Synthesis of *cyclo*-Diglycyl-L-tyrosyl-diglycyl-L-tyrosyl and Hydrolysis by Chymotrypsin

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A cyclic hexapeptide, *cyclo*-(diglycyl-L-tyrosyl)$_2$, was synthesized by several different routes; the cyclization reactions of a tripeptide active ester, a tripeptide azide, and a hexapeptide azide produced the desired cyclic hexapeptide. The cyclic peptide was hydrolyzed slowly by chymotrypsin to a tripeptide diglycyl-tyrosine, whereas a synthetic linear hexapeptide, diglycyl-L-tyrosyl-diglycyl-L-tyrosine, was hydrolyzed very rapidly to the tripeptide. Since the synthetic *cyclo*-(diglycyl-L-phenylalanyl)$_2$ was insoluble in a buffer, it was not clear whether an incubation mixture of the substrate and enzyme yielded diglycyl-phenylalanine.

For a study of the mode of action of proteolytic enzymes on cyclic peptides, several compounds have been synthesized in this laboratory. In a previous paper, it was shown that *cyclo*-(Gly$_5$-Lys)[3] was completely hydrolyzed to H-Gly$_5$-Lys-OH by trypsin.[4] It was also observed that *cyclo*-(Gly$_2$-Lys)$_2$ was hydrolyzed to H-Gly$_2$-Lys-OH by trypsin though the rate of hydrolysis was extremely slow as compared with that of H-(Gly$_2$-Lys)$_2$-OH.[5] It is known that chymotrypsin hydrolyzes predominantly a peptide bond at the linkage of phenylalanine and tyrosine carbonyl.[6,7] Therefore, it was expected that a cyclic peptide containing Phe or Tyr residue might be hydrolyzed by chymotrypsin.

The present paper describes the syntheses of *cyclo*-(Gly$_2$-Tyr)$_2$ and a linear hexapeptide, H-(Gly$_2$-Tyr)$_2$-OH, and the mode of action of chymotrypsin on these peptides.

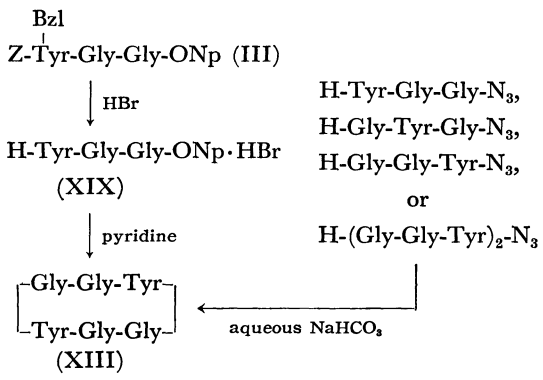

Fig. 1. Synthesis of the cyclic hexapeptide by various reaction sequences.

Five different routes shown in Fig. 1 were undertaken for the synthesis of the cyclic hexapeptide (XIII). In one route, XIII was synthesized *via* a linear tripeptide active ester (XIX), in which Tyr residue occupies the *N*-terminus of a peptide sequence. XIX was treated with a large amount of pyridine, and a pure cyclic hexapeptide was isolated easily though the yield was only 9%. The product was proved to be a dimer by molecular weight determination.

The cyclic peptide was also synthesized *via* linear tripeptide azides, in which Tyr residue occupies three different positions (Fig. 1). Crude cyclic peptide was isolated after the cyclization reaction of the azide in a large amount of water containing sodium bicarbonate. A linear hexapeptide azide was also subjected to the cyclization reaction (Fig. 1). Although the synthetic procedure in azide method to yield a crude cyclic peptide was simpler than that in an active ester method, treatment by a Sephadex LH-20 column with crude cyclic peptide was necessary to isolate a pure cyclic hexapeptide.

TABLE 1. YIELD AND SPECIFIC ROTATION OF CYCLIC HEXAPEPTIDES OBTAINED BY VARIOUS REACTIONS

Starting compound	*cyclo*-(Gly$_2$-Tyr)$_2$[a] Yield, %	$[\alpha]_D^{20}$[b]
Bzl Z-Tyr-Gly$_2$-ONp	9	−63.2°
H-Tyr-Gly$_2$-NHNH$_2$	6	−63.0°
H-Gly-Tyr-Gly-NHNH$_2$	17	−63.5°
H-Gly$_2$-Tyr-NHNH$_2$	15	−62.5°
H-(Gly$_2$-Tyr)$_2$NHNH$_2$	20	−63.1°

a) All samples showed only one spot (R_f 0.80) on the paper chromatogram, and the same mp (decomp.) of 250—255°C. All air-dried samples were assigned to cyclic hexapeptide trihydrate by elemental analyses and molecular weight determinations within experimental errors.
b) *c* 0.2, DMF.

Yields of the cyclic hexapeptide by different routes are summarized in Table 1. It appears that the position of Tyr residue in a tripeptide active ester or azide has an influence on the yield of the cyclic hexapeptide isolated; a lower yield was observed when a bulky Tyr residue occupies *N*-terminus in a tripeptide intermediate.

An incubation mixture of the cyclic hexapeptide (XIII) was analyzed by paper chromatography to check how the susceptibility of XIII toward chymo-

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3) Abbreviation: Z, benzyloxycarbonyl; ONp, *p*-nitrophenyl ester; TsOH, *p*-toluenesulfonic acid; TEA, triethylamine; DMF, dimethylformamide; Tyr (Bzl), *O*-benzyl-L-tyrosine residue. Amino acid symbol except Gly denotes L configuration.
4) M. Ohno and N. Izumiya, This Bulletin, **38**, 1831 (1965).
5) O. Abe, H. Takiguchi, M. Ohno, S. Makisumi, and N. Izumiya, *ibid.*, **40**, 1945 (1967).
6) H. Neurath and G. W. Schwert, *Chem. Rev.*, **46**, 69 (1950).
7) N. Yoshida, T. Yamamoto, and N. Izumiya, *Arch. Biochem. Biophys.*, **123**, 165 (1968).

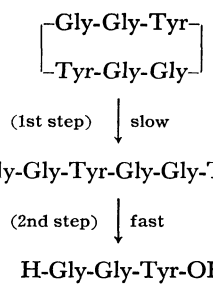

Fig. 2. Hydrolysis of the cyclic hexapeptide by chymotrypsin.

trypsin. It was observed that XIII was completely hydrolyzed to the tripeptide, H-Gly$_2$-Tyr-OH, after some 6 hr (see Fig. 4). A synthetic linear hexapeptide H-(Gly$_2$-Tyr)$_2$-OH was also subjected to an enzymatic experiment. The rate of its hydrolysis to the tripeptide was much greater than that of XIII;[8] the linear hexapeptide was completely hydrolyzed after 10 min with the same amount of enzyme used for the cyclic hexapeptide (see Fig. 5). Thus it was deduced that the hydrolysis of the cyclic hexapeptide by the enzyme might proceed in the two steps shown in Fig. 2; the first step, from *cyclo*-(Gly$_2$-Tyr)$_2$ to H-(Gly$_2$-Tyr)$_2$-OH, was supposed to be rate-determining.

It was expected that *cyclo*-(Gly$_2$-Phe)$_2$ (XVIII) would be synthesized more easily than *cyclo*-(Gly$_2$-Tyr)$_2$ and XVIII would hydrolyzed by chymotrypsin to H-Gly$_2$-Phe-OH. Since XVIII was insoluble in pH 8.0 buffer, it was not clear whether an incubation mixture of this cyclic hexapeptide and the enzyme yielded the expected tripeptide H-Gly$_2$-Phe-OH.

Experimental

A spot of material on a paper or plate was detected by spraying ninhydrin, Pauli reagent for Tyr residue or butyl hypochlorite reagent for peptide bond.[9] A spot of an amino group-blocked material on a plate was also detected by spraying 47% by hydrobromic acid and ninhydrin. The R_f refers to paper chromatography on Toyo Roshi No. 52 with a solvent of *n*-butanol-acetic acid-pyridine-water (4:1:1:2, vol). Purity of material was also checked by thin-layer chromatography with the same solvent or chloroform-methanol (5:1, vol).

Z-Tyr(Bzl)-Gly$_2$-OBzl (I). To a solution of Z-Tyr (Bzl)-ONp[10] (8.4 g) and H-Gly$_2$-OBzl·TsOH[11] (6.3 g) in chloroform (100 m*l*) was added TEA (2.4 m*l*) at room temperature. The mixture was left to stand overnight, evaporated *in vacuo*, and the oily residue was dissolved in ethyl acetate. It was washed with 2% hydrochloric acid, 4% sodium bicarbonate, and water, successively, dried over sodium sulfate and evaporated. The oil was solidified by the addition of petroleum ether. It was recrystallized from ethyl acetate-ether-petroleum ether; yield, 10.7 g (88%); mp 116—118°C; $[\alpha]_D^{15}$ +4.2° (*c* 1, acetic acid).

Found: C, 68.96; H, 5.77; N, 6.80%. Calcd for $C_{35}H_{35}O_7N_3$: C, 68.95; H, 5.79; N, 6.89%.

Z-Tyr(Bzl)-Gly$_2$-OH (II). To a solution of I (6.1 g) in a mixture of dioxane-water (2:1; 300 m*l*) was added 2N sodium hydroxide (6 m*l*). After 24 hr, 2N hydrochloric acid (6 m*l*) was added and the solution was evaporated. The residual crystals were collected with the aid of water, and recrystallized from dioxane-ether-petroleum ether; yield, 4.47 g (86%); mp 145—147°C; $[\alpha]_D^{15}$ —21.4° (*c* 1, DMF).

Found: C, 64.42; H, 5.82; N, 7.89. Calcd for $C_{28}H_{29}O_7N_3$: C, 64.73; H, 5.63; N, 8.09%.

Z-Tyr(Bzl)-Gly$_2$-ONp (III). To a solution of II (1.15 g) in pyridine (7 m*l*) was added di-*p*-nitrophenyl sulfite (3.2 g). After 24 hr, it was evaporated and the residual solid was collected with the aid of a mixture of ether-petroleum ether. The yield was 1.36 g (106%); the *p*-nitrophenyl ester content was estimated to be 93%.[12] The product was used for the preparation of the cyclic peptide (XIII).

Z-Tyr(Bzl)-Gly$_2$-NHNH$_2$ (IV). A solution of I (1.35 g) and hydrazine hydrate (2.2 m*l*) in DMF (15 m*l*) was allowed to stand for 24 hr, and evaporated *in vacuo*. After the addition of water, the crystals were collected by filtration; yield, 1.35 g (88%); mp 209—212°C; $[\alpha]_D^{21}$ —21.6° (*c* 1, DMF).

Found: C, 62.78; H, 5.92; N, 12.78%. Calcd for $C_{28}H_{31}O_6N_5$: C, 63.02; H, 5.86; N, 13.13%.

H-Tyr-Gly$_2$-NHNH$_2$·2HCl (V·2HCl). Compound IV (0.89 g, 2 mmol) suspended in 0.43N methanolic hydrogen chloride (7.1 m*l*) was treated with hydrogen in the presence of palladium black. With the progress of reaction the suspended material dissolved into the solution. The filtrate from the catalyst was evaporated; yield of the residual hygroscopic crystal, 0.76 g (approximately 100%); *Rf* 0.50. This was used for the cyclization reaction.

Z-Gly-Tyr-Gly-OBzl (VI). To a chilled solution of Z-Gly-Tyr-NHNH$_2$[13] (2.32 g, 6 mmol) in a mixture of acetic acid (25 m*l*) —N hydrochloric acid (12 m*l*) was added an aqueous solution of sodium nitrite (0.62 g, 9 mmol). After 15 min, water (150 m*l*) was added and the azide was extracted with ethyl acetate. The organic layer was washed with 10% sodium bicarbonate and dried, and the filtrate was added to a mixture of H-Gly-OBzl·TsOH (2.02 g, 6 mmol) and TEA (0.84 m*l*) in chloroform (24 m*l*). After 3 days at O°C, the solution was evaporated, and the oily residue was solidified by trituration with 2% hydrochloric acid and 4% sodium bicarbonate. It was recrystallized from ethyl acetate-ether-petroleum ether; yield, 2.1 g (70%); mp 89—91°C; $[\alpha]_D^{18}$ —5.3° (*c* 1, DMF).

Found: C, 63.69; H, 5.90; N, 8.18%. Calcd for $C_{28}H_{29}O_7N_3 \cdot 1/2H_2O$: C, 63.63; H, 5.72; N, 7.95%.

Z-Gly-Tyr-NHNH$_2$ (VII). This was prepared from VI in the same manner as for the preparation of IV; yield, 63%; mp 181—183°C; $[\alpha]_D^{18}$ —10.4° (*c* 1, DMF).

Found: C, 56.64; H, 5.72; N, 15.77%. Calcd for $C_{21}H_{25}O_6N_5$: C, 56.87; H, 5.68; N, 15.79%.

H-Gly-Tyr-Gly-NHNH$_2$·2HCl (VIII·2HCl). A solution of VII (1.05 g) in 0.38N methanolic hydrogen chloride (15 m*l*) was hydrogenated as for the preparation of V·2HCl; yield of hygroscopic crystal, 0.89 g (98%).

H-Gly$_2$-Tyr-NHNH$_2$·2HCl (IX·2HCl). Z-Gly$_2$-Tyr-NHNH$_2$[7] (4.56 g) was hydrogenated; yield of hygroscopic crystal, 3.88 g (100%).

Z-(Gly$_2$-Tyr)$_2$-OEt (X). To azide prepared from Z-Gly$_2$-Tyr-NHNH$_2$ (1.19 g) was coupled with H-Gly$_2$-Tyr-OEt·HCl[14] (0.97 g) as described for the preparation of VI.

8) It is of interest to note that H-Gly$_2$-Tyr-Gly$_3$-OH or H-Gly$_2$-Tyr-Gly$_4$-OH was hydrolyzed by chymotrypsin faster than other homologues, H-Gly$_2$-Tyr-Gly$_n$-OH (n=1 and 2).[7]

9) M. Kimura, K. Murayama, M. Nomoto, and Y. Fujita, *J. Chromatogr.*, **41**, 458 (1969).

10) M. Bodanszky and V. du Vigneaud, *J. Amer. Chem. Soc.*, **81**, 5688 (1959).

11) T. Yamashita, *J. Biochem.*, **48**, 651 (1960).

12) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **40**, 624 (1957).

13) K. Hofmann, A. Lindenmann, M. Z. Magee, and N. H. Khan, *J. Amer. Chem. Soc.*, **74**, 470 (1952).

The product was recrystallized from hot ethanol; yield, 0.82 g (41%); mp 124—126°C; $[\alpha]_D^{22}$ +19.0° (*c* 1, acetic acid).

Found: C, 57.04; H, 5.76; N, 11.06%. Calcd for $C_{36}H_{42}O_{11}N_6$: C, 57.44; H, 5.89; N, 11.17%.

Z-(Gly_2-Tyr)$_2$-NHNH$_2$ (XI). Compound X (0.82 g) was treated with hydrazine hydrate (1.12 m*l*) in DMF (10 m*l*) as described for the preparation of IV; yield, 0.64 g (80%); mp 151—153°C; $[\alpha]_D^{15}$ −6.4° (*c* 0.5, DMF).

Found: C, 54.12; H, 5.81; N, 14.79%. Calcd for $C_{34}H_{40}O_{10}N_8 \cdot 2H_2O$: C, 53.96; H, 5.86; N, 14.81%.

H-(Gly_2-Tyr)$_2$-NHNH$_2$·2HCl (XII·2HCl). This was prepared from XI (607 mg); yield of hygroscopic crystal, 570 mg (102%)

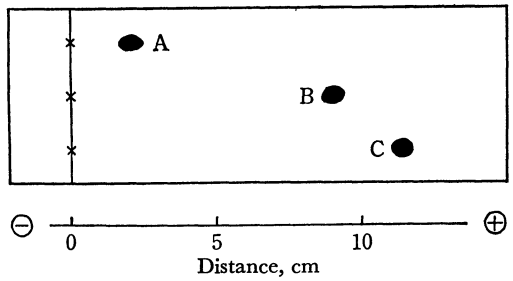

Fig. 3. Paper electrophoresis of *cyclo*-(Gly_2-Tyr)$_2$ (A), H-(Gly_2-Tyr)$_2$-OH (B) and H-Gly_2-Tyr-OH (C). Solvent, 0.01M citrate buffer (pH 6.7); 600 V/30 cm; 3 hr.

cyclo-*(Gly_2-Tyr)$_2$ (XIII)* *(a) From Z-Tyr(Bzl)-Gly_2-ONp (III):* III (1.35 g, 2.21 mmol) was dissolved in 25% hydrogen bromide in acetic acid (8 m*l*) at room temperature. After 2 hr, the solution was evaporated and the oily residue (1.1 g), H-Tyr-Gly_2-ONp·HBr (XIX), was dissolved in DMF (15 m*l*). It was added to pyridine (600 m*l*) at 55—60°C.[5] After evaporation, the residue was dissolved in a mixture (140 m*l*) of dioxane-methanol-water (7:2:1), and the solution was passed successively through columns of Amberlite IR4B (OH^- form, 1.8×8 cm) and Dowex 50 (H^+ form, 1.8×15 cm). The eluate was evaporated, and the residual crystals were collected by filtration with the aid of ether. It was recrystallized from methanol-ether; yield of an air-dried material, 53 mg (9% from III). Its homogeneity was established by paper electrophoresis (Fig. 3) and paper chromatography (*Rf* 0.80; Fig. 4). The value of specific rotation is shown in Table 1.

Found: C, 51.31; H, 5.90; N, 13.70%; mol wt, 605.[15] Calcd for $C_{26}H_{30}O_8N_6 \cdot 3H_2O$: C, 51.31; H, 5.96; N, 13.81%; mol wt, 609. The air-dried material lost 8.95% of its weight after being dried for 2 hr at 70°C *in vacuo*. Calcd for $3H_2O$: 8.88%.

(b) From Tripeptide Hydrazide (V, VIII or IX): A cyclization reaction of H-Gly-Tyr-Gly-NHNH$_2$ (VIII) is described as an example as follows. To a chilled solution of VIII·2HCl (816 mg, 2.14 mmol) in 0.1N hydrochloric acid (21.4 m*l*), an aqueous solution of sodium nitrite (162 mg, 2.35 mmol) was added. After 15 min, the solution was poured into cold water (1000 m*l*) containing sodium bicarbonate (2.7 g). After 3 days at 0—4°C, the solution was neutralized with 1N hydrochloric acid and evaporated. The residue was collected by filtration and washed with water. The product was dissolved in a mixture of dioxane-methanol-water and passed through the columns as described above. Evaporation of the eluate yielded a powder (305 mg) with pale yellow color.[16] A part (100 mg) of the powder dissolved in methanol (1 m*l*) was applied to a column (1.8×35 cm) with Sephadex LH-20, and the development was continued with methanol. Fractions from 39 to 48 m*l* were evaporated, and the crystals were collected with the aid of water; yield of an air-dried product, 40 mg (17%). Data of this product are shown in Table 1.

(c) From Hexapeptide Hydrazide (XII): XII·2HCl (591 mg) was treated in the same manner as described above; yield of an air-dried product, 104 mg (20%). Its properties agreed with those of the products obtained from tripeptide active ester or azides (see Table 1).

Z-(Gly_2-Tyr)$_2$-OBzl (XIV). Z-Gly_2-Tyr-OBzl[17] (780 mg, 1.5 mmol) was treated with 25% hydrogen bromide in acetic acid (4 m*l*) as described for the preparation of XIX; the H-Gly_2-Tyr-OBzl·HBr (XX) (0.7 g) was obtained as an oil. The azide from Z-Gly_2-Tyr-NHNH$_2$ (443 mg, 1 mmol) was coupled with XX (0.7 g) as described for the preparation of VI. The product was recrystallized from DMF-ethyl acetate; yield, 509 mg (64%), mp 115—116°C; $[\alpha]_D^{25}$ −5.0°

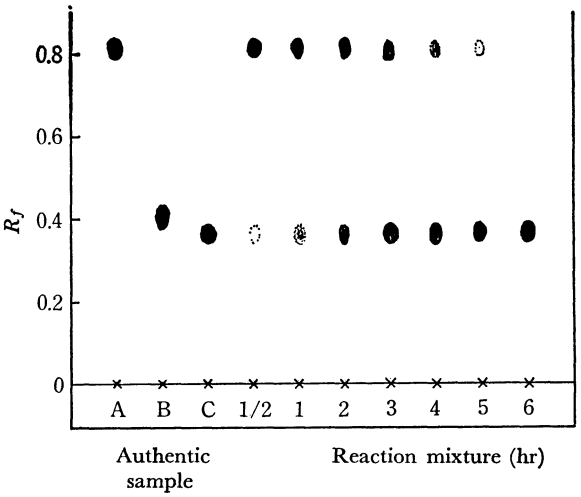

Fig. 4. Paper chromatogram of a reaction mixture (No. 1 of Table 2) of *cyclo*-(Gly_2-Tyr)$_2$ and chymotrypsin. A,B, and C, see Fig. 3; solvent, *n*-butanol-acetic acid-pyridine-water (4:1:1:2).

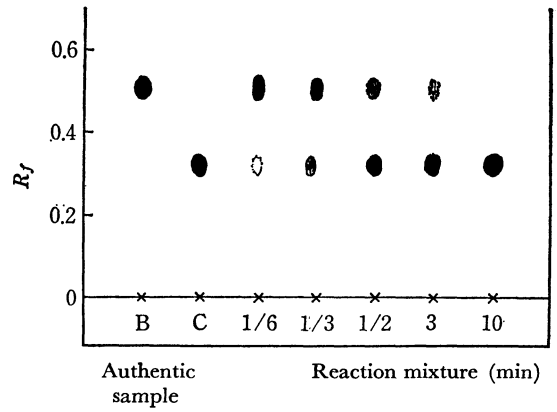

Fig. 5. Paper chromatogram of a reaction mixture (No. 2 of Table 2) of H-(Gly_2-Tyr)$_2$-OH and chymotrypsin. B and C, see Fig. 3; solvent, *n*-butanol-acetic acid-pyridine-water (15:3:10:12).

14) T. Yamashita, *J. Biochem.*, **48**, 846 (1960).

15) Methanol was used as a solvent for Hitachi Osmometer, type 115.

16) Recrystallization from metahnol - ether gave no analytically pure cyclic hexapeptide.

17) N. Izumiya and H. Uchio, *J. Biochem.*, **46**, 235 (1959).

TABLE 2. COMPOSITION OF MIXTURE OF THE ENZYMATIC REACTION

No.	Substrate	Weighed amount (0.04 mmol) of substrate	0.2M Tris buffer, pH 8.0	Weighed amount of chymotrypsin	Total volume
1	*cyclo*-$(Gly_2\text{-}Tyt)_2 \cdot 3H_2O$	24 mg	1.0 m*l*	10 mg	2.0 m*l*
2	H-$(Gly_2\text{-}Tyr)_2$-OH$\cdot 4H_2O$	26	1.0	10	2.0

(*c* 1, DMF).

Found: C, 59.75; H, 5.64; N, 10.15%. Calcd for $C_{41}H_{44}O_{11}N_4 \cdot 2/3H_2O$: C, 59.77; H, 5.75; N, 10.20%.

H-(Gly$_2$-Tyr)$_2$-OH (XV). Compound XIV (240 mg) suspended in a mixture (5 m*l*) of acetic acid - methanol - water (6:3:1) was hydrogenated, and the filtrate was evaporated. The crystals were collected by filtration with the aid of acetone, and recrystallized from water-ethanol; yield, 135 mg (85%); mp 238—242°C (decomp.); $[\alpha]_D^{20}$ −2.8° (*c* 1, water).

Found: C, 48.04; H, 6.07; N, 12.84%. Calcd for $C_{26}H_{32}O_9N_6 \cdot 4H_2O$: C, 48.44; H, 6.25; N, 13.04%.

Action of Chymotrypsin on cyclo-*(Gly$_2$-Tyr)$_2$ (XIII) and H-(Gly$_2$-Tyr)$_2$-OH (XV).* α-Chymotrypsin was salt free, crystalline sample from Worthington Biochemical Corp., U.S.A. H-Gly_2-Tyr-OH was prepared as described in literature.[17] Enzymatic experiments were carried out at pH 8.0 and 30°C. The progress of the reactions was checked by paper chromatography as a function of time. Table 2 shows the composition of the reaction mixture. The control experiments showed that no hydrolysis of XIII and XV occurred in the absence of the enzyme. In the presence of the enzyme, XIII was completely hydrolyzed to linear tripeptide after 6 hr, as can be seen in Fig. 4. On the other hand, the linear hexapeptide (XV) was hydrolyzed to the tripeptide within only 10 min in the same condition (Table 2) as seen in Fig. 5.

Z-Gly$_2$-Phe-OBzl (XVI). This was prepared from Z-Gly_2-OH (2.66 g) and H-L-Phe-OBz·TsOH (4.27 g) by the mixed anhydride method as described previously[5] yield, 3.2 g (64%); mp 81—82°C; $[\alpha]_D^{23}$ +7.0° (*c* 1, acetic acid).

Found: C, 65.59; H, 5.94; N, 8.13%. Calcd for $C_{28}H_{29}O_6N_3 \cdot 1/2H_2O$: C, 65.61; H, 5.90; N, 8.20%.

Z-Gly$_2$-Phe-NHNH$_2$ (XVII). This was prepared from XVII as described for the preparation of IV; yield, 68%; mp 134—136°C; $[\alpha]_D^{20}$ +13.0° (*c* 1, acetic acid).

Found: C, 58.55; H, 6.00; N, 16.12%. Calcd for $C_{21}H_{25}O_5N_5 \cdot 1/4H_2O$: C, 58.39; H, 5.95; N, 16.21%.

cyclo-*(Gly$_2$-Phe)$_2$ (XVIII).* Compound XVII (1.07 g, 2.5 mmol) was hydrogenated as described for the preparation of V·2HCl, and the resulting H-Gly_2-Phe-$NHNH_2$·2HCl (0.90 g, 98%) was obtained as hygroscopic crystals. This product (0.9 g) was subjected to cyclization reaction as described for the preparation of XIV from H-Gly-Tyr-Gly-$NHNH_2$. The crude product dissolved in a mixture of dioxane-methanol-water was passed through the columns of Dowex 1 and Dowex 50. The eluate was evaporated, and the residual nice crystals were collected by filtration with the aid of water; yield of an air-dried material, 164 mg (25%); mp 280—285°C (decomp.); $[\alpha]_D^{20}$ −52.0° (*c* 0.5, DMF); R_f 0.93. The material contained no water of crystallization.

Found: C, 59.66; H, 5.89; N, 15.96%; mol wt, 530. Calcd for $C_{26}H_{30}O_6N_6$: C, 59.76; H, 5.79; N, 16.08%; mol wt, 523. This material (XVIII) did not dissolve in pH 8.0 buffer even at a level of 0.002M. It was therefore, not clear whether an incubation mixture of XVIII and chymotrypsin yielded H-Gly_2-Phe-OH by means of paper chromatography.

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