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t-Amyloxycarbonyl as a New Protecting Group in Peptide Synthesis. II. Synthesis of a Hexapeptide Amide Related to Eledoisin by the *t*-Amyloxycarbonyl Method

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The synthesis of a hexapeptide amide, L-lysyl-L-phenylalanyl-L-isoleucylglycyl-L-leucyl-L-methionine amide, has been carried out as a demonstration of the *t*-amyloxycarbonyl method. This peptide is known to possess strong biological activity which is similar to that of eledoisin and bradykinin. During the synthesis, no complication occurred involving the *t*-amyloxycarbonyl group, and the final product had the expected activity. Therefore, it is considered that the *t*-amyloxycarbonyl method is useful for the synthesis of complicated peptides.

In the preceding paper,[1] the syntheses and properties of *t*-amyloxycarbonyl (AOC) amino acids were reported, and it was suggested that the use of the AOC *N*-protecting group should be valuable in peptide synthesis, because this group has similar properties to the *t*-butoxycarbonyl (BOC) group and preparation of AOC-amino acids is as easy as that of carbobenzoxy derivatives. In the present work, a hexapeptide amide (X), L-lysyl-L-phenylalanyl-L-isoleucylglycyl-L-leucyl-L-methionine amide, was synthesized to test the efficiency of the AOC-method, and a part of the compound was also synthesized by the classical method for comparison.

The syntheses of eledoisin and its analogues have already been reported by many workers,[2,3] and the hexapeptide amide X was found by Bernardi et al.[3] to possess high biological activity. In those works, mentioned above, the BOC-method was used for the synthesis together with the carbobenzoxy group to avoid an undesirable side reaction with the methionine residue. In the present work, di-AOC-lysyl-phenylalanyl-isoleucyl-glycine (IV) was first prepared from glycine benzyl ester by the stepwise elongation method[4] as shown in Scheme 1.

AOC-Ileu-OH + H-Gly-OBz
67% ↓ DCCD in THF+$CHCl_3$
AOC-Ileu-Gly-OBz (Ia)
81% ↓ 1) CF_3COOH 2) AOC-Phe-ONP
AOC-Phe-Ileu-Gly-OBz (IIa)
88% ↓ 1) CF_3COOH 2) di-AOC-Lys-ONP in DMF
di-AOC-Lys-Phe-Ileu-Gly-OBz (IIIa)
98% ↓ H_2/Pd in MeOH
di-AOC-Lys-Phe-Ileu-Gly-OH (IVa)

Scheme 1.

The classical method for synthesizing this compound is shown in Scheme 2.

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2) E. Sandrin and R. A. Boissonnas, *Experientia*, **18**, 59 (1962); B. Camerino, G. De Caro, R. A. Boissonnas, E. Sandrin and E. Stürmer, ibid., **19**, 339 (1963); E. Sandrin and R. A. Boissonnas, *Helv. Chim. Acta*, **46**, 1637 (1963); E. Schröder and K. Lübke, *Experientia*, **20**, 19 ((1964); E. Stürmer, E. Sandrin and R. A. Boissonnas, ibid., **20**, 303 (1964); E. Sandrin and R. A. Boissonnas, *Helv. Chim. Acta*, **47**, 417 (1964); K. Lübke, E. Schröder, R. Schmiechen and H. Gibian, *Ann.*, **679**, 195 (1964); F. Chillemi, *Gazz. Chim. Ital.*, **93**, 1079 (1963); L. Bernardi, G. Bosisio, R. De Castiglione, O. Goffredo and F. Chillemi, ibid., **94**, 853 (1964); F. Chillemi and O. Goffredo, ibid., **94**, 866 (1964); R. De Castiglione, F. Chillemi, L. Bernardi and O. Goffredo, ibid., **94**, 875 (1964); F. Chillemi, L. Bernardi and G. Bosisio, ibid., **94**, 891 (1964); E. Schröder, K. Lübke and R. Hempel, *Experientia*, **21**, 70 (1965).

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Z-Ileu-ONP + H-Gly-OEt
87% ↓ in $CHCl_3$
Z-Ileu-Gly-OEt (Ib)
78% ↓ 1) HBr in AcOH 2) Z-Phe-ONP in DMF
Z-Phe-Ileu-Gly-OEt (IIb)
73% ↓ 1) HBr in AcOH 2) di-AOC-Lys-ONP in DMF
di-AOC-Lys-Phe-Ileu-Gly-OEt (IIIb)
82% ↓ NaOH in pyridine
di-AOC-Lys-Phe-Ileu-Gly-OH (IVb)

Scheme 2.

During the synthesis shown in Scheme 1, no complications occurred in relation to the AOC-group; that is, removal of the AOC-groups with trifluoroacetic acid was almost quantitative, as was expected, and the crystallizability of the product at each step was quite satisfactory. On the other hand, trouble arose over the last stage of Scheme 2, and saponification of the ester group in the AOC-tetrapeptide ethyl ester (IIIb) gave only a colored product with a low melting point. Further, AOC-leucyl-methionine amide (VI) was prepared without any trouble by the active ester method, as shown in Scheme 3, and then compound VI was converted to leucyl-methionine amide (VII) using 2N hydrogen chloride in acetic acid. Condensation of the AOC-tetrapeptide IV with dipeptide amide VII was carried out using *p*-nitrophenyl trifluoroacetate as the condensing reagent according to the description of Sakakibara and Inukai.[5] In this case, AOC-tetrapeptide *p*-nitrophenylester was isolated and then coupled with compound VII (Scheme 3). Finally, the protected hexapeptide amide IX obtained was treated with 2 N hydrogen chloride in acetic acid to get the hexapeptide amide X, and it was confirmed that the removal of the AOC-groups was as smooth as that of BOC groups.

The homogeneity of the resulting hexapeptide amide X was judged by paper chromatography and by paper electrophoresis, and no contamination was detected by those procedures. The amino acid ratio in the acid hydrolyzate of compound X was determined using an automatic amino acid analyzer, and the result was also quite satisfactory. Furthermore, it was found that this compound was completely digestible by leucine aminopeptidase to give the expected amount of each amino acid, indicating that no racemization occurred during the synthesis. The biological activity of compound X was determined by the contraction test with guinea-pig ileum,[6] and the activity was 2.5 times higher than that of bradykinin which was prepared in this laboratory.[7] In conclusion, it can be asserted that the AOC method is useful in place of the BOC method for synthesis of complicated peptides.

Incidentally, during the course of the synthesis, carbobenzoxy-L-isoleucine was obtained as crystals for the first time in this laboratory.[8] Of the many carbobenzoxy derivatives of common L-amino acids, carbobenzoxy-L-leucine and this material were thought not to be crystallizable and, moreover, commercially available L-isoleucine used to be contaminated with *allo*-isoleucine and with leucine. Therefore, the crystallization of carbobenzoxy-L-isoleucine is a great advantage in the

AOC-Leu-ONP + H-Met-OMe
89% ↓ in DMF
AOC-Leu-Met-OMe (V)
72% ↓ NH_3 in MeOH
AOC-Leu-Met-NH_2 (VI)
92% ↓ 2N HCl in AcOH
H-Leu-Met-NH_2·HCl (VII)

IVa 98% + IVb 65% → CF_3COONP
di-AOC-Lys-Phe-Ileu-Gly-ONP (VIII)

83.6% ↓ in DMF
di-AOC-Lys-Phe-Ileu-Gly-Leu-Met-NH_2 (IX)
↓ 2 N HCl in AcOH
2HCl·Lys-Phe-Ileu-Gly-Leu-Met-NH_2 (X)

Scheme 3.

5) S. Sakakibara and N. Inukai, This Bulletin, **34**, 1979 (1965).
6) T. Suzuki, Y. Mizushima, T. Sato and S. Iwanaga, *J. Biochem.* (*Japan*), **57**, 14 (1965).
7) S. Sakakibara and N. Inukai, This Bulletin, in preparation.
8) S. Inoue, "The 3rd Symposium on Peptide Chemistry (Japan)," Inst. for Protein Research, Osaka University, Osaka (1964), p. 89.

purification of this compound. The purity of the crystallized product and an oily material which was obtained from the mother liquor after recrystallization were tested with an automatic amino acid analyzer after removal of the *N*-protecting group, and only a trace of contamination was detected in the crystallized product, whereas the *allo*-isoleucine and leucine contaminating the original material were both found to be concentrated in the oily residue.

Experimental*

Crystallization of Carbobenzoxy-L-isoleucine.[8] —Crude carbobenzoxy-L-isoleucine, which was prepared from L-isoleucine[9] (131 g., 1 mol.) by the usual procedure, was purified once by sodium bicarbonate extraction, and then the oily material was treated with petroleum ether (60—80°C) at −20°C. After each extraction the upper layer was discarded, and the inside-surface of the vessel was scratched with a glass bar under fresh petroleum ether at −20°C. Then the mixture was stored in a deep freezer for about one week and a small crop of crystals were obtained in the syrup. The remaining oil was then crystallized completely by triturating the crystals thoroughly and then storing them again in the deep freezer for two or three more days. If crystal-seed is available, crude material can be crystallized directly by triturating it with the seeds at −20°C. The crude crystals thus obtained were collected by filtration at −20°C using a pre-cooled funnel. Then, the product was dissolved in a mixture of toluene (250 ml.) and petroleum ether (250 ml.), and the solution was seeded with a few crystals and kept in a deep freezer. After one or two days, about 30% of the compounds separated as crystals, while the rest separated as an oil. Then, the mixture was gradually warmed to room temperature to re-dissolve the oily part, the crystals were triturated well, and the mixture was restored in the deep freezer. After two more days, this procedure was repeated, and finally the crystals formed were collected by filtration. Filtration, washing and drying procedures in vacuo were carried out at below 10°C, and the yield of dried crystals was about 60% (157 g.); m. p. 44—46°C, $[\alpha]_D^{26}$ +6.5° (*c* 6, ethanol).

Found: C, 63.48; H, 7.24; N, 5.25. Calcd. for $C_{14}H_{19}O_4N$: C, 63.38; H, 7.22; N, 5.28%.

***t*-Amyloxycarbonyl-L-isoleucylglycine Benzyl Ester (Ia).**—A solution of *t*-amyloxycarbonyl-L-isoleucine (4.92 g., 0.02 mol.) in tetrahydrofuran (40 ml.) was cooled to −5°C, and dicyclohexylcarbodiimide (4.12 g., 0.02 mol.) was added to the solution with stirring. After about 10 min., a solution of glycine benzyl ester tosylate (6.74 g., 0.02 mol.) and triethylamine (2.8 ml., 0.02 mol.) in chloroform (20 ml.) was added to the reaction mixture. The mixture was allowed to react for one hour at −5°C, and then for additional 10 hr. at 10°C. Dicyclohexylurea formed was removed by filtration, and the filtrate was concentrated to syrup which was dissolved again in ethyl acetate (100 ml.). Then, the solution was washed thoroughly with 0.5 N hydrochloric acid, 5% sodium bicarbonate and water, and the washed solution was dried over anhydrous sodium sulfate. The dried solution was then concentrated to syrup, and the residue was redissolved to a mixture of ethyl acetate (5 ml.) and petroleum ether (60—80°C, 40 ml.) to precipitate a small amount of insoluble materials which was filtered off. The final solution was concentrated to dryness, and the residue was crystallized gradually as colorless needles. Recrystallization from a mixture of ether (1 ml.) and petroleum ether (30 ml.) gave 5.0 g. (67%) of product; m. p. 68—70.5°C, $[\alpha]_D^{22}$ −25.7° (*c* 2.0, methanol).

Found: C, 64.28; H, 8.32; N, 7.09. Calcd. for $C_{21}H_{32}O_5N_2$: C, 64.26; H, 8.22; N, 7.14%.

Carbobenzoxy-L-isoleucylglycine Ethyl Ester (Ib).—Carbobenzoxy-L-isoleucine *p*-nitrophenyl ester[4] (3.86 g., 0.01 mol.) was dissolved in a solution of glycine ethyl ester hydrochloride (1.39 g., 0.01 mol.) and triethylamine (1.4 ml., 0.01 mol.) in chloroform (20 ml.), and the mixture was allowed to react at room temperature for 20 hr. The solvent was distilled off in vacuo, and the residue was dissolved in ethyl acetate. The ethyl acetate solution was washed well with N ammonia, N hydrochloric acid and water. After drying it over anhydrous sodium sulfate, the solvent was removed by distillation under reduced pressure and the product was obtained as crystals; wt. 3.20 g. (91%), m. p. 150—152°C. Recrystallization from ethyl acetate (25 ml.) gave 2.8 g. (87%) of product; m. p. 153.5—154.5°C, $[\alpha]_D^{24}$ −26.0° (*c* 1, acetic acid); Bernardi et al.[10] reported m. p. 156—159°C.

Found: C, 62.01; H, 7.38; N, 8.06. Calcd. for $C_{18}H_{26}O_5N_2$; C, 61.70; H, 7.48; N, 8.00%.

***t*-Amyloxycarbonyl-L-phenylalanyl-L-isoleucylglycine Benzyl Ester (IIa).**—Compound Ia (4.32 g., 0.011 mol.) was dissolved in anhydrous trifluoroacetic acid (10 ml.) and the solution was allowed to stand at room temperature for 20 min. The trifluoroacetic acid was then evaporated off in vacuo, and the remaining oil was dried by flushing with toluene followed by storage over sodium hydroxide in vacuo. The residue was dissolved in dimethylformamide (25 ml.), neutralized with triethylamine (1.6 ml., 0.011 mol.) and subjected to reaction with AOC-L-phenylalanine *p*-nitrophenyl ester[1] (4.4 g., 0.011 mol.). After standing overnight at room temperature, the solution was diluted with water (60 ml.) to precipitate the product as crystals which were separated by filtration, washed well with N ammonia and with water, and dried; wt. 5.84 g. (98%). The crude material was recrystallized from 60% methanol; wt. 4.85 g. (81%), m. p. 142—144°C, $[\alpha]_D^{22}$ −13.8° (*c* 1.9, dimethylformamide), −33.3° (*c* 2, methanol.)

Found: C, 66.65; H, 7.82; N, 7.87. Calcd. for $C_{30}H_{41}O_6N_3$: C, 66.77; H, 7.66; N, 7.79%.

Carbobenzoxy-L-phenylalanyl-L-isoleucylglycine Ethyl Ester (IIb).—Compound Ib (3.5 g., 0.01 mol.) was treated with 31% hydrogen bromide in acetic acid (10.8 g.) for 40 min., and the product was precipitated by adding dry ether (150 ml.) to the reaction mixture. The separated product was washed with ether by decantation, and dried over sodium hydroxide in vacuo.

* All melting points given are uncorrected.

9) This material was obtained from Tanabe Pharmaceutical Co., Ltd., Osaka.

10) L. Bernardi, G. Bosisio, R. De Castiglione, O. Goffredo and F. Chillemi, *Gazz. Chim. Ital.*, 853 **94**, (1964).

The dried powder was dissolved in dimethylformamide, and triethylamine (1.80 ml.) was added to the solution. The triethylamine hydrobromide formed was filtered off, and the filtrate was subjected to reaction with carbobenzoxy-L-phenylalanine *p*-nitrophenyl ester (4.0 g., 0.01 mol.) for 24 hr. at room temperature. Water (100 ml.) was added to the mixture, and crystalline precipitates which appeared were filtered, washed well with N ammonia, and dried. The product was recrystallized from ethanol; wt. 3.9 g. (78%), m. p. 179.5—180.5°C, $[\alpha]_D^{24}$ −21.0° (*c* 1.7, acetic acid). Bernardi et al.[10] reported m. p. 175—176°C.

Found: C, 65.31; H, 6.90; N, 8.31. Calcd. for $C_{27}H_{33}O_6N_3$: C, 65.44; H, 6.71; N, 8.48%.

Di-*t*-amyloxycarbonyl-L-lysyl-phenylalanyl-L-isoleucylglycine Benzyl Ester (IIIa).—Compound IIa (3.78 g., 0.007 mol.) was dissolved in anhydrous trifluoroacetic acid (8 ml.), and the solution was allowed to stand at room temperature for 25 min. The trifluoroacetic acid was then evaporated to dryness in vacuo, and the residue was treated with a mixture of ether and petroleum ether to obtain crystals. The supernatant was removed by decantation, and the product was dried over sodium hydroxide in vacuo at room temperature; wt. 3.75 g., m. p. 202—203°C. This material was dissolved in dimethylformamide (30 ml.), neutralized with triethylamine (0.93 ml.) and then subjected to reaction with di-AOC-L-lysine *p*-introphenyl eater[1] (3.22 g., 0.0065 mol.). After standing overnight at room temperature, the solution was diluted with water (50 ml.) to precipitate the product, which was separated by filtration, washed well with N ammonia and with water, and dried. The product was recrystallized from 60% ethanol; wt. 4.47 g. (88%), m. p. 151—152°C, $[\alpha]_D^{22}$ −17.5° (*c* 2, dimethylformamide).

Found: C, 64.78; H, 8.13; N, 8.85. Calcd. for $C_{42}H_{63}O_9N_5$: C, 64.50; H, 8.12; N, 8.95%.

Di-*t*-amyloxycarbonyl-L-lysyl-L-phenylalanyl-L-isoleucylglycine Ethyl Ester (IIIb).—Compound IIb (1.25 g., 0.0025 mol.) was treated with 31% hydrogen bromide in acetic acid (2.6 g.) for 40 min., and the product was precipitated by adding dry ether (70 ml.) to the reaction mixture. It was separated by decantation, washed well with dry ether and dried over sodium hydroxide in vacuo. The dried powder was dissolved in dimethylformamide, and then triethylamine (0.38 ml.) was added to the solution. The triethylamine hydrobromide formed was filtered off, and the filtrate was subjected to reaction with di-*t*-amyloxycarbonyl-L-lysine *p*-nitrophenyl ester[1] (1.24 g., 0.0025 mol.) for 24 hr. at room temperature. Water (40 ml.) was added to the reaction mixture, and the precipitate which appeared was filtered, washed well with N ammonia and dried; wt. 1.64 g. (91%). This material was recrystallized from 60% ethanol; wt. 1.31 g. (73%), m. p. 162—163°C, $[\alpha]_D^{21}$ −18.5° (*c* 1.5, dimethylformamide).

Found: C, 61.71; H, 8.46; N, 9.75. Calcd. for $C_{37}H_{61}O_9N_5$: C, 61.72; H, 8.54; N, 9.73%.

Di-*t*-amyloxycarbonyl-L-lysyl-L-phenylalanyl-L-isoleucylglycine (IV).—a) Hydrogen was passed through a solution of IIIa (3.13 g., 0.004 mol.) in methanol (150 ml.) containing a small amount of palladium black for about 3 hr. The catalyst was then removed by filtration, and the filtrate was concentrated to dryness under reduced pressure. The residue was dissolved in a mixture of methanol and ethyl acetate (30 ml. each), and the product was precipitated as crystals by adding petroleum ether (60—80°C, 60 ml.). Recrystallization of the crude product from 50% methanol gave 2.74 g. (98%) of product which was dried over phosphorus pentoxide in vacuo at 60°C for 8 hr.; m. p. 127—133°C (decomp.), $[\alpha]_D^{21.5}$ −17.2° (*c* 2, dimethylformamide).

Found: C, 59.93; H, 8.48; N, 9.98. Calcd. for $C_{35}H_{57}O_9N_5 \cdot 1/2\ H_2O$: C, 59.99; H, 8.34; N, 9.99%.

b) A solution of IIIb (0.94 g., 0.0013 mol.) in pyridine (5 ml.) was treated with N sodium hydroxide solution (1.5 ml.) at room temperature for 3 hr., and then the solution was neutralized with N hydrochloric acid (1.5 ml.). The solvent was evaporated out under reduced pressure, and the residue was washed with ether and then with water. Then, the residue was dissolved in hot ethanol (10 ml.) and reprecipitated by adding water (30 ml.). It was collected by filtration and reprecipitated again from hot 50% methanol: wt. 0.74 g. (82%) after drying in vacuo at 60°C for 8 hr., m. p. 123—131°C (decomp.).

Found: C, 60.48; H, 8.41; N, 10.29. Calcd. for $C_{35}H_{57}O_9N_5 \cdot 1/2H_2O$: C, 59.99; H, 8.34; N, 9.99%.

***t*-Amyloxycarbonyl-L-leucyl-L-methionine Methyl Ester (V).**—Triethylamine (1.1 ml.) was added to a solution of L-methionine methyl ester hydrochloride (1.39 g., 0.007 mol.) in dimethylformamide (15 ml.), and the crystals formed were filtered off. The *p*-nitrophenyl ester of *t*-amyloxycarbonyl-L-leucine[1] (2.56 g., 0.007 mol.) was added to the filtrate prepared as described above, and the mixture was allowed to stand at room temperature for 20 hr. Water (120 ml.) was then added to the reaction mixture, and an oil separated which was extracted well with ethyl acetate (about 100 ml.). The ethyl acetate extract was washed thoroughly in succession with N ammonia, 0.5 N hydrochloric acid and water, and dried over anhydrous sodium sulfate. On removal of the solvent a crystalline residue was obtained, which was recrystallized from a mixture of ethyl acetate and petroleum ether; wt. 2.73 g. (89%), m. p. 92—93°C, $[\alpha]_D^{22}$ −39.5° (*c* 2.1, methanol).

Found: C, 55.53; H, 8.89; N, 7.29; S, 8.15. Calcd. for $C_{18}H_{34}O_5N_2S$: C, 55.35; H, 8.78; N, 7.17; S, 8.21%.

***t*-Amyloxycarbonyl-L-leucyl-L-methionine Amide (VI).**—A solution of V (2.0 g., 0.0051 mol.) in methanol (50 ml.) was saturated with dry ammonia gas at 0°C, and the solution was allowed to stand for 24 hr. at room temperature. The reaction mixture was concentrated to dryness under reduced pressure, and the residue was crystallized from a mixture of aqueous ethanol and acetone. The yield of product was 1.38 g. (72%); m. p. 104—106°C, $[\alpha]_D^{22}$ −37.8° (*c* 1.1, methanol).

Found: C, 54.50; H, 8.90; N, 11.36; S, 8.53. Calcd. for $C_{17}H_{33}O_4N_3S$: C, 54.37: H, 8.85; N, 11.19; S, 8.53%.

Di-*t*-amyloxycarbonyl-L-lysyl-L-phenylalanyl-L-isoleucylglycyl-L-leucyl-L-methionine Amide (IX).—Compound IVa (1.038 g., 0.0015 mol.) was dried over phosphorus pentoxide in vacuo at 80°C for 8 hr. and then dissolved in dry pyridine (3.0 ml.). *p*-Nitrophenyl trifluoroacetate (0.494 g., 0.0021 mol.) was added to the solution and the mixture was allowed to react overnight at room temperature. Then ice-cold water (20 ml.) was added to the mixture, and the precipitate

formed was separated by filtration. The product was then resuspended in water (20 ml.), collected by filtration, and dried in vacuo over phosphorus pentoxide; wt. 1.20 g. (98%), m. p. 171—173°C (decomp.). This material VIIIa was used in the following reaction without further purification. The same type compound VIIIb was prepared from IVb in 90% yield; m. p. 165—168°C (decomp.) which had to be recrystallized from 60% methanol for the following reaction; yield 65%, m. p. 176—179°C (decomp.). Compound VI (0.563 g., 0.0015 mol.) was treated with 2 N hydrogen chloride in acetic acid (5 ml.) at room temperature for 40 min., and then the reaction product was precipitated by adding ice-cold ether (30 ml.). The precipitate was collected by filtration, washed well with ether and dried over sodium hydroxide in vacuo. The product which corresponded to leucylmethionine amide hydrochloride VII (0.44 g.) was added to a solution of VIIIa (1.2 g., 0.0015 mol.) in dimethylformamide (5 ml.) together with triethylamine (0.21 ml.), and the mixture was allowed to react overnight at room temperature. Water was added in the reaction mixture to precipitate the product, which was then collected by filtration, washed well with N ammonia and with water, and dried. Repeated precipitation from dimethylformamide - ethyl acetate and then from dimethylformamide-water gave 1.16 g. (82%) of product; m. p. 233—234°C, $[\alpha]_D^{21.5}$ −26.5° (*c* 1, dimethylformamide).

Found: C, 58.91; H, 8.36; N, 12.10; S, 3.24. Calcd. for $C_{46}H_{78}O_{10}N_8S$: C, 59.07; H, 8.41; N, 11.98; S, 3.43%.

Compound VIIIb was also subjected to the same reaction with VII, and the same product was finally obtained in 85% yield; m. p. 233—234°C, $[\alpha]_D^{21.5}$ −25.9° (*c* 0.9, dimethylformamide).

L-Lysyl-L-phenylalanyl-L-isoleucylglycyl-L-leucyl-L-methionine Amide Dihydroc hloride Dihydrate (X).—Compound IX (0.2 g.) was dissolved in 2 N hydrogen chloride in acetic acid (5 ml.), and the solution was allowed to react at room temperature for 40 min. The product was precipitated by adding ice-cold ether (20 ml.) at 0°C, and the precipitate was collected by filtration washed well with ether and dried over sodium hydroxide in vacuo. The residue was dissolved in 60% methanol (4 ml.), and insoluble material was filtered off. The filtrate was concentrated to dryness over phosphorus pentoxide in vacuo to obtain the final product; wt. 0.17 g., m. p. 240—243°C (decomp.), $[\alpha]_D^{23}$ −16.8° (*c* 0.8, acetic acid); Bernardi et al. reported[3] m. p. 240—243°C (decomp.), $[\alpha]_D^{21}$ −17° (*c* 0.8, 95% acetic acid).

Found:* C, 50.00; H, 7.96; N, 13.63. Calcd. for $C_{34}H_{58}O_6N_8S \cdot 2HCl \cdot 2H_2O$: C, 50.05; H, 7.91; N, 13.73%.

The R_f values of the product in *n*-butanol : acetic acid : water (4 : 1 : 1) and *n*-butanol : pyridine : water (1 : 1 : 1) were 0.62 and 0.74 respectively (R_f values of an authentic leucine in the same solvent systems were 0.61 and 0.54 respectively); paper electrophoresis showed this compound to be homogeneous at pH 2.3 and 4.75, respectively. The amino acid ratios in the acid hydrolyzate were Lys 1.03, Phe 1.00, Ileu 0.98, Gly 1.06, Leu 1.01, Met 0.87. The amino acid ratios in the leucine-aminopeptidase digest were Lys 1.06, Phe 0.97, Ileu 0.98, Gly 1.06, Leu 1.00,Met 1.00. The biological activity, determined by the contraction test with guineapig ileum[6] was 2.5 times more than that of bradykinin prepared in this laboratory.[7]

The authors wish to express their thanks to Professor Tomoji Suzuki for determining the biological activity of the synthetic material.

* This material was dried over phosphorus pentoxide in vacuo for 10 hr. at 80°C.