# *Syntheses of Peptides Containing Proline. I. Syntheses of Glycyl-L-prolyl-L-leucyl-glycyl-L-proline and its Related Compounds*

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Collagenase is a proteinase with a very high degree of substrate specificity, and its nature has been the object of keen interest of workers both in the fields of biochemistry and medicine. Very recently several preliminary reports[1-4] have been published and we seem to have some clue to look into the whole picture of the action of collagenase.

It had already been shown by several workers[3,5-8] that when collagen was digested with collagenase, most of the *N*-terminal groups belonged to glycine and several amino acids were found at the *C*-terminal ends. Nagai and Noda[2] found that poly-(L-prolyl-L-leucyl-glycyl)[9] was entirely hydrolyzed by collagenase, mostly into tripeptides, glycyl-prolyl-leucine and peptides from both the ends, prolyl-leucyl-glycyl-prolyl-leucine and glycyl-prolyl-leucyl-glycine. Tripeptides similar to the above were also isolated from collagenase digested collagen[7,10]. From these results and also from the accumulated informations[4,6,11-13] of oligopeptides unsusceptible to the action of collagenase, it was contemplated that the substrate specificity of collagenase requires the presence of two proline residues at a proper distance.

The syntheses of peptides containing two proline residues in the sequence of X-Pro-Leu-Gly-Pro-Y which would be a possible good substrate were carried out in our laboratory. The pentapeptide amide, glycyl-L-prolyl-L-leucyl-glycyl-L-proline amide was split by collagenase into two peptides, glycyl-prolyl-leucine and glycyl-proline amide[2]. This pentapeptide amide was the first example of the synthetic oligo-peptide as a good substrate of collagenase, and furnished a good starting inertia for us to elucidate the substrate specificity of the enzyme. Subsequently, many kinds of peptide containing proline, hydroxyproline or sarcosine were synthesized along this line and a preliminary report on an enzymatic test of those peptides has already been published[14].

In the present series of reports, the synthetic studies of those peptides containing proline will be described successively.

## Syntheses of Peptides

In the planning of the synthetic routes of the pentapeptide, a precaution was taken not to use routes passing through dipeptide esters containing proline because free esters of proline dipeptides are considered to have a great tendency for diketopiperazine formation[15]. In the present study, almost all of the possible routes which conformed with the above precaution, as indicated befow, were tried, and the yield, crystallizability and specific rotation of products were compared for each route. The synthetic routes tested were as follows*:

(1) **a.** Z-Gly-$N_3$ + H-Pro-OEt
$\longrightarrow$ {Z-Gly-Pro-OEt}
$\longrightarrow$ Z-Gly-Pro-OH (I)

**b.** Z-Gly-OH + H-Pro-OEt
M.A.
$\longrightarrow$ {Z-Gly-Pro-OEt}
$\longrightarrow$ Z-Gly-Pro-OH (I)

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* Z = Carbobenzoxy; M. A. = isovaleroyl mixed anhydride method; { } shows a material failed to crystallize, the purity of which is not confirmed by elementary analysis; [ ] shows a material failed to crystallize but obtained as a powder, the purity of which is confirmed by elementary analysis.

(2) Z-Leu-OH + H-Gly-OEt
$\xrightarrow{\text{M.A.}}$ Z-Leu-Gly-OEt (II)
$\longrightarrow$ Z-Leu-Gly-NH-$NH_2$ (III)
II $\longrightarrow$ Z-Leu-Gly-OH (IV)

(3) **a.** Z-Leu-Gly-$N_3$ ($\longleftarrow$ III)
+H-Pro-OEt
$\longrightarrow$ Z-Leu-Gly-Pro-OEt (V)

**b.** IV + H-Pro-OEt $\xrightarrow{\text{M.A.}}$ V

(4) Z-Pro-OH + H-Leu-Gly-OEt ($\longleftarrow$ II)
$\xrightarrow{\text{M.A.}}$ Z-Pro-Leu-Gly-OEt (VI)
VI $\longrightarrow$ Z-Pro-Leu-Gly-NH-$NH_2$ (VII)

(5) **a.** Z-Pro-OH + H-Leu-Gly-Pro-OEt
($\longleftarrow$ V)
$\xrightarrow{\text{M.A.}}$ {Z-Pro-Leu-Gly-Pro-OEt} (VIII)

**b.** Z-Pro-Leu-Gly-$N_3$
($\longleftarrow$ VII) + H-Pro-OEt
$\longrightarrow$ {Z-Pro-Leu-Gly-Pro-OEt} (VIII)
VIII $\longrightarrow$ [Z-Pro-Leu-Gly-Pro-OH]
(IX) $\longrightarrow$ [H-Pro-Leu-Gly-Pro-OH] (X)

(6) **a.** Z-Gly-$N_3$ + H-Pro-Leu-Gly-OEt
($\longleftarrow$ VI)
$\longrightarrow$ Z-Gly-Pro-Leu-Gly-OEt (XI)

**b.** I + H-Leu-Gly-OEt ($\longleftarrow$ II) $\xrightarrow{\text{M.A.}}$
XI $\longrightarrow$ Z-Gly-Pro-Leu-Gly-OH (XII)
XI $\longrightarrow$ Z-Gly-Pro-Leu-Gly-NH-$NH_2$ (XIII)

(7) **a.** Z-Gly-$N_3$ + H-Pro-Leu-Gly-Pro-OEt
($\longleftarrow$ VIII)
$\longrightarrow$ {Z-Gly-Pro-Leu-Gly-Pro-OEt} (XIV)

**b.** I + H-Leu-Gly-Pro-OEt ($\longleftarrow$ V)
$\xrightarrow{\text{M.A.}}$ {Z-Gly-Pro-Leu-Gly-Pro-OEt} (XIV)

**c.** XII + H-Pro-OEt
$\xrightarrow{\text{M.A.}}$ {Z-Gly-Pro-Leu-Gly-Pro-OEt} (XIV)

**d.** Z-Gly-Pro-Leu-Gly-$N_3$
($\longleftarrow$ XIII) + H-Pro-OEt
$\longrightarrow$ {Z-Gly-Pro-Leu-Gly-Pro-OEt} (XIV)

XIV $\longrightarrow$ Z-Gly-Pro-Leu-Gly-Pro-OH (XV)
$\longrightarrow$ [H-Gly-Pro-Leu-Gly-Pro-OH] (XVI)
XIV $\longrightarrow$ [Z-Gly-Pro-Leu-Gly-Pro-$NH_2$] (XVII)
$\longrightarrow$ [H-Gly-Pro-Leu-Gly-Pro-$NH_2$] (XVIII)

Methods of peptide-bond formation employed in the present study, were the azide method and the isovaleroyl mixed anhydride method[16]. The azide method is well known to be suitable for keeping the optical purity of peptides in the course of the reaction[17]. The mixed anhydride method is a comparatively simple method of the activation of the carboxyl component, and had been used successfully by Ressler and du Vigneaud[18] for the coupling reaction of carbobenzoxy-L-proline and L-leucyl-glycine ethylester. In every case of the azide procedure used in our present study, it was to be noted that carbobenzoxypeptide azide containing proline residue was insoluble in ether, and chloroform was the most suitable solvent for extracting the azide formed. In regard to the acylation of the proline imino group, the azide method was superior to the mixed anhydride method in view of the synthetic yield, crystallizability and melting point of the products. For example, carbobenzoxyglycyl-L-proline (I) which was prepared by the azide method from carbobenzoxyglycine hydrazide and L-proline ethylester was sufficiently pure without recrystallization, and the yield was also satisfactory (m. p. 155°C, yield 75%). On the other hand, the mixed anhydride method gave the substance I with a lower melting point (153°C) in poorer yield (42%). Similar results were obtained in cases of coupling reactions of carbobenzoxy-L-leucyl-glycine with L-proline ethylester, and of carbobenzoxyglycyl-L-prolyl-L-leucyl-glycine with L-proline ethylester. Recently, Bodanszky and du Vigneard[19] have published an interesting communication, in which they proposed the use of carbobenzoxyamino acid *p*-nitrophenylesters for acylation of peptide esters. If their new procedures would be employed in parts of our procedures, better results might be obtained.

Among the synthetic routes for the carbobenzoxypentapeptide ester (XIV), the best result was obtained by route 7-d. Since the

16) J. R. Vaughan, Jr. and R. L. Osato, *J. Am. Chem. Soc.*, 73, 5553 (1951).
17) M. B. North and G. T. Young, *Chemistry & Industry*, 1567 (1955).
18) C. Ressler and V. du Vigneaud, *J. Am. Chem. Soc.*, 76, 3107 (1954).
19) M. Bodanszky and V. du Vigneaud, ibid., 81, 5688 (1959).

substance XIV has not been heretofore crystallized, the synthetic yields for those procedures, 7-b, c and d, were compared with each other on the saponified product XV. The route 7-a was eliminated, since the substance VIII which was used as the intermediate in the route was not crystallized, and purification of which was unsuccessful. Substance XV prepared by route 7-d was readily crystallized under the presence of ethyl acetate, but the same substance XV prepared by route 7-b or 7-c was hard to crystallize. The route 7-b was abandoned for the reason that the crystallizability of the intermediate V was poor and of which yield was rather low. Although both intermediate substances XII and XIII were easily crystallized and were obtained from XI in good yields, mixed anhydride route 7-c was somewhat inferior to azide route 7-d as described above. The intermediate XI was prepared through two routes 6-a and 6-b, and the former was somewhat superior to the latter in synthetic yield. In regard to the optical purity, every preparations (XV) obtained by routes 7-b, c and a showed the same specific rotation after recrystallization.

Carbobenzoxy-L-prolyl-L-leucyl-glycyl-L-proline ethylester (VIII) was prepared not only as an intermediate for the substance XIV, but also as a substrate for collagenase. In the synthesis of the substance VIII, two routes, mixed anhydride procedure (5-a) and azide procedure (5-b), were taken into consideration. Since the substances VIII and its saponified product IX were not crystallized, formation of by-products, separation of which seemed to be difficult, was particularly undesirable. In the similar cases of 7-b and 7-d, which corresponded respectively to 5-a and 5-b, it was observed previously that the former was inferior to the latter in the purity and yield of the product. Then, the route 5-a was not explored in this case. Carbobenzoxy-L-prolyl-L-leucyl-glycine hydrazide (VII), which was used in route 5-b, was water-soluble substance and it was very interesting, that this substance was crystallizable only from toluene and this crystal contained crystallization toluene, which could be removed completely when the compound was heated at 50°C in vacuo.

Peptides or their derivatives containing proline residue in general are hydrophilic and peptides containing two or more proline residues, especially, were hard to be crystallized because of the effect of humidity. It was, however, found that carbobenzoxyglycyl-L-prolyl-L-leucyl-glycyl-L-proline (XV) was an exceptional example of readily crystallizable substance as described above. In this case, both ethyl acetate and water were indispensable for crystallization. When the peptide XV was recrystallized from ethyl acetate which contains equimolar amount of water to the peptide, it formed prisms. The crystal contained one mole each of water, and ethyl acetate per mole of the peptide as crystallization solvent and was fairly stable even when it was kept in the open air. In turn, when the same peptide was recrystallized from water-saturated ethyl acetate, different crystals (plates) were obtained, and they were unstable in the open air and gradually transformed into opaque crystals. A crystallographic study on these phenomena is now in progress by Sasada and Kakudo in our laboratory with X-ray diffraction technique.

As described above, the interesting phenomenon that some kinds of peptide derivatives (VII and XV) have affinity with specific substances (toluene or ethyl acetate) seemed to suggest a possible mode of combination in the enzyme-substrate complex formation. Similarly, the penta-peptide amide (XVIII) which was dried azeotropically by using toluene or ethanol occluded the solvents firmly, and complete removal of the solvents was very hard.

The above mentioned carbobenzoxypentapeptide XV was the best substrate for collagenase as described in the previous communication[14]. The substances IX, XV, XVI and XVIII were all hydrolyzed completely by collagenase within a comparatively short period of time. Studies on the enzymatic digestion of these peptides will be published elsewhere in the near future.

## Experimental*

**Carbobenzoxyglycyl-L-proline (I). — a.** Carbobenzoxyglycine hydrazide (11.2 g., 0.05 mol.) was dissolved in a mixture of acetic acid (40 ml.) and water (80 ml.), and 6 N hydrochloric acid (17 ml., 0.1 mol.) was added into the solution. The mixture was cooled to −5°C, shaken well with a small amount of chloroform and carefully treated with sodium nitrite (3.8 g., 0.055 mol.). The carbobenzoxyglycine azide formed was extracted three times with cold chloroform (total 150 ml.). The combined extract was washed with ice water (50 ml.) and acids for a short time over anhydrous sodium sulfate at −5°C. The dried solution was filtered into a mixture of proline ethylester hydrochloride (10 g., 0.055 mol.) and triethylamine (5.6 g., 0.055 mol.) in chloroform (50 ml.) at −5°C, and allowed to stand overnight at 0∼4°C. The reaction mixture was washed successively with 0.5 N hydrochloric acid, water and 5% sodium bicarbonate solution and dried over anhydrous magnesium sulfate. The dried solution was concentrated to syrup under reduced pressure and the residue was redissolved in methanol (100 ml.), and then 2 N sodium hydroxide (25 ml., 0.05 M) was added. After the solution was kept for 2 hr. at room temperature, it was neutralized with 6 N hydrochloric acid, and the methanol

* All melting points were uncorrected.

was removed under reduced pressure. As soon as methanol was removed, colorless prisms appeared. After being stored overnight in a refrigerator, the crystals were filtered off, washed with water and ethyl acetate, and dried in a desiccator. The yield of I with m. p. 154.5~155°C was 75% (11.5 g.). This substance was pure enough without recrystallization for preparing higher peptides. Recrystallization from ethyl acetate of the product (1.4 g.) gave prisms (1.2 g.); m. p. 154.5~155°C, $[\alpha]_D^{15}$ −70.4° (c. 6, ethanol); Bergmann et al.[20] reported m. p. 156°C (corr.).

**b.** Carbobenzoxyglycine (2.1 g., 0.01 M) was dissolved in chloroform (40 ml.) by adding triethylamine (1.0 g., 0.01 M), and then toluene (40 ml.) was added into the solution. The mixture was cooled to −10°C and isovaleroyl chloride (1.2 g., 0.01 M) was added slowly into the solution under stirring. After about 1.5 hr. a solution of proline ethylester hydrochloride (2.16 g., 0.012 M) and triethylamine (1.2 g., 0.012 M) in chloroform (40 ml.) was added into the mixed anhydride solution, and the mixture was allowed to stand overnight at 4°C. The reaction mixture was treated as described above. Yield of I was 42% (1.3 g.); m. p. 152~154°C. Recrystallization from ethyl acetate of the product (1.3 g.) gave fine prisms (1.1 g.); m. p. 153~154°C, $[\alpha]_D^{15}$ −70.2° (c. 5, ethanol).

**Carbobenzoxy-L-leucyl-glycine Ethylester (II).**—This substance was prepared according to the procedures of Vaughan and Osato[16]; yield 70%, m. p. 102~104°C, $[\alpha]_D^{15}$ −26.8° (c. 5.5, ethanol).

**Carbobenzoxy-L-leucyl-glycine Hydrazide (III).**—Into a solution of II (10 g.) in ethanol (40 ml.) was added hydrazine hydrate (8 ml.), and then the mixture was allowed to stand overnight at room temperature. The reaction mixture was concentrated to dryness under reduced pressure and the residual crystalline mass was recrystallized from ethanol and ether to give needles; m. p. 130~132°C, yield 89% (8.5 g.).

Found: C, 56.97; H, 7.29; N, 16.71. Calcd. for $C_{16}H_{24}O_4N_4$: C, 57.13; H, 7.19; N, 16.66%.

**Carbobenzoxy-L-leucyl-glycine (IV).**—This substance was prepared from II according to procedures of Bergmann et al.[21]; m. p. 114~115°C, yield 90%, $[\alpha]_D^{15}$ −26.3° (c. 8, ethanol).

**Carbobenzoxy-L-leucyl-glycyl-L-proline Ethylester (V).**—**a.** A solution of carbobenzoxy-L-leucyl-glycine azide in chloroform, which was prepared from III (3.4 g.) as in the case of I-a was added into a mixture of proline ethylester hydrochloride (2 g.) and triethylamine (1.1 g.) in chloroform (20 ml.) at −5°C, and the mixture was allowed to stand overnight. The reaction mixture was washed successively with water, 1 N hydrochloric acid, water, 5% sodium bicarbonate and water again, and dried. The dried solution was concentrated to syrup and the residue was crystallized in a refrigerator after treatment with petroleum benzine. The crude crystals formed were triturated with a small amount of ethyl acetate and carefully recrystallized with a small amount of petroleum benzine. The crystals were filtered off, washed with ether and dried in a desiccator in vacuo to give fine needles; m. p. 81~85°C, yield 80% (3.6 g.). Recrystallization of the product from ethyl acetate-petroleum benzine gave needles with m. p. 89~91°C; yield 44% (2.0 g.), $[\alpha]_D^{17}$ −78.0° (c. 6, ethanol). A second crop of the recrystallization was obtained in 11% yield (0.5 g.); m. p. 88~91°C.

Found: C, 61.75; H, 7.25; N, 9.64. Calcd. for $C_{23}H_{33}O_6N_3$: C, 61.72; H, 7.43; N, 9.36%.

**b.** Carbobenzoxy-L-leucyl-glycine (IV) (3.2 g.) was dissolved in chloroform (50 ml.) by adding triethylamine (1 g.) and then toluene (50 ml.) was added to the solution. The mixture was cooled to −10°C and isovaleroyl chloride (1.2 g.) was added slowly to the solution under stirring. After about 1.5 hr. a solution of proline ethylester hydrochloride (2.16 g.) and triethylamine (1.2 g.) in chloroform (40 ml.) was added to the mixed anhydride solution, and then the mixture was allowed to stand overnight at 4°C. The reaction mixture was treated as described above to give a crude product; yield 67% (3 g.). Recrystallization from ethyl acetate-petroleum benzine gave fine needles; m. p. 88~91°C, yield 46% (2.1 g.), $[\alpha]_D^{17}$ −77.8° (c. 4, ethanol).

**Carbobenzoxy-L-prolyl-L-leucyl-glycine Ethylester (VI).**—This substance was prepared according to the procedures of Ressler and du Vigneaud[18]; m.p. 148.5~150.5°C, yield 88~90%. Recrystallization from ethanol-water gave 70% yield, m. p. 150~151.5°C, $[\alpha]_D^{15}$ −81.9° (c. 2, ethanol); second crop from the mother liquor gave 13% yield, m. p. 149~150°C.

**Carbobenzoxy-L-prolyl-L-leucyl-glycine Hydrazide (VII).**—Hydrazine hydrate (2 ml.) was added to a solution of VI (2.24 g.) in ethanol (30 ml.) and then the mixture was allowed to stand overnight at room temperature. The reaction mixture was concentrated to syrup under reduced pressure and the residual syrup was dried by the addition of benzene followed by evaporation under reduced pressure. After the drying procedures were repeated, the residue was crystallized by trituration with toluene (about 10 ml.) and dried over sodium hydroxide at room temperature to give fine needles; yield 91% (2.4 g.), m. p. 76~80°C. This substance contained about one mole of crystallization toluene.

Found: C, 62.82; H, 7.44; N, 14.14. Calcd. for $C_{21}H_{31}O_5N_5 \cdot 3/4C_7H_8$: C, 62.73; H, 7.42; N, 13.94%.

After dryness in vacuo at 50°C, the crystallization toluene was removed.

Found: C, 58.27; H, 7.26; N, 16.40. Calcd. for $C_{21}H_{31}O_5N_5$: C, 58.18; H, 7.21; N, 16.16%.

**Carbobenzoxy-L-prolyl-L-leucyl-glycyl-L-proline (IX).**—**b.** A carbobenzoxy-L-prolyl-L-leucyl-glycine azide solution, which was prepared from VII (toluene-free 2.15 g.) as in the case of I-a, was added to a mixture of proline ethylester hydrochloride (1 g.) and triethylamine (0.56 g.) in chloroform (20 ml.) at −5°C. After being kept overnight at 4°C, the solution was washed successively with water, 1 N hydrochloric acid, water, 5% sodium bicarbonate solution and again with water, and dried. The dried solution was concentrated to syrup under reduced pressure and the residue was

20) M. Bergmann et al., *Ber.*, **65**, 1192 (1932); *Z. physiol. Chem.*, **212**, 72 (1932).

21) M. Bergmann et al., *J. Biol. Chem.*, **111**, 225 (1935).

redissolved in methanol (30 ml.). 1 N Sodium hydroxide (8 ml.) was added into the methanol solution, and the mixture was allowed to stand for about 3 hr. at room temperature. After neutralizing the reaction mixture with 6 N hydrochloric acid, it was concentrated to syrup under reduced pressure and the remaining oily material was dissolved in 5% sodium bicarbonate solution. The bicarbonate solution was washed well with ethyl acetate and then acidfied with 6 N hydrochloric acid. The separated oil was extracted with chloroform. On evaporation of the solvent a syrup was obtained and was solidified by trituration with petroleum benzine. After dryness in vacuo at 80°C, the amorphous powder amounted to 54% yield (1.4 g.); m.p. 90~120°C (sintered at 90°C), $[\alpha]_D^{17}$ −106.1° (c. 5.5, ethanol).

Found: C, 61.20; H, 7.29; N, 10.38. Calcd. for $C_{26}H_{36}O_7N_4$: C, 60.45; H, 7.02; N, 10.85%.

**L-Prolyl-L-leucyl-glycyl-L-proline (X).**—Palladium-charcoal (5%, 1 g.) was added to a solution of IX (1 g.) in a mixture of methanol (20 ml.) and water (30 ml.), and hydrogen was introduced into the suspension for 3 hr. under stirring. The catalyst was filtered off and washed three times with hot water (total 30 ml.), and then the combined filtrate was concentrated under reduced pressure. The residual syrup was dissolved in small amount of water, and the solution was filtered. The clear solution was concentrated to syrup and the residue was dried by the addition of benzene followed by evaporation under reduced pressure, and then pulverized. After dryness in vacuo at 110°C for about 3 hr., the product amounted to an 80% yield (0.6 g.); deliquescent powder, m. p. 170~180°C (sintered at 147~150°C), $[\alpha]_D^{15}$ −121.5° (c. 5, water).

Found: C, 55.94; H, 7.86; N, 14.32. Calcd. for $C_{19}H_{30}O_5N_4 \cdot 1/6H_2O$: C, 56.09; H, 7.93; N, 14.54%.

Complete dryness of this substance failed because the substance was partially charred when heated at 135°C for 3 hr. in vacuo.

**Carbobenzoxyglycyl-L-prolyl-L-leucyl-glycine Ethylester (XI).**—**a.** Palladium-charcoal (5%, 5 g.) and 6 N hydrochloric acid (6 ml.) was added to a solution of VI (13.4 g.) in ethanol (200 ml.), and hydrogen was introduced into the suspension for 3 hr. under stirring. The catalyst was filtered off and washed three times with ethanol (total 60 ml.), and then the combined filtrate was concentrated under reduced pressure. The residual syrup was dried by the addition of toluene followed by evaporation under reduced pressure. A carbobenzoxyglycine azide solution, which was prepared from carbobenzoxyglyciues hydrazide (6.7 g.) according to the procednre in the case of I-a was added into a mixture of the prolyl-leucyl-glycine ethylester hydrochloride and triethylamine (3 g.) in chloroform (100 ml.). After storage overnight in a refrigerator at 4°C, the reaction mixture was washed successively with 1 N hydrochloric acid, water, 5% sodium bicarbonate solution and water again, and dried. Evaporation of the solvent gave a syrupy material which was crystallized by the addition of chloroform and petroleum benzine. The yield of the crude product was 80% (12 g.); m. p. 96~101°C (after dryness in vacuo at 80°C). This substance was used for the preparation of XII and XIII without recrystallization. Melting point of this substance rose to 101~104°C after two recrystallization from chloroform and petroleum benzene; $[\alpha]_D^{15}$ −69.9° (c. 5, ethanol).

Found: C, 59.47; H, 7.10; N, 11.15. Calcd. for $C_{25}H_{36}O_7N_4$: C, 59.51; H, 7.19; N, 11.10%.

**b.** The substance II (10 g.) was hydrogenated in ethanol (50 ml.) containing 6 N hydrochloric acid (5 ml.) with 5% palladium-charcoal (4 g.). The catalyst was filtered off and washed three times with ethanol, and then the combined filtrate was concentrated under reduced pressure. The residual syrup was dried by the addition of toluene followed by evaporation under reduced pressure. A mixture of the leucyl-glycine ethylester hydrochloride and triethylamine (3 g.) in chloroform (30 ml.) was added to a mixed anhydride solution at −10°C, which was prepared from I (8 g.), triethylamine (2.65 g.) and isovaleroyl chloride (3.15 g.) in a mixture of chloroform (100 ml.) and toluene (100 ml.) as in the case of I-b. After being kept overnight at 4°C, the reaction mixture was treated in a similar manner to the case of XI-a and a crude product was obtained in a 78% yield (10.3 g.); m. p. 96~101°C (dried in vacuo at 80°C). After several recrystallizations from chloroform-petroleum benzine, this substance melted at 101~105°C; $[\alpha]_D^{15}$ −70.5° (c. 5, ethanol).

**Carbobenzoxyglycyl-L-prolyl-L-leucyl-glycine (XII).**—The crude product of XI-a (5 g.) was dissolved in methanol (50 ml.) and 1 N sodium hydroxide (11 ml.) was added into the solution, and then the mixture was allowed to stand at room temperature for 2 hr. The mixture was neutralized with 6 N hydrochloric acid, and the methanol was distilled under reduced pressure. The crystals formed were filtered off, washed with water and ethyl acetate, and dried; m. p. 184~187°C, yield 87% (4.1 g.). After recrystallization from ethanol-water (1 : 1), this substance melted at 185~188°C; $[\alpha]_D^{15}$ −60.4° (c. 6.5, pyridine).

Found: C, 58.14; H, 6.90; N, 11.76. Calcd. for $C_{23}H_{32}O_7N_4$: C, 57.97; H, 6.77; N, 11.76%.

This substance was prepared from XI-b (3.2 g.) in a yield of 86% (2.6 g.); m. p. 184~188°C, $[\alpha]_D^{15}$ −60.3° (c. 5, pyridine).

**Carbobenzoxyglycyl-L-prolyl-L-leucyl-glycine Hydrazide (XIII).**—The crude XI-a (10.1 g.) was dissolved in ethanol (50 ml.) and hydrazine hydrate (5 ml.) was added to the solution. After being kept overnight at room temperature, the reaction mixture was concentrated to dryness, and the residual syrup was crystallized from ethanol and ether to give long needles; m. p. 155~157°C, yield 97% (9.5 g.). Recrystallization from ethanol and petroleum benzine gave a 94% yield (9.2 g.); m. p. 157~159°C.

Found: C, 56.34; H, 7.04; N, 17.24. Calcd. for $C_{23}H_{34}O_6N_6$: C, 56.31; H, 6.99; N, 17.13%.

This substance was prepared from XI-b (8.8 g.) in a yield of 94% (8.1 g.); m. p. 157~159°C.

**Carbobenzoxyglycyl-L-prolyl-L-leucyl-glycyl-L-proline (XV).**—**d.** An azide solution which was prepared from XIII (8 g.) as in the case of I-a,

was added to a solution of proline ethylester hydrochloride (3.2 g.) and triethylamine (1.8 g.) in chloroform (20 ml.) at −5°C. The reaction mixture was allowed to stand overnight at 4°C, and was then washed successively with water, 1 N hydrochloric acid, water, 5% sodium bicarbonate solution and water again, and dried. The dried solution was concentrated to syrup under reduced pressure and the residue was redissolved in methanol (100 ml.). 1 N sodium hydroxide (20 ml.) was added into the methanol solution and the mixture was allowed to stand for about 3 hr. at room temperature. After neutralizing the reaction mixture with 6 N hydrochloric acid, it was concentrated to dryness under reduced pressure and the remaining syrup was dissolved in saturated sodium bicarbonate solution. The bicarbonate solution was washed with ethyl acetate and then acidified with 6 N hydrochloric acid. The separated oil was extracted with chloroform and the extract was concentrated to syrup, which was crystallized by trituration with ethyl acetate containing a small amount of water. After storage overnight in a refrigerator, the crystals were filtered off and dried in vacuo at 80°C for about 3 hr. to give 5.0 g. of the first crop. On evaporation of the ethyl acetate washing described above, a slight amount of residue was obtained. The residue was again saponified in the same manner and a second crop (0.6 g.) was obtained. The total yield was 60%. Recrystallization of the product from ethyl acetate containing an equimolar amount of water to the peptide, gave prisms (6 g.) with m. p. 72°C (dec. at 75~77°C), which were dried in the open air. This material contained one mole of ethyl acetate and one mole of water as crystallization solvents.

Found: C, 56.55; H, 7.44; N, 10.39. Calcd. for $C_{28}H_{39}O_8N_5 \cdot C_4H_8O_2 \cdot H_2O$: C, 56.54; H, 7.27; N, 10.30%.

The crystallization solvents could be removed at 80°C in vacuo to give an amorphous powder; m. p. 130~135°C, $[\alpha]_D^{15}$ −128.2° (c. 4, ethanol).

Found: C, 58.49; H, 6.84; N, 12.23. Calcd. for $C_{28}H_{39}O_8N_5$: C, 58.62; H, 6.85; N, 12.21%.

The route XIV-b-XV gave 35% yield of the amorphous powder; $[\alpha]_D^{15}$ −128.2° (c. 4, ethanol). The route XIV-c-XV gave a 45% yield of the same substance; $[\alpha]_D^{15}$ −128.2° (c. 4, ethanol).

**Glycyl-L-prolyl-L-leucyl-glycyl-L-proline (XVI).** — Palladium-charcoal (5%, 1 g.) was added to a solution of XV (1.7 g.) (amorphous powder) in methanol (20 ml.) and water (30 ml.), and hydrogen was introduced into the suspension for 3 hr. under stirring. The catalyst was filtered off and washed three times with hot water (total 30 ml.), and then the combined filtrate was concentrated under reduced pressure. The residual syrup was dissolved in a small amount of water, and the solution was filtered. The clear solution was concentrated to syrup and the residue was dried by the addition of toluene followed by evaporation under reduced pressure, and then pulverized. After dryness in vacuo at 110°C for about 3 hr., the product amounted to a 51% yield (0.8 g.) of deliquescent powder; m. p. 160~167°C, $[\alpha]_D^{15}$ −160.1° (c. 5.5, water.)

Found: C, 53.72; H, 7.65; N, 15.71. Calcd. for $C_{20}H_{33}O_6N_5 \cdot 1/2H_2O$: C, 53.56; H, 7.64; N, 15.62%.

**Carbobenzoxyglycyl-L-prolyl-L-leucyl-glycyl-L-proline Amide (XVII).**—The syrupy material XIV which was prepared from XIII (4.9 g.) according to the procedure XV-d was dissolved in methanol (40 ml.). After being saturated with dry ammonia gas at 0°C, the solution was allowed to stand one week at room temperature in a sealed tube. The reaction mixture was concentrated to syrup under reduced pressure and the residue was dried by the addition of benzene followed by evaporation under reduced pressure. The dried residue was dissolved in hot ethyl acetate and reprecipitated by the addition of petroleum benzine, and dried over phosphrous pentoxide in vacuo at 80°C; yield 63% (3.6 g.), m. p. 118~128°C (sintered at 102~118°C), $[\alpha]_D^{17}$ −117° (c. 4.5, ethanol).

Found: C, 58.73; H, 7.23; N, 14.60. Calcd. for $C_{28}H_{40}O_7N_6$: C, 58.72; H, 7.04; N, 14.68%.

**Glycyl-L-prolyl-L-leucyl-glycyl-L-proline Amide (XVIII).**—Palladium-charcoal (5%, 1 g.) was added to a solution of XVII (1.5 g.) in ethanol (20 ml.) and water (20 ml.), and hydrogen was introduced into the suspension for 3 hr. under stirring. The catalyst was filtered off and washed three times with hot water (total 30 ml.), and then the combined filtrate was concentrated under reduced pressure. The residual syrup was dissolved in a small amount of water, and the solution was filtered. The clear solution was concentrated to syrup and the residue was dried by the addition of ethanol followed by evaporation under reduced pressure, and then pulverized. This material was dried at 110°C in vacuo for 3 hr. before analysis; deliquescent powder, yield of 82% (0.95 g.), m. p. 135~155°C (sintered at 110~115°C), $[\alpha]_D^{17}$ −147.2°C (c. 3.3, ethanol).

Found: C, 54.25; H, 7.76; N, 19.04. Calcd. for $C_{20}H_{34}O_5N_6$: C, 54.78; H, 7.82; N, 19.17%.

## Summary

Glycyl-L-prolyl-L-leucyl-glycyl-L-proline and its related compounds were synthesized for elucidating the substrate specificity of collagenase. In the synthetic study, almost all of the possible routes were tried and the yield, crystallizability and specific rotation of products were compared for each route. Peptides or their derivatives containing two proline residues in general were hydrophilic and hard to be crystallized, but it was found, that carbobenzoxyglycyl-L-prolyl-L-leucyl-glycyl-L-proline was an exceptional example of a readily crystallizable substance and a good substrate for collagenase.

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