared by solution method

		Type of condensation	
		Papain-catalyzed condensation (XIX-pa)	HOBt/DCCI (XIX)
MP (°C)		186—195	189—195
$[\alpha]_D$ (c 1.0)	DMF	−27.3°	−26.5°
	MeOH	−48.3°	−48.4°
R_f (TLC)	system I	0.62	0.63
	system II	0.56	0.55

It is apparent that the proteolytic enzymes, papain, nagarse and microbial metalloenzyme, are capable of synthesizing oligopeptide by fragment condensation. The oligopeptide formation can be attributed to the insolubility of the products, which influences the equilibrium in favor of the peptide bond formation.

In view of the low solubility of the protected oligopeptides, the application of this enzymatic procedure, enzymatic procedure, along with a combination of the solution method, promises a new possibility of facile preparation of the peptide.

Experimental

The melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. The optical rotations were measured with a Yanagimoto automatic polarimeter OR-50 type. The NMR spectra were recorded on a Varian HA-100 High Resolution NMR spectrometer, using tetramethylsilane as an internal or external standard. The thin layer chromatography was carried out on precorted plates of silica gel G (E. Merck). The developing solvents commonly used were 2-butanol–3% ammonia water (8: 3, v/v) (system I) and 1-butanol–acetic acid–water (4: 1: 5, v/v) (system II).

Enzymes. All the proteolytic enzymes used in this investigation were commercial preparations; papain (Midori Juji Co., E-2 1200 U/g); nagarse (Nagase Sangyo Co., 50×10^4 PUN/g); microbial metalloenzyme *(St. caespitosus)* (Kyowa Hakko Kogyo Co.). The proteolytic activities of the papain, nagarse and microbial metalloenzyme, assayed by the casein digestion method of Tsuru, Yamamoto, and Fukumoto[19] with the exception that the papain was preincubated with cysteine, were 2.20×10^5, 1.15×10^6 and 2.64×10^5 PU/g, respectively. The microbial metalloenzyme seems to be contaminated with alkaline proteinases, since the proteolytic activity is inhibited by not only EDTA but also diisopropyl fluorophosphate.[7]

BOC-Val-Tyr(BZL)-OMe (I). A mixture of BOC–Val–OH (10.9 g, 50 mmol) and H–Tyr(BZL)-OMe·HCl (16.1 g, 50 mmol) in 120 ml of *N,N*-dimethylformamide was cooled at −5 °C with stirring. To the chilled solution were added *N*-methylmorpholine (5.1 g, 50 mmol) and 1-hydroxybenzotriazole (6.8 g, 50 mmol), followed by a solution of dicyclohexylcarbodiimide (10.7 g, 52 mmol) in 50 ml of *N,N*-dimethylformamide. Stirring was continued at the same temperature

for 1 h and then at room temperature for 8 h. After removal of the urea by filtration the filtrate was evaporated to dryness *in vacuo* and the residue was treated with water. The solid product was collected by filtration and washed with 5% sodium hydrogencarbonate, water, 1 M hydrochloric acid and water. Recrystallization twice from methanol–water yielded 20.3 g (83.7%) of I; mp 114—116 °C; $[\alpha]_D$ −13.0° (*c* 1.0, methanol).

Found: C, 67.17; H, 7.31; N, 5.79%. Calcd for $C_{27}H_{36}N_2O_6$: C, 66.92; H, 7.49; N, 5.78%.

BOC-Val-Tyr(BZL)-OH (II). A solution of I (14.5 g, 30 mmol) in methanol was treated with 1 M sodium hydroxide (33 ml) at room temperature for 2 h. After removal of methanol *in vacuo*, the aqueous solution was acidified with 2 M hydrochloric acid under ice-cooling. The precipitate was collected by filtration and washed with water, and then recrystallized from methanol–water; 11.6 g (82.2%); mp 142—145 °C; $[\alpha]_D$ +0.2° (*c* 1.0, methanol). A sample was further recrystallized from a small volume of methanol for analysis; mp 143—145 °C.

Found: C, 66.51; H, 7.01; N, 6.07%. Calcd for $C_{26}H_{34}N_2O_6$: C, 66.36; H, 7.28 N, 5.95%.

H-Val-Tyr(BZL)-OMe·HCl (III). Eighty milliliter of 5 M hydrogen chloride in ethyl acetate was added to a suspension of I (9.69 g, 20 mmol) in 40 ml of ethyl acetate. The substance dissolved immediately to form a clear solution. After being left to stand for 2 h at room temperature, the solution was evaporated to dryness *in vacuo*. The residue was crystallized by addition of ether; 8.10 g (96.2%); mp 175—179 °C; $[\alpha]_D$ +29.2° (*c* 1.0, methanol).

Found: C, 62.94; H, 6.82; N, 6.45%. Calcd for $C_{22}H_{29}N_2O_4Cl$: C, 62.77; H, 6.94; N, 6.66%.

2,4,6-Trimethylbenzyl 2,4,5-Trichlorophenyl Carbonate. 2,4,6-Trimethylbenzyl alcohol (26.2 g, 174 mmol) was allowed to react with 2,4,5-trichlorophenyl chloroformate (45.2 g, 174 mmol) in 200 ml of dichloromethane in the presence of quinoline (23.2 g, 180 mmol), by the same procedure as that for the preparation of *t*-butyl 2,4,5-trichlorophenyl carbonate.[12] Recrystallization from ligroine yielded colorless needles of the carbonate; 43.0 g (66.1%); mp 113—115 °C.

Found: C, 54.41; H, 4.18; Cl, 28.73%. Calcd for $C_{17}H_{15}O_3Cl_3$: C, 54.64; H, 4.05; Cl, 28.47%.

TMZ-Arg(NO$_2$)-OH (IV). Twenty-eight milliliter of 40% benzyltrimethylammonium hydrxide was added to a suspension of H–Arg(NO$_2$)–OH (10.6 g, 48.5 mmol) in 100 ml of methanol and the resulting mixture was stirred at 55—60 °C until solution was complete (2 h). The solvent was removed *in vacuo* and replaced by a mixture of 200 ml of dioxane and 50 ml of water. To the solution obtained was added 2,4,6-trimethylbenzyl 2,4,5-trichlorophenyl carbonate (18.7 g, 50.0 mmol) and the solution was stirred for 6 h at 55 °C. After removal of the solvent *in vacuo*, the residue was taken up in 250 ml of water and the aqueous solution was washed three times with 100 ml of ether. IV was precipitated by bringing the pH of the solution to 4—5 with citric acid. The crude material was collected, washed with water, and recrystallized from ethanol–ether; 17.2 g (89.7%); mp 176—178 °C; $[\alpha]_D$ +20.7° (*c* 1.0, methanol) and +0.8° (*c* 1.0, tetrahydrofuran).

Found: C, 51.52; H, 6.36; N, 17.48%. Calcd for $C_{17}H_{25}N_5O_6$: C, 51.63; H, 6.37; N, 17.71%.

TMZ-Arg(NO$_2$)-Val-Tyr(BZL)-OMe (V). Condensation of III (4.21 g, 10 mmol) with IV (3.95 g, 10 mmol) was carried out as described for I. Recrystallization of the product twice from methanol–water yielded V as a colorless powder; 5.97 g (78.4%); mp 191—192 °C; $[\alpha]_D$ +3.0° (*c* 1.0, *N,N*-dimethylformamide).

Found: C, 61.33; H, 6.67; N, 12.80%. Calcd for $C_{39}H_{51}N_7O_9$: C, 61.48; H, 6.75; N, 12.87%.

H-Arg(NO$_2$)-Val-Tyr(BZL)·OMe·HCl (VI). Deprotection of V (5.20 g, 6.83 mmol) with 5 M hydrogen chloride in ethyl acetate was carried out in the way as in the preparation of III. The solid product obtained on evaporation was recrystallized from methanol–ether; 4.16 g (98.0%); mp 220—223 °C; $[\alpha]_D$ +25.7° (*c* 1.0, methanol); thin layer chromatography, R_f 0.59 (system I).

Found: C, 53.95; H, 6.43; N, 15.82%. Calcd for $C_{28}H_{40}N_7O_7Cl$: C, 54.05; H, 6.48; N, 15.76%.

This compound was found to be identical with that obtained from the corresponding BOC-derivative (amorphous powder) by comparison of the melting point, optical rotation and R_f value of thin layer chromatography: VI obtained from BOC-derivative; mp 219—223 °C, no depression on admixture with above product; $[\alpha]_D$ +25.4° (*c* 1.0, methanol): thin layer chromatography of a mixed sample, R_f 0.60 (system I).

BOC-Asn-Arg(NO$_2$)-Val-Tyr(BZL)-OMe (VII). To a chilled (−5 °C) solution of BOC–Asn–OH (5.8 g, 25 mmol) and VI (15.6 g, 25 mmol) in 120 ml of *N,N*-dimethylformamide were added *N*-methylmorpholine (2.5 g, 25 mmol) and 1-hydroxybenzotriazole (3.4 g, 25 mmol), followed by dicyclohexylcarbodiimide (5.4 g, 26 mmol) in 20 ml of *N,N*-dimethylformamide. The mixture was stirred at −5 °C for 2 h and then at room temperature for 10 h. The mixture, after removal of urea, was evaporated to dryness *in vacuo* and the residue was treated with 80 ml of water. The resulting solid product was collected by filtration and washed with 5% sodium hydrogencarbonate, water, 1 M hydrochloric acid and water. Recrystallization from *N,N*-dimethylformamide—water gave 16.9 g (85.5%) of VII, mp 198—201 °C. For analysis, a small amount of the product was further recrystallized from *N,N*-dimethylformamide–methanol–water; mp 201—203 °C; $[\alpha]_D$ −5.2° (*c* 1.0, *N,N*-dimethylformamide).

Found: C, 55.80; H, 6.41; N, 15.59%. Calcd for $C_{37}H_{53}N_9O_{11}$: C, 55.56; H, 6.68; N, 15.76%.

BOC-Asn-Arg(NO$_2$)-Val-Tyr(BZL)-OH (VIII). To a stirred solution of VII (1.0 g, 1.25 mmol) in 20 ml of *N,N*-dimethylformamide was added dropwise 0.1 M sodium hydroxide until the pH became 11. The solution was maintained at this pH by gradual addition of sodium hydroxide over a period of 1 h (the total amount of sodium hydroxide required was 30 ml). After stirring for 2 h at room temperature, 4 ml of 1 M hydrochloric acid was added with stirring over 15 min under ice-cooling. The mixture was concentrated *in vacuo* nearly to dryness and the residue was triturated with water. The resulting solid product was collected by filtration, washed thoroughly with water, and recrystallized from ethanol–water; 0.87 g (88.6%); mp 167—170 °C; $[\alpha]_D$ −5.0° (*c* 0.5, methanol); thin layer chromatography, R_f 0.57 (system I) and 0.69 (system II).

Found: C, 54.82; H, 6.49; N, 16.04%. Calcd for $C_{36}H_{51}N_9O_{11}$: C, 55.02; H, 6.54; N, 16.04%.

TMZ-Pro-OH (IX). The 2,4,6-trimethylbenzyloxycarbonylation of H–Pro–OH (11.5 g, 100 mmol) with 2,4,6-trimethylbenzyl 2,4,5-trichlorophenyl carbonate (39.2 g, 105 mmol) was carried out as described for IV. The oily material which separated on acidification of the aqueous solution was extracted with 400 ml of ethyl acetate, the ethyl acetate solution being washed with water and dried over sodium sulfate. Removal of solvent gave 28.6 g (98.2%) of IX as a colorless viscous oil; $[\alpha]_D$ −31.4° (*c* 2.03, methanol). The homogeneity of this product was established by thin layer chromatography, R_f 0.47 (system I) and 0.79 (system II). The oily product was further characterized by the preparation of the corresponding dicyclohexylamine salt; mp 165—168 °C; $[\alpha]_D$ −20.0° (*c* 1.0, methanol).

Found: C, 71.32; H, 9.54; N, 5.82%. Calcd for $C_{25}H_{44}N_2O_4$: C, 71.15; H, 9.38; N, 5.93%.

TMZ-Pro-Phe-OEt (X). Condensation of IX (23.6 g, 81 mmol) with H-Phe-OEt·HCl (18.6 g, 81 mmol) in the presence of *N*-methylmorpholine (8.2 g, 81 mmol), 1-hydroxybenzotriazole (10.9 g, 81 mmol) and dicyclohexylcarbodiimide (17.1 g, 83 mmol) was carred out as described for I. The product was recrystallized twice from ethyl acetate–hexane; 34.5 g (85.9%); mp 97—99 °C; $[\alpha]_D$ −46.0° (*c* 1.0, ethanol).

Found: C, 69.56; H, 7.13; N, 5.95%. Calcd for $C_{27}H_{34}N_2O_5$: C, 69.50; H, 7.35; N, 6.01%.

H-Pro-Phe-OEt·HCl (XI). X (10.0 g, 21 mmol) was treated with 5 M hydrogen chloride in ethyl acetate by the same procedure as for III. The product obtained on evaporation of the reaction mixture was recrystallized from ethanol–ether; 6.51 g (94.9%); mp 151—153 °C; $[\alpha]_D$ −35.0° (*c* 1.0, ethanol).

Found: C, 58.77; H, 7.10; N, 8.62%. Calcd for $C_{16}H_{23}N_2O_3Cl$: C, 58.80; H, 7.09; N, 8.57%.

This compound was found to be identical with that obtained from BOC-Pro-Phe-OEt (oily material) by comparison of melting point, optical rotation and thin layer chromatography: XI obtained from BOC-derivative; mp 151—153 °C, no depression on admixture with a sample prepared above; $[\alpha]_D$ −35.1° (*c* 1.0, ethanol); thin layer chromatography of a mixed sample gave a single spot, R_f 0.68 (system I).

TMZ-His(BZL)-OH (XII). H-His(BZL)-OH (12.3 g, 50 mmol) was allowed to react with 2,4,6-trimethylbenzyl 2,4,5-trichlorophenyl carbonate (19.4 g, 52 mmol) as in the preparation of IV. The product was recrystallized from methanol–water; 17.8 g (84.5%); mp 181—183 °C; $[\alpha]_D$ +34.2° (*c* 1.0, methanol) and +12.0° (*c* 1.0, *N,N*-dimethylformamide).

Found: C, 68.49; H, 6.40; N, 9.98%. Calcd for $C_{24}H_{27}N_3O_4$: C, 68.39; H, 6.46; N, 9.97%.

TMZ-His(BZL)-Pro-Phe-OEt (XIII). A solution of XII (13.1 g, 31.0 mmol) and XI (10.0 g, 30.6 mmol) in 80 ml of *N,N*-dimethylformamide was cooled at −5 °C with stirring. To this solution were added *N*-methylmorpholine (3.10 g, 30.6 mmol) and 1-hydroxybenzotriazole (4.13 g, 30.6 mmol), followed by a solution of dicyclohexylcarbodiimide (6.40 g, 31.0 mmol) in 30 ml of *N,N*-dimethylformamide. Stirring was continued for 2 h at this temperature and 8 h at room temperature. After the urea was filtered off, the filtrate was evaporated *in vacuo* and the residue was taken up in 300 ml of ethyl acetate. The ethyl acetate solution was washed with 7% aqueous ammonia and water, dried over sodium sulfate, and then evaporated to dryness *in vacuo*. The resulting semi-solid product was purified by dissolution in 200 ml of hot ethyl acetate, treated with charcoal, and concentration to a small volume followed by the addition of 100 ml of petroleum ether to yield XIII as an amorphous powder; 20.8 g (97.8%); $[\alpha]_D$ −37.2° (*c* 1.0, methanol). The homogeneity was confirmed by thin layer chromatography; R_f 0.84 (system I) and 0.72 (system II).

Found: C, 68.81; H, 6.68; N, 10.08%. Calcd for $C_{40}H_{47}N_5O_6$: C, 69.24; H, 6.83; N, 10.09%.

All attempts to crystallize the product were unsuccessful. It was thus used for the reaction without further purification.

H-His(BZL)-Pro-Phe-OEt·2HCl (XIV). Fifty milliter of 5 M hydrogen chloride in ethyl acetate was added to a solution of XIII (8.20 g, 11.8 mmol) in 25 ml of ethyl acetate. After being left to stand for 2 h at room temperature, the solution was evaporated *in vacuo* to afford a solid product which was collected by filtration with the aid of ether and washed thoroughly with ether. Recrystallization from ethanol gave colorless needles of XIV; 6.62 g (95.1%); mp 215—218 °C; $[\alpha]_D$ −6.3° (*c* 1.0, methanol).

Found: C, 58.88; H, 6.36; N, 11.89%. Calcd for $C_{29}H_{37}N_5O_4Cl_2$: C, 59.08; H, 6.33; N, 11.88%.

BOC-Val-His(BZL)-Pro-Phe-OEt·2H₂O (XV). BOC-Val-OH (3.74 g, 17.2 mmol) was coupled with XIV (9.98 g, 16.9 mmol) in the presence of *N*-methylmorpholine (3.42 g, 33.8 mmol), 1-hydroxybenzotriazole (2.28 g, 16.9 mmol) and dicyclohexylcarbodiimide (3.51 g, 17.0 mmol) by the same procedure as for XIII. Removal of urea by filtration followed by concentration of the filtrate *in vacuo* gave an oily residue, which was taken up in 200 ml of ethyl acetate. The ethyl acetate solution, after being washed with 7% aqueous ammonia, was dried over sodium sulfate and evaporated to dryness *in vacuo*. Trituration of the residue with a mixture of ether and petroleum ether afforded a solid material. Recrystallization from ethanol–ether gave XV as an amorphous powder with two molecules of the water of crystallization; 11.2 g (88.0%); mp 111—116 °C; $[\alpha]_D$ −36.0° (*c* 1.0, methanol); thin layer chromatography, R_f 0.82 (system I) and 0.70 (system II).

Found: C, 61.97; H, 7.25; N, 11.24%. Calcd for $C_{39}H_{52}N_6O_7\cdot 2H_2O$: C, 62.21; H, 7.49; N, 11.16%.

H-Val-His(BZL)-Pro-Phe-OEt·2HCl (XVI). A suspension of XV (9.03 g, 12.0 mmol) in 40 ml of ethyl acetate was treated with 96 ml of 5 M hydrogen chloride in ethyl acetate. After being left to stand for 2 h at room temperature the solution was evaporated *in vacuo* and the residue was taken up in 150 ml of water. The aqueous layer was washed three times with 50 ml of ethyl acetate, followed by treatment with charcoal. Removal of the solvent by azeotropic distillation *in vacuo* gave an oily material. This was solidified by triturating with ethanol–ether and recrystallized from the same solvent; 7.40 g (89.4%); mp 158—162 °C; $[\alpha]_D$ −15.9° (*c* 1.0, methanol) and −10.4° (*c* 1.0, ethanol).

Found: C, 58.96; H, 6.98; N, 12.33%. Calcd for $C_{34}H_{46}N_6O_5Cl_2$: C, 59.21; H, 6.72; N, 12.19%.

BOC-Val-Tyr(BZL)-Val-His(BZL)-Pro-Phe-OEt·1/2 H₂O (XVII) as an Authentic Sample. To a cooled solution (−5 °C) of II (2.35 g, 5.00 mmol) and XVI (3.45 g, 5.00 mmol) in 50 ml of *N,N*-dimethylformamide were added *N*-methylmorpholine (1.01 g, 10.0 mmol) and 1-hydroxybenzotriazole (0.68 g, 5.00 mmol) with stirring, followed by a solution of dicyclohexylcarbodiimide (1.05 g, 5.10 mmol) in 5 ml of *N,N*-dimethylformamide. The mixture was stirred for 2 h at −5 °C and for 36 h at room temperature. Removal of urea by filtration followed by concentration of the filtrate *in vacuo* gave a solid material, which was collected by suction with water, washed with 7% aqueous ammonia and water, and then dissolved in 20 ml of a mixture of *N,N*-dimethylformamide and water (5: 1, v/v). A small amount of insoluble substance was removed by filtration, the filtrate being passed through a column of Sephadex LH-20. The effluent was evaporated to dryness *in vacuo*, and the product was suspended in water, collected by filtration, and dried. Recrystallization twice from methanol–water gave XVII as a colorless powder; 3.89 g (71.5%); mp 164—173 °C; $[\alpha]_D$ −59.2° (*c* 1.0, methanol) and −35.3° (*c* 1.0, *N,N*-dimethylformamide). The homogeneity of this product was confirmed by thin layer chromatography, R_f 0.79 (system I) and 0.75 (system II). Prior to analysis, the compound was dried over phosphorus pentaoxide at 70 °C and 2 mmHg.

Found: C, 66.77; H, 7.09; N, 10.32%. Calcd for $C_{60}H_{76}N_8O_{10}\cdot 1/2\ H_2O$: C, 66.82; H, 7.19; N, 10.39%.

BOC-Val-Tyr(BZL)-Val-His(BZL)-Pro-Phe-OH·H₂O (XVIII) as an Authentic Sample. To a stirred solution of XVII (2.17 g, 2.0 mmol) in 40 ml of ethanol was added 48 ml of 0.1 M sodium hydroxide dropwise at room temperature

over a period of 1 h. After being stirred for 2 h the reaction mixture was neutralized with Dry Ice according to the method of Schwyzer, *et al.*,[20] and evaporated to a small volume *in vacuo*. The residue obtained was taken up in 40 ml of water and the solution was acidified with citric acid to yield a solid product. Recrystallization from ethanol gave XVIII as colorless needles with one molecule of the water of crystallization; 1.29 g (62.3%); mp 172—179 °C; $[\alpha]_D$ −28.7° (*c* 1.0, *N,N*-dimethylformamide); thin layer chromatography, R_f 0.41 (system I) and 0.63 (system II).

Found: C, 65.86; H, 6.92; N, 10.69%. Calcd for $C_{58}H_{72}N_8O_{10}\cdot H_2O$: C, 65.76; H, 7.04; N, 10.58%.

BOC-Asn-Arg(NO_2)-Val-Tyr(BZL)-Val-His(BZL)-Pro-Phe-OEt·2H_2O(XIX) as an Authentic Sample. *N*-Methylmorpholine (2.02 g, 20 mmol) and 1-hydroxybenzotriazole (1.35 g, 10 mmol) were added with stirring to a cooled (−5 °C) solution of VIII (7.86 g, 10 mmol) and XVI (6.70 g, 10 mmol) in 80 ml of *N,N*-dimethylformamide. To this was added in one portion a solution of dicyclohexylcarbodiimide (2.27 g, 11 mmol) in 10 ml of *N,N*-dimethylformamide. After being stirred for 48 h at room temperature, the reaction mixture was worked up by the same procedure for XVII. Recrystallization from ethanol–water gave XIX as a colorless powder with two molecules of the water of crystallization; 10.6 g (74.3%); mp 189—195 °C; $[\alpha]_D$ −48.4° (*c* 1.0, methanol) and −26.5° (*c* 1.0, *N,N*-dimethylformamide). Only one spot was obtained in thin layer chromatography, R_f 0.63 (system I) and 0.55 (system II).

Found: C, 59.07; H, 6.72; N, 14.75%. Calcd for $C_{70}H_{93}N_{15}O_{15}\cdot 2H_2O$: C, 59.17; H, 6.88; N, 14.79%.

Enzymatic Syntheses of the Protected Valine-5 Angiotensin II 3—8 Fragment. *i) Papain-catalyzed Condensation of II with XVI (XVII-pa)*: Papain (150 mg) and 0.1 ml of 2-mercaptoethanol were added to a solution of II (471 mg, 1.0 mmol) and XVI (690 mg, 1.0 mmol) in a mixture of 15 ml of McIlvaine buffer (pH 5.5) and 15 ml of methanol containing 2 ml of 1 M sodium hydroxide. After being incubated at 38 °C for 2 h, the product precipitated was collected by filtration, washed with 7% aqueous ammonia, 2% citric acid and water, and then dried. Recrystallization from methanol–water gave the hexapeptide ester (XVII-pa) as colorless crystals with a half molecule of the water of crystallization; 615 mg (57.0%); mp 167—173 °C, no depression on admixture with XVII prepared by the solution method; $[\alpha]_D$ −36.0° (*c* 1.0, *N,N*-dimethylformamide) and −58.5° (*c* 1.0, methanol). Only one spot was obtained in thin layer chromatography, R_f 0.79 (system I) and 0.74 (system II).

Found: C, 66.72; H, 7.16; N, 10.55%. Calcd for $C_{60}H_{76}N_8O_{10}\cdot 1/2\ H_2O$: C, 66.82; H, 7.19; N, 10.39%.

The NMR spectrum in DMSO-d_6 showed the following signals in ppm: 0.74 (12H; $-CH_3$ of Val), 1.05 (3H: $-CH_3$ of OEt), 1.36 (9H; $-CH_3$ of BOC), 1.75 (4H; β,γ-CH_2- of Pro), 1.91 (2H; β-CH- of Val), 2.78—3.28 (8H; δ-CH_2- of Pro and β-CH_2- of Phe, His and Tyr), 3.92 (2H; $-CH_2-$ of OEt), 4.98—5.05 (4H; $-CH_2-$ of BZL in His and Tyr), 6.79 and 7.02 (4H; $-C_6H_4-$ of Tyr), and 7.18—7.34 (15H; $-C_6H_5$ of Phe and BZL in His and Tyr).

ii) Nagarse-catalyzed Condensation of II with XVI (XVIII-na): Nagarse (40 mg) was added to a suspension of II (282 mg, 0.60 mmol) and XVI (414 mg, 0.60 mmol) in 8.0 ml of McIlvaine buffer of pH 7.5 containing 1.2 ml of 1 M sodium hydroxide. After being incubated at 38 °C for 6 h, the product precipitated was collected by filtration, washed with 2% citric acid and water, and then dried. The product was purified by solution in 40 ml of hot ethanol, treatment with charcoal, and concentration into a small volume, followed by the addition of ethyl acetate which gave the hexapeptide acid (XVIII-na) as a colorless powder with two molecules of the water of crystallization; 476 mg (73.7%); mp 165—169 °C; $[\alpha]_D$ −27.4° (*c* 1.0, *N,N*-dimethylformamide); thin layer chromatography, R_f 0.41 (system I) and 0.64 (system II).

Found: C, 64.87; H, 6.90; N, 10.52%. Calcd for $C_{58}H_{72}N_8O_{10}\cdot 2H_2O$: C, 64.66; H, 7.11; N, 10.40%.

The NMR spectrum of XVIII-na in DMSO-d_6 showed the following signals in ppm: 0.75 (12H; $-CH_3$ of Val), 1.36 (9H; $-CH_3$ of BOC), 1.69 (4H; β,γ-CH_2- of Pro), 1.92 (2H; β-CH- of Val), 2.80—3.11 (8H; δ-CH_2- of Pro and β-CH_2- of Phe, His and Tyr), 4.97 and 5.07 (4H; $-CH_2-$ of BZL in His and Tyr), 6.79 and 7.01 (4H; $-C_6H_4-$ of Tyr), and 7.18—7.33 (15H; $-C_6H_5$ of Phe and BZL in His and Tyr). No signals of ethyl ester were observed in this spectrum.

iii) Microbial Metalloenzyme-catalyzed Condensation of II with XVI (XVIII-me): Microbial metalloenzyme (50 mg) was added to a suspension of II (235 mg, 0.50 mmol) and XVI (345 mg, 0.50 mmol) in 8.0 ml of Veronal buffer of pH 7.0 containing 1.0 ml of 1 M sodium hydroxide. After being incubated at 38 °C for 6 h, the reaction mixture was worked up in the same way as for XVIII-na. XVIII-me was obtained as a colorless powder with two molecules of the water of crystallization; 222 mg (41.2%); mp 164—169 °C, no depression on admixture with XVIII-na; $[\alpha]_D$ −27.9° (*c* 0.5, *N,N*-dimethylformamide); thin layer chromatography, R_f 0.42 (system I) and 0.65 (system II).

Found: C, 64.89; H, 7.01; N, 10.32%. Calcd for $C_{58}H_{72}N_8O_{10}\cdot 2H_2O$: C, 64.66; H, 7.11; N, 10.40%.

The NMR spectral data of XVIII-me were consistent with those of XVIII-na.

Enzymatic Synthesis of the Protected Valine-5 Angiotensin II Amide-1. Papain-catalyzed Condensation of VIII with XVI (XIX-pa): Papain (100 mg) and 0.07 ml of 2-mercaptoethanol were added to a solution of VIII (472 mg, 0.60 mmol) and XVI (414 mg, 0.60 mmol) in a mixture of 10 ml of McIlvaine buffer (pH 5.5) and 10 ml of methanol containing 1.2 ml of 1 M sodium hydroxide. After being incubated at 38 °C for 2 h, the product precipitated was collected by filtration, washed 7% with aqueous ammonia, 2% citric acid and water. Recrystallization carried out by evaporating an ethanol solution of the product gave a colorless powder of the octapeptide ester (XIX-pa) with two molecules of the water of crystallization; 685 mg (78.1%); mp 186—195 °C, no depression on admixture with XIX prepared by solution method; $[\alpha]_D$ −27.3° (*c* 1.0, *N,N*-dimethylformamide) and −48.3° (*c* 1.0, methanol). Only one spot was obtained in thin layer chromatography, R_f 0.62 (system I) and 0.56 (system II).

Found: C, 59.11; H, 6.62; N. 14.92%. Calcd for $C_{70}H_{93}N_{15}O_{15}\cdot 2H_2O$: C, 59.17; H, 6.88; N, 14.79%.

The NMR spectrum of XIX-pa in DMSO-d_6 showed following signals in ppm: 0.75 (12H; $-CH_3$ of Val), 1.08 (3H; $-CH_3$ of OEt), 1.36 (9H; $-CH_3$ of BOC), 1.50—1.80 (8H; β,γ-CH_2- of Pro and Arg), 3.45 (2H; δ-CH_2- of Arg), 3.92 (2H; $-CH_2-$ of OEt), 4.98—5.21 (4H; $-CH_2-$ of BZL in His and Tyr), 6.80 and 7.12 (4H, $-C_6H_4-$ of Tyr), and 7.20—7.32 (15H; $-C_6H_5$ of Phe, and BZL in His and Tyr).

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