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Synthesis of *cyclo*-Diglycyl-L-lysyl-diglycyl-L-lysyl and Hydrolysis by Trypsin

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A protected cyclic hexapeptide (XVI), *cyclo*-(diglycyl-ε-benzyloxycarbonyl-L-lysyl)$_2$, was synthesized in three different ways; the cyclization reactions of a protected tripeptide azide, a protected tripeptide active ester, and a protected hexapeptide active ester produced the protected cyclic hexapeptide (XVI) in fairly good yields. The product (XVI) was then converted by hydrogenolysis to the cyclic hexapeptide (XVII), *cyclo*-(diglycyl-L-lysyl)$_2$. It was found that the cyclic hexapeptide was hydrolyzed slowly by trypsin to a tripeptide diglycyl-lysine, whereas a synthetic hexapeptide, diglycyl-lysyl-diglycyl-lysine, was hydrolyzed very rapidly to the tripeptide.

In a previous paper from this laboratory, it was shown that L-valyl-L-lysine anhydride and the other dipeptide anhydrides which contain L-lysine residue were not hydrolyzed by trypsin at the lysine carbonyl linkage.[1] On the other hand, it was observed that *cyclo*-(pentaglycyl-L-lysyl) was completely hydrolyzed by trypsin.[2] From these results, it was concluded that a certain minimum ring size may be necessary for the hydrolysis of cyclic peptides by the enzyme. For the study of the mode of action of proteolytic enzymes

1) N. Izumiya, T. Kato, Y. Fujita, M. Ohno and M. Kondo, This Bulletin, **37**, 1809 (1964).
2) M. Ohno and N. Izumiya, *ibid.*, **38**, 1831 (1965).

on cyclic peptides, several compounds of various ring sizes are being synthesized in this laboratory. The present paper will describe the synthesis of *cyclo*-(diglycyl-L-lysyl)$_2$ and the mode of action of trypsin on the cyclic hexapeptide.

Three different routes, shown in Figs. 1, 2, and 3, were undertaken for the synthesis of the cyclic benzyloxycarbonyl-substituted hexapeptide, *cyclo*-[Gly-Gly-Lys(ε-Z)]$_2$ (XVI).

Figure 1 indicates the sequence of reactions *via* the linear benzyloxycarbonyl-substituted tripeptide azide. The product was obtained in a 16% yield from IV by the cyclization reaction of the azide in a large amount of water containing sodium bicarbonate. It is of interest to note that the cyclization reactions of the linear benzyloxycarbonyl-substituted hexapeptide azides, H-Gly$_3$-Lys(ε-Z)-Gly$_2$-N$_3$ and H-Gly$_5$-Lys(ε-Z)-N$_3$, gave only traces of the protected cyclic hexapeptide.[2] The product obtained in the 16% yield was proved by the molecular weight determination to be a dimeric compound, indicated as XVI.

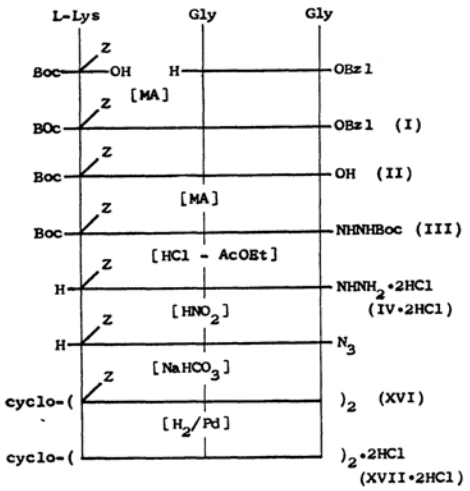

Fig. 1. Cyclization of the linear tripeptide azide.
Z-, Benzyloxycarbonyl; Boc-, *t*-butyloxycarbonyl; -OBzl, benzyl ester

Since the tripeptide hydrazide (IV) might be derived from BOC-Lys(ε-Z)-Gly$_2$-NHNH$_2$ (XXVI) by the treatment of hydrogen chloride in ethyl acetate, we tried to prepare XXVI by the reaction of I and hydrazine under various conditions; it was found that the yield of XXVI in this process was very poor. Therefore, a similar compound, III, which can easily be converted to IV, was prepared in a good yield by the reaction of the mixed anhydride derived from II and *t*-butyloxycarbonyl hydrazide.

Figure 2 shows the sequence of reactions *via* the linear tripeptide active ester (X), in which the ε-benzyloxycarbonyl-lysine residue occupies

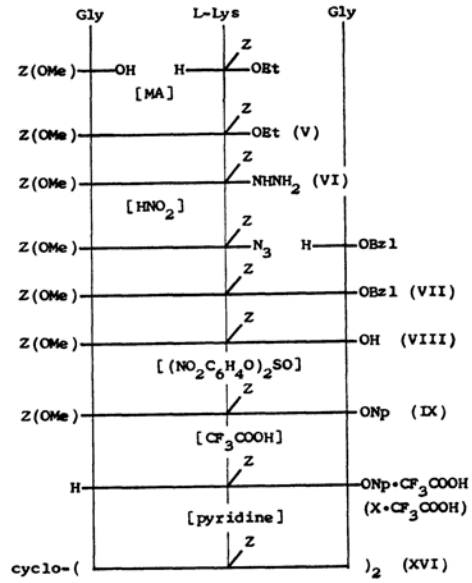

Fig. 2. Cyclization of the linear tripeptide active ester.
Z(OMe)-; *p*-methoxybenzyloxycarbonyl

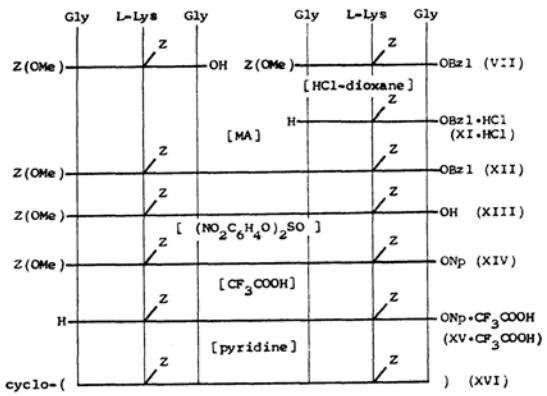

Fig. 3. Cyclization of the linear hexapeptide active ester.

the middle position of the peptide sequence. The active ester (X) was treated with a large amount of pyridine, and the cyclic peptide isolated in a good yield (41% as calculated from VIII) was again found to be the dimer (XVI).

In the route of Fig. 3 the above method was also used, but the cyclization was tried from the linear hexapeptide active ester (XV). The yield of the cyclic hexapeptide (XVI) was 20% from XIII.

The final product, *cyclo*-(Gly-Gly-Lys)$_2$·2HCl (XVII·2HCl), was obtained as hygroscopic crystals by the hydrogenolysis of the protected cyclic peptide (XVI) in the presence of palladium black; the homogeneity of the product (XVII·2HCl) was established by the chromatographies and by amino acid analysis.

During the hydrogenolysis of *cyclo*-[Gly-Gly-Lys(ε-Z)]$_2$ (XVI), the progress of the reaction was checked by thin-layer chromatography in the course of time; the intermediate reaction solution revealed three spots, with R_f values of 0.78, 0.33, and 0.04, as may be seen in Fig. 6. The spot with 0.78 was proved to be of the starting material (XVI), while that with 0.04 was of the final product (XVII). The product indicating R_f 0.33 was considered to be a cyclic hexapeptide, in which one of the ε-amino groups was protected by the benzyloxycarbonyl group and the other, not. At the completion of the hydrogenation, only one spot, with an R_f value of 0.04, was observed on the chromatogram.

Figure 4 shows the syntheses of the linear hexapeptide, H-(Gly-Gly-Lys)$_2$-OH, as a substrate for trypsin, and the linear tripeptide, H-Gly-Gly-Lys-OH, as an authentic material on the enzymic experiment.

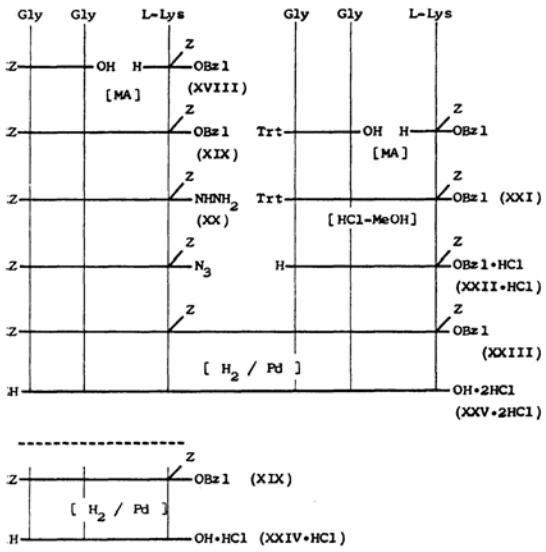

Fig. 4. Syntheses of the linear tri- and hexapeptides. Trt-, trityl

Crystalline trypsin was added to a 0.01 M solution at pH values of 8.0 for both *cyclo*-(Gly-Gly-Lys)$_2$ and H-(Gly-Gly-Lys)$_2$-OH. It was observed that both the compounds were completely hydrolyzed to the tripeptide, H-Gly-Gly-Lys-OH, as may be seen in Figs. 8 and 9. This agreed well with the fact that a peptide containing the L-lysine residue was susceptible to the hydrolytic action of trypsin at the linkage of lysine carbonyl.[3] However, paper chromatography indicated that the rate of the hydrolysis of H-(Gly-Gly-Lys)$_2$-OH to the tripeptide was much faster than that of *cyclo*-(Gly-Gly-Lys)$_2$. Therefore, it was deduced that

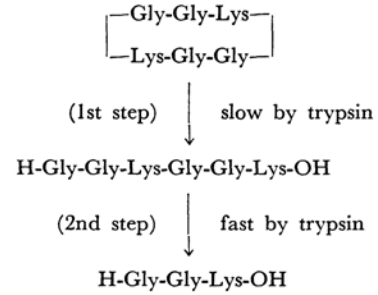

Fig. 5. Hydrolysis of the cyclic hexapeptide by trypsin.

the hydrolysis of the cyclic hexapeptide by trypsin proceeded in the two steps shown in Fig. 5; the first step, from *cyclo*-(Gly-Gly-Lys)$_2$ to H-(Gly-Gly-Lys)$_2$-OH, seemed to be the rate-determining step. Attempts to detect the spot corresponding to H-(Gly-Gly-Lys)$_2$-OH during the incubation of *cyclo*-(Gly-Gly-Lys)$_2$ with trypsin failed. The fact might be explained by the rapid hydrolysis of H-(Gly-Gly-Lys)$_2$-OH by the enzyme. In this connection, it is of interest to note that Yamamoto and Izumiya synthesized a number of H-Gly$_2$-Lys-Gly$_n$-OH, wherein *n* was 1, 2, 3, or 4, and found that H-Gly$_2$-Lys-Gly$_3$-OH, a hexapeptide, was hydrolyzed by trypsin faster than other homologues.[4]

Experimental

All the melting points are uncorrected. The optical rotations were measured on a Yanagimoto Photometric Direct Reading Polarimeter OR-20. Prior to analysis, the compounds were dried over phosphorus pentoxide to a constant weight at 80°C and 2 mmHg.

***t*-Butyloxycarbonyl-ε-benzyloxycarbonyl-L-lysyl-diglycine Benzyl Ester (I).** To a chilled solution of *t*-butyloxycarbonyl-ε-benzyloxycarbonyl-L-lysine[5] (2.81 g, 7.39 mmol) and triethylamine (1.03 m*l*, 7.39 mmol) in tetrahydrofuran (22 m*l*), isobutyl chloroformate (0.97 m*l*, 7.39 mmol) was added at −5°C. After 15 min, a mixture of diglycine benzyl ester *p*-toluenesulfonate (2.92 g, 7.39 mmol) and triethylamine (1.03 m*l*, 7.39 mmol) in chloroform (22 m*l*) was added. The mixture was left to stand overnight at room temperature and then evaporated *in vacuo*. The residual oil was solidified by adding water. The product was collected by filtration, washed successively with 0.5 M citric acid, 0.5 M sodium bicarbonate, and water, and dried. Recrystallization from ethyl acetate-ether yielded 3.86 g (89%); mp 99—101°C; $[\alpha]_D^{20}$ −2.8° (*c* 2, dimethylformamide).

Found: C, 61.44; H, 6.72; N, 9.48%. Calcd for $C_{30}H_{40}O_8N_4$: C, 61.62; H, 6.91; N, 9.58%.

***t*-Butyloxycarbonyl-ε-benzyloxycarbonyl-L-lysyl-diglycine (II).** To a solution of I (1.17 g, 2 mmol) in methanol (10 m*l*), N sodium hydroxide (2.4 m*l*,

3) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

4) T. Yamamoto, and N. Izumiya, *Arch. Biochem. Biophys.*, **120**, 497 (1967).

5) G. W. Anderson and A. C. McGregor, *J. Am. Chem. Soc.*, **79**, 6180 (1957).

2.4 mmol) was added. After 37 hr at 0°C, the solution was concentrated *in vacuo* to remove the methanol, and the residual solution was diluted with water and acidified with 10% citric acid in an ice bath. The mixture was extracted with ethyl acetate (100 m*l*), and the organic layer was dried over anhydrous sodium sulfate and then evaporated to dryness *in vacuo*. The residual oil was solidified by the addition of ether. Yield, 0.81 g (82%); mp 113—114°C; $[\alpha]_D^{20}$ −4.7° (*c* 2, dimethylformamide).

Found: C, 55.48; H, 7.12; N, 11.11%. Calcd for $C_{23}H_{34}O_8N_4$: C, 55.85; H, 6.93; N, 11.33%.

***t*-Butyloxycarbonyl-ε-benzyloxycarbonyl-L-lysyl-diglycine *t*-Butyloxycarbonyl Hydrazide (III).** The mixed anhydride prepared from II (3.96 g, 8 mmol), triethylamine (1.12 m*l*, 8 mmol), and isobutyl chloroformate (1.04 m*l*, 8 mmol) in tetrahydrofuran (24 m*l*) was coupled with *t*-butyloxycarbonyl hydrazide[6] (1.05 g, 8 mmol) dissolved in a chilled mixture of triethylamine (1.12 m*l*, 8 mmol) and chloroform (24 m*l*). Then the reaction mixture was treated as has been described for the preparation of I. The product was recrystallized from ethyl acetate-ether. Yield, 4.27 g (88%); mp 80—83°C; $[\alpha]_D^{20}$ −4.6° (*c* 2, dimethylformamide).

Found: C, 55.07; H, 7.45; N, 13.63%. Calcd for $C_{28}H_{44}O_9N_6$: C, 55.24; H, 7.30; N, 13.81%.

ε-Benzyloxycarbonyl-L-lysyl-diglycine Hydrazide Dihydrochloride (IV·2HCl). To a solution of III (1.82 g, 3 mmol) in a mixture of ethyl acetate (20 m*l*) and methanol (0.2 m*l*), 2.6 N dry hydrogen chloride in ethyl acetate (11.5 m*l*) was added. The solution was then allowed to stand at room temperature. After 2 hr, the solution was evaporated *in vacuo* to afford crystals, which were filtered off in the cold room with the aid of ether. The yield of the hygroscopic crystals was 1.36 g (94%); R_f 0.57.[7]

***p*-Methoxybenzyloxycarbonylglycyl-ε-benzyloxycarbonyl-L-lysine Ethyl Ester (V).** *p*-Methoxybenzyloxycarbonylglycine[8] (7.18 g, 30 mmol), was coupled with ε-benzyloxycarbonyl-L-lysine ethyl ester *p*-toluenesulfonate (14.4 g, 30 mmol), following the procedure employed for the preparation of I. The reaction mixture was allowed to stand overnight and then evaporated to dryness *in vacuo*. The oily residue was dissolved in ethyl acetate (150 m*l*); then the solution was washed successively with 4% sodium bicarbonate, 10% citric acid, and water, and dried over sodium sulfate. The filtrate was evaporated *in vacuo*. The oily residue was solidified by the addition of petroleum ether. The product was recrystallized from ethyl acetate-ether; yield, 10.3 g (65%); mp 98—99°C; $[\alpha]_D^{20}$ −6.5° (*c* 2, dimethylformamide).

Found: C, 61.34; H, 6.70; N, 7.98%. Calcd for $C_{27}H_{35}O_8N_3$: C, 61.22; H, 6.67; N, 7.94%.

***p*-Methoxybenzyloxycarbonylglycyl-ε-benzyloxycarbonyl-L-lysine Hydrazide (VI).** A solution of V (1.06 g, 2 mmol) and hydrazine hydrate (2 m*l*, 40 mmol) in dimethylformamide (15 m*l*) was allowed to stand at room temperature for 48 hr. The excess hydrazine hydrate was evaporated *in vacuo*, and then water was added to the residue. The resulting crystals were collected by filtration; yield, 1.01 g (98%); mp 165—166°C;[$\alpha]_D^{20}$ −4.5° (*c* 2, dimethylformamide).

Found: C, 58.44; H, 6.69; N, 13.55%. Calcd for $C_{25}H_{33}O_7N_5$: C, 58.24; H, 6.45; N, 13.59%.

***p*-Methoxybenzyloxycarbonylglycyl-ε-benzyloxycarbonyl-L-lysyl-glycine Benzyl Ester (VII).** To a cold solution of VI (2.58 g, 5 mmol) in acetic acid (100 m*l*) and 2 N hydrochloric acid (6.3 m*l*), a cold aqueous solution of sodium nitrite (0.828 g, 6 mmol) was added. After the solution had been allowed to stand for 5 min at −5°C, it was diluted with cold water (150 m*l*) and the protected tripeptide azide was extracted two times with 60-m*l* portions of ethyl acetate. The combined ethyl acetate solution was washed twice with a sodium bicarbonate solution and with water, dried over sodium sulfate, and then added to a cold solution of glycine benzyl ester *p*-toluenesulfonate (1.69 g, 5 mmol) in triethylamine (0.7 m*l*, 5 mmol) and dimethylformamide (20 m*l*). After the mixture had been stirred for 3 days at 0°C, the insoluble material was removed by filtration and the filtrate was evaporated *in vacuo*. The crystals were filtered with the aid of ether, and washed successively with a 10% citric acid solution, a 4% sodium bicarbonate solution, and water. The product was recrystallized from dioxane-methanol (1 : 1) and ether; yield, 5.68 g (95%); mp 153—154°C; $[\alpha]_D^{20}$ −6.9° (*c* 2, dimethylformamide).

Found: C, 63.10; H, 6.25; N, 8.43%. Calcd for $C_{34}H_{40}O_9N_4$: C, 62.94; H, 6.23; N, 8.64%.

***p*-Methoxybenzyloxycarbonylglycyl-ε-benzyloxycarbonyl-L-lysyl-glycine (VIII).** To a solution of VII (1.30 g, 2 mmol) in dioxane-methanol (1 : 1, 20 m*l*), 2 N sodium hydroxide (1.2 m*l*, 2.4 mmol) was added. After 5 hr, the solution was concentrated *in vacuo*, and the residue was dissolved in water and acidified with 0.5 M citric acid under cooling. The resulting precipitate was collected by filtration and washed with water. The product was recrystallized from dioxane-methanol (1 : 1) and ether; yield, 1.01 g (90%); mp 162—163°C (decomp.); $[\alpha]_D^{20}$ −3.8° (*c* 2, dimethylformamide).

Found: C, 58.03; H, 6.33; N, 10.30%. Calcd for $C_{27}H_{34}O_9N_4$: C, 58.05; H, 6.14; N, 10.03%.

***p*-Methoxybenzyloxycarbonylglycyl-ε-benzyloxycarbonyl-L-lysyl-glycine *p*-Nitrophenyl Ester (IX).** A solution of VIII (279 mg, 0.5 mmol) and di-*p*-nitrophenyl sulfite (648 mg, 2.0 mmol) in pyridine (6 m*l*) was allowed to stand overnight at room temperature. The solvent was then removed *in vacuo*, and the oily residue was solidified by adding a mixture of ether and petroleum ether (1 : 1). The product was collected by filtration, and washed with a mixture of ether and petroleum ether until no yellow color could be discerned upon the addition of a sodium hydroxide solution to the filtrate. The yield was 304 mg; the *p*-nitrophenyl ester content in the product was estimated to be 82% spectrophotometrically by measuring the optical density of the solution of the compound in dimethylformamide - N sodium hydroxide (1 : 1) at 412 mμ.[9]

6) L. A. Carpino, *ibid.*, **82**, 2725 (1960).

7) The R_f value was obtained by thin-layer chromatography with Merck silica gel G and the *n*-butanol - acetic acid - pyridine - water (4 : 1 : 1 : 2, v/v) system. Compounds possessing a free amino group were detected by spraying them with a ninhydrin reagent, and those with blocked amino groups, by spraying them with 47% hydrobromic acid and then the ninhydrin reagent.

8) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962).

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

Glycyl-ε-benzyloxycarbonyl-L-lysyl-glycine *p*-Nitrophenyl Ester Trifluoroacetate (X·CF_3COOH). To IX (300 mg, 0.37 mmol), anisole (0.5 m*l*) and trifluoroacetic acid (2 m*l*) were added at −5°C. After 20 min, the solution was evaporated *in vacuo* at 0°C, and the residue was triturated with ether. The product solidified was collected by filtration and washed with ether; it was used for the cyclization reaction without further purification.

Glycyl-ε-benzyloxycarbonyl-L-lysyl-glycine Benzyl Ester Hydrochloride (XI·HCl). To a solution of VII (260 mg) in dioxane (4 m*l*), 4.02 N hydrogen chloride in dioxane (2.0 m*l*) was added at room temperature. After 4 hr, the solution was evaporated to dryness *in vacuo*, and the residue was collected with the aid of ether. Yield, 182 mg (90%); mp 177—182°C.

Found: C, 57.55; H, 6.43; N, 11.00%. Calcd for $C_{25}H_{33}O_6N_4Cl$: C, 57.62; H, 6.40; N, 10.75%.

***p*-Methoxybenzyloxycarbonylglycyl-ε-benzyloxycarbonyl-L-lysyl-diglycyl-ε-benzyloxycarbonyl-L-lysyl-glycine Benzyl Ester (XII).** The mixed anhydride prepared from VIII (1.68 g, 3 mmol) was coupled with XI·HCl (1.56 g, 3 mmol) as has been described in the preparation of V. Yield, 2.90 g (94%); mp 171—175°C; $[\alpha]_D^{20}$ −10.3° (*c* 2, dimethylformamide).

Found: C, 60.52; H, 6.33; N, 11.21%. Calcd for $C_{52}H_{64}O_{14}N_8$: C, 60.91; H, 6.30; N, 10.93%.

***p*-Methoxybenzyloxycarbonylglycyl-ε-benzyloxycarbonyl-L-lysyl-diglycyl-ε-benzyloxycarbonyl-L-lysyl-glycine (XIII).** To a solution of XII (1.54 g, 1.5 mmol) in a mixture of dioxane and methanol (1 : 1, 100 m*l*), 2 N sodium hydroxide (0.9 m*l*, 1.8 mmol) was added. The solution was allowed to stand for 5 hr at 30°C, and then overnight at room temperature. The reaction mixture was evaporated *in vacuo*, and the oily residue was diluted with water and acidified with 0.5 M citric acid under cooling. After the solution had been kept in a refrigerator overnight, the precipitate was collected by filtration, washed with water, and dried. Recrystallization from dioxane-methanol-ether gave 1.30 g (93%); mp 174—176°C; $[\alpha]_D^{20}$ −8.3° (*c* 2, dimethylformamide).

Found: C, 57.52; H, 6.30; N, 11.80%. Calcd for $C_{45}H_{58}O_{14}N_8$: C, 57.80; H, 6.26; N, 11.99%.

***p*-Methoxybenzyloxycarbonylglycyl-ε-benzyloxycarbonyl-L-lysyl-diglycyl-ε-benzyloxycarbonyl-L-lysyl-glycine *p*-Nitrophenyl Ester (XIV).** A solution of XIII (1.12 g, 1.2 mmol) and di-*p*-nitrophenyl sulfite (3.89 g, 12 mmol) in pyridine (30 m*l*) was treated as has been described in the preparation of IX. The powder (XIV) obtained was used for the next step without further purification.

Glycyl-ε-benzyloxycarbonyl-L-lysyl-diglycyl-ε-benzyloxycarbonyl-L-lysyl-glycine *p*-Nitrophenyl Ester Trifluoroacetate (XV·CF_3COOH). To a mixture of XIV (1.27 g) and anisole (1 m*l*), trifluoroacetic acid (5.2 m*l*) was added at −5°C. After 10 min, the solution was evaporated *in vacuo* at 0°C and the residue was triturated with ether. The hexapeptide *p*-nitrophenyl ester trifluoroacetate was collected by filtration in a cold room, washed with ether, and used for the cyclization reaction without further purification.

***cyclo*-(Diglycyl-ε-benzyloxycarbonyl-L-lysyl)$_2$ (XVI).** *XVI from IV.* To a cold solution of IV·2HCl (721 mg, 1.5 mmol) in a mixture of N hydrochloric acid (1.5 m*l*, 1.5 mmol) and water (13.5 m*l*), a solution of sodium nitrite (10.4 mg, 1.5 mmol) in water (1 m*l*) was added. After having stood for 10 min at 0°C, the solution was poured into 1000 m*l* of cold water containing sodium bicarbonate (1.9 g).[10] The solution was kept at 4°C for 48 hr, and then the pH was adjusted to 5 with 2 N hydrochloric acid. After the solution had been evaporated *in vacuo*, the residue was collected by filtration with the aid of water. The product was dissolved in a mixture (100 m*l*) of dioxane, methanol, and water (1 : 2 : 1). The solution was passed successively through columns (2×10 cm, each) of Dowex-1 (OH^- form) and Dowex 50 (H^+ form). The filtrate and washings were combined and evaporated to dryness *in vacuo* (101 mg). Recrystallization from dioxane-ether gave 90 mg (16%); mp 263—265°C (decomp.); $[\alpha]_D^{20}$ −13.5° (*c* 2, dimethylformamide).

Found: C, 56.50; H, 6.41; N, 14.64%. Calcd for $C_{36}H_{48}O_{10}N_8 \cdot \frac{1}{2}H_2O$: C, 56.75; H, 6.50; N, 14.71%.

The air-dried compound lost 1.15% of its weight after being dried for 2 hr at 130°C, 2 mmHg. Calcd for $1/2H_2O$: 1.18%.

The molecular weight of the XVI which was derived from IV was determined by a Hitachi Osmometer, type 115 (solvent: dimethylformamide).

Found: 755. Calcd for $C_{36}H_{48}O_{10}N_8 \cdot \frac{1}{2}H_2O$: 762.

XVI derived from X. X·CF_3COOH (280 mg) was dissolved in dimethylformamide (5 m*l*) containing glacial acetic acid (0.2 m*l*). The solution was stirred, drop by drop into pyridine (200 m*l*) with the temperature kept at 55—60°C over a period of 6 hr. The solution was stirred at this temperature for an additional 2 hr and then evaporated to dryness *in vacuo*. The residue was dissolved in a mixture (250 m*l*) of dioxane, methanol, and water (1 : 2 : 1), and insoluble materials were filtered off. The filtrate was treated with the columns of Dowex-1 and Dowex-50 as has been described above. The filtrate and washings were then combined and evaporated to dryness *in vacuo*. Recrystallization from dioxane-ether gave 75 mg (41% from VIII); mp 256—260°C; $[\alpha]_D^{20}$ −13.5° (*c* 2, dimethylformamide). The molecular weight of the XVI derived from X was determined by the procedure described above (solvent: dimethylformamide). Found: 750. Calcd: 762.

XVI derived from XV. XV·CF_3COOH (1.21 g) was treated with pyridine (800 m*l*) as has been described above. Yield of XVI, 185 mg (20% from XIII); mp 258—260°C (decomp.); $[\alpha]_D^{20}$ −13.5° (*c* 2, dimethylformamide).

***cyclo*-(Diglycyl-L-lysyl)$_2$ Dihydrochloride (XVII·2HCl).** XVI (75.3 mg, 0.1 mmol) was suspende in a mixture of N hydrochloric acid (0.22 m*l*) and methanol (0.5 m*l*), and the suspension was treated with hydrogen in the presence of palladium black (about 30 mg). As hydrogenolysis had proceeded, the suspended material went into the solution within about half an hour. The progress of the reaction was checked by thin-layer chromatography as a function of the time; the results are shown in Fig. 6. After the completion of hydrogenolysis, the filtrate from the catalyst was evaporated *in vacuo* to dryness. The residual oil was changed to hygroscopic crystals by treatment with acetone; yield, 48 mg (86%). Elemental analysis was

10) Instead of cold water, the authors used dioxane-water (1 : 1) or dimethylformamide-water (1 : 1). These solvents gave almost the same yields of XVI as that when water was used.

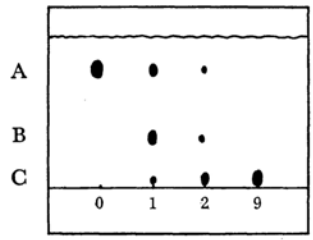

Fig. 6. Thin-layer chromatogram of the solution of hydrogenolysis of the protected cyclic hexapeptide.
A, *cyclo*-(Gly-Gly-Lys(ε-Z))$_2$
B, presumably *cyclo*-(Gly-Gly-Lys-Gly-Gly-Lys(ε-Z))
C, *cyclo*-(Gly-Gly-Lys)$_2$

not performed because of its hygroscopic charater, but the homogeneity of this cyclic peptide hydrochloride was established by paper chromatography (R_f 0.22[11]), paper electrophoresis (Fig. 7), and amino acid analysis.

The paper electrophoresis was carried out under the following conditions: voltage gradient, 500V/30 cm, and solvent system, formic acid - acetic acid - methanol - water (1 : 3 : 6 : 10, v/v) (pH 1.8).

Amino acid analysis was performed as follows. XVII·2HCl (0.05 mg) was hydrolyzed with 6 N hydrochloric acid for 24 hr. The hydrolysate was dissolved in 0.1 m*l* of a 0.2 M sodium carbonate buffer of pH 9.2, the solution was applied to a column (0.9×5 cm) with Aminex Q-150 (31—45 microns, Na^+ form), and development was continued with the same solvent. Elution was carried out at room temperature with a flow rate of 20—25 m*l* per hr, and a 1 m*l* fraction was collected. The amino-acid content in the fraction was determined by the ninhydrin method described by Yemm and Cocking.[12] When the optical density is plotted against the test tube number, the glycine peak appears at tube number from 3 to 5 and the lysine peak, from 7 to 11. The molar ratio of lysine to glycine was 1.00 : 2.01.

ε-Benzyloxycarbonyl-L-lysine Benzyl Ester *p*-Toluenesulfonate (XVIII). The general procedure[13] for the synthesis of an amino acid benzyl ester toluenesulfonate was used in the preparation of XVIII. A mixture of ε-benzyloxycarbonyl-L-lysine (5.60 g, 20 mmol), *p*-toluenesulfonic acid monohydrate (4.56 g, 25 mmol), benzyl alcohol (10 m*l*), and benzene (20 m*l*) was heated with a Dean and Stark apparatus. After the reaction had been completed, the reaction mixture was treated with ether-petroleum ether (1 : 1). The product collected was recrystallized from ethanol-ether-petroleum ether; yield, 8.7 g (80%); mp 110—112°C; $[\alpha]_D^{20}$ −5.4° (*c* 2, dimethylformamide).

Found: C, 62.13; H, 6.52; N, 20.91%. Calcd for $C_{29}H_{34}O_7N_2S$: C, 61.96; H, 6.33; N, 20.64%.

Benzyloxycarbonyldiglycyl-ε-benzyloxycarbonyl-L-lysine Benzyl Ester (XIX). Benzyloxycarbonylglycylglycine (0.80 g, 3 mmol) was coupled with XVIII (1.63 g, 3 mmol) as has been described in the preparation of V. The product was recrystallized from ethyl acetate-ether; yield, 1.86 g (57%); mp 99—100°C; $[\alpha]_D^{20}$ −11.3° (*c* 2, dimethylformamide).

Found: C, 63.87; H, 6.06; N, 9.29%. Calcd for $C_{33}H_{38}O_8H_4$: C, 64.05; H, 6.20; N, 9.06%.

Benzyloxycarbonyldiglycyl-ε-benzyloxycarbonyl-L-lysine Hydrazide (XX). XIX (619 mg, 1 mmol) was converted to the hydrazide (XX) as in the preparation of VI. Yield, 385 mg (71%); mp 174—175°C; $[\alpha]_D^{20}$ −4.2° (*c* 2, dimethylformamide).

Found: C, 57.24; H, 6.46; N, 15.19%. Calcd for $C_{26}H_{34}O_7N_6$: C, 57.54; H, 6.33; N, 15.49%.

Trityldiglycyl - ε - benzyloxycarbonyl - L - lysine Benzyl Ester (XXI). Tritylglycylglycine (1.12 g, 3 mmol) was coupled with XVIII as in the preparation of V. The product was recrystallized from ethyl acetate - petroleum ether; yield, 1.37 g (63%); mp 115—117°C; $[\alpha]_D^{20}$ −10.0° (*c* 2, dimethylformamide).

Found: C, 63.87; H, 6.06; N, 9.29%. Calcd for $C_{33}H_{38}O_8N_4$: C, 64.05; H, 6.20; N, 9.06%.

Diglycyl - ε - benzyloxycarbonyl - L - lysine Benzyl Ester Hydrochloride (XXII·HCl). A solution of XXV (724 mg, 1 mmol) in 0.2 N hydrogen chloride in methanol (6 m*l*) was boiled for 2 min in a water bath. After the solution had then been concentrated *in vacuo*, ether was added to the residue. The resulting crystals were collected by filtration and washed with ether. The product was recrystallized from ethanol-ether; yield, 438 mg (84%); mp 155—160°C.

Found: C, 57.32; H, 6.51; N, 10.93%. Calcd for $C_{25}H_{33}O_6N_4Cl$: C, 57.62; H, 6.40; N, 10.75%.

Benzyloxycarbonyldiglycyl-ε-benzyloxycarbonyl-L-lysyl - diglycyl - ε - benzyloxycarbonyl - L - lysine Benzyl Ester (XXIII). XX (271 mg, 0.5 mmol) in glacial acetic acid (12 m*l*) and 2 N hydrochloric acid (0.63 m*l*) was treated with sodium nitrite (41.4 mg, 0.6 mmol). After 10 min, cold water (60 m*l*) was added to the solution. The azide, which precipitated as a white mass, was collected by filtration, washed with 0.5 M sodium bicarbonate and water, and then dried in an evacuated desiccator. The azide was added to a solution of XXII·HCl (270 mg, 0.5 mmol) and triethylamine (0.07 m*l*, 0.5 mmol) in dimethylformamide (10 m*l*). The mixture was stirred for 2 days at 4°C and then evaporated *in vacuo*. The resulting product was collected by filtration with the aid of water and washed with 0.5 M citric acid. It was then recrystallized from dioxane - methanol - ether - petroleum ether. Yield, 420 mg (84%); mp 148—153°C; $[\alpha]_D^{20}$ −11.6° (*c* 2, dimethylformamide).

Found: C, 61.25; H, 6.29; N, 11.28%. Calcd for $C_{51}H_{62}O_{13}N_8$: C, 61.55; H, 6.29; N, 11.26%.

Diglycyl - L - lysine Monohydrochloride (XXIV·HCl). A solution of XIX (62 mg, 0.1 mmol) in a mixture of acetic acid (1.2 m*l*), methanol (0.6 m*l*), and water (0.2 m*l*) was treated with hydrogen in the presence of palladium black. The filtrate from the catalyst was evaporated *in vacuo* to dryness. The residue was dissolved in 0.1 N hydrochloric acid (1.1 m*l*), and the solution was again evaporated *in vacuo*. The hygroscopic crystals were collected with the aid of a mixture of ethanol and acetone; yield, 23 mg (80%).

11) The R_f value refers to the paper chromatography on Toyo Roshi No. 52 with the *n*-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2, v/v) system.
12) E. Yemm and E. C. Cocking, *Analyst*, **80**, 209 (1955).
13) N. Izumiya and S. Makisumi, *Nippon Kagaku Zasshi (J. Chem. Soc. Japan, Pure Chem. Sect.)*, **78**, 1768 (1957).

TABLE 1. COMPOSITION OF THE MIXTURE OF THE ENZYME REACTION

No.	Substrate	Weighed amount of substrate	0.2 M Tris buffer (pH 8.0)	Weighed amount of trypsin	Total volume
1	—	—	0.50 ml	2.0 mg	1.0 ml
2	*cyclo*-(Gly-Gly-Lys)$_2$ (XVII)	0.01 mmol	0.50	2.0	1.0
3	ditto	0.01	0.50	—	1.0
4	H-(Gly-Gly-Lys)$_2$-OH (XXV)	0.01	0.50	2.0	1.0
5	ditto	0.01	0.50	—	1.0

Its homogeneity was established by paper chromatography (R_f 0.16[10]), paper electrophoresis (Fig. 7), and amino acid analysis (the ratio of lysine to glycine, 1.00 : 1.98).

Uchio and Izumiya prepared this compound (XXIV·HCl) by means of the hydrogenation of diglycyl-ε-benzyloxycarbonyl-L-lysine.[14]

Diglycyl - L - lysyl-diglycyl - L - lysine Dihydrochloride (XXV·2HCl). XXIII (99 mg, 0.1 mmol) was converted to the dihydrochloride (XXV·2HCl) as above. A minor change was the use of 2.2 ml of 0.1 N hydrochloric acid. The yield of the hygroscopic crystal was 72 mg (90%); R_f 0.09.[10] Amino acid analysis gave the ratio of lysine to glycine as 1.00 : 2.01. XXV in paper electrophoresis gave a single spot, while the spots produced by XXV and the tripeptide, XXIV, could not be separated from each other, as Fig. 7 shows. XXIV and XXV were, however, distinguished from each other upon paper chromatography, as is shown in Fig. 8.

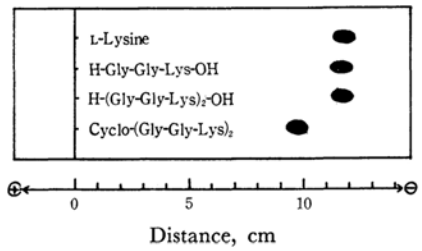

Fig. 7. Paper electrophoresis of the cyclic hexapeptide and the related compounds.

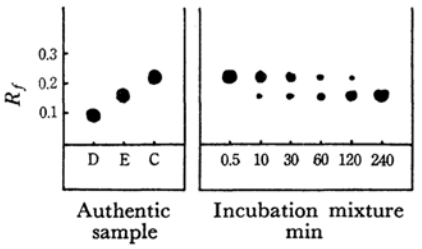

Fig. 8. Paper chromatogram of the incubation mixture of *cyclo*-(Gly-Gly-Lys)$_2$ and trypsin (No. 2 in Table 1).
Authentic sample:
C, *cyclo*-(Gly-Gly-Lys)$_2$
D, H-(Gly-Gly-Lys)$_2$-OH
E, H-Gly-Gly-Lys-OH

Action of Trypsin on *cyclo*-(Gly-Gly-L-Lys)$_2$ and Linear H-(Gly-Gly-L-Lys)$_2$-OH. Hydrolysis experiments on substrates using trypsin were carried out at pH 8.5 and 30°C, the initial concentration of the substrates was 0.01 M. The progress of the reactions was checked by paper chromatographies[11] as a function of the time. Table 1 shows the composition of the reaction mixture. The control experiments (Nos. 1, 3 and 5) showed that neither the hydrolysis of the cyclic peptides and the linear hexapeptide in the absence of the enzyme nor the autolysis of trypsin occurred. After incubation for 4 hr, the cyclic hexapeptide (XVII) was completely hydrolyzed to the linear tripeptide, as may be seen in Fig. 8. The fact that the product of this enzymatic reaction was the corresponding linear tripeptide, H-Gly-Gly-Lys-OH, was proved by chromatographic comparison with an authentic sample of XXIV·HCl. On the other hand, the linear hexapeptide XXV was completely hydrolyzed to the linear tripeptide immediately after trypsin was added (Fig. 9).

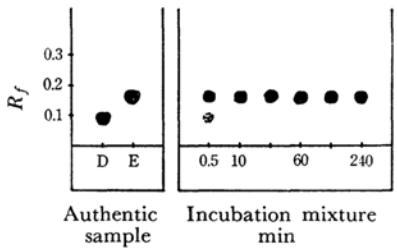

Fig. 9. Paper chromatogram of the incubation mixture of H-(Gly-Gly-Lys)$_2$-OH and trypsin (No. 4 in Table 1). D and E, see Fig. 8.

14) H. Uchio and N. Izumiya, *Memoris of the Faculty of Science, Kyushu University, Ser. C, Chemistry*, **3**, 5 (1958).