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Studies on Peptides. XVIII.[1-3] Catalytic Hydrogenation of Methionine-containing Peptides

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Under anhydrous conditions, the N^{α}-benzyloxycarbonyl group attached to methionine was quantitatively removed by catalytic hydrogenation over a palladium catalyst in the presence of boron trifluoride etherate in methanol. Application of this procedure for the synthesis of methionine-peptides was demonstrated by the successful preparation of the C-terminal pentapeptides of eledoisin and physalaemin. Phenylalanylisoleucylglycylleucylmethionine amide and phenylalanyltyrosylglycylleucylmethionine amide were prepared by hydrogenation of the corresponding N^{α}-benzyloxycarbonyl derivatives in quantitative yield. It was shown also that arginylmethionine methyl ester could be prepared by catalytic hydrogenation of N^{α}-benzyloxycarbonyl-N^{G}-nitroarginine methyl ester. Partial methylation of the free carboxyl group is one of the side reaction in this procedure. Scope and limitation of catalytic hydrogenation of the benzyloxycarbonyl group in the presence of boron halides in anhydrous organic solvents were examined.

Removal of the benzyloxycarbonyl group from peptides containing the methionine residue by catalytic hydrogenation is not generally used in the peptide synthesis because of the poisoning action of the sulfur atom of methionine to the catalyst. In a few instances, the removal of the benzyloxycarbonyl group was achieved by catalytic hydrogenation in the presence of barium sulfate,[5-7] however wide applications of this procedure have not appeared in the literature so far. Recently Medziharadszky and Schweiger[8] reported the use of cyclohexylamine, instead of barium sulfate and found it effective for the removal of the benzyloxycarbonyl group by catalytic hydrogenation from the peptides containing methionine in a position other than the N-terminus. This condition suffers some limitation as regards to the position of the methionine residue in the peptide chain and such basic condition may cause the formation of diketopiperazine when peptide alkyl esters are hydrogenated.

Generally, removal of the N^{α}-benzyloxycarbonyl group from amino acids or peptides by catalytic hydrogenation is performed in the presence of appreciable amount of acetic acid, formic acid or hydrogen chloride. As early as 1949, Fruton *et al.*[9] achieved debenzyloxycarbonylation of methionine-peptides by prolonged hydrogenation over a palladium catalyst

1) The Part XVII of this series: *Chem. Pharm. Bull.* (Tokyo), **16**, 1379 (1968).
2) The preliminary communication of this paper has appeared in this Bulletin, **15**, 1621 (1967).
3) Amino acids, peptides and their derivatives mentioned in this communication are of the L-configuration and their abbreviated designation are those recommended by IUPAC-IUB commission for biological nomenclature in July, 1965 and 1966; *Biochemistry*, **5**, 1445, 2485 (1966); **6**, 362 (1967).
4) Location: *a*) *Sakyo-ku, Kyoto*; *b*) *Yamashina, Kyoto*.
5) K. Hofmann, A. Jöhl, A.E. Furlenmeier, and H. Kappeler, *J. Am. Chem. Soc.*, **79**, 1636 (1957).
6) C.H. Li, J. Meienhofer, E. Schnabel, D. Chung, T.B. Lo, and J. Ramachandran, *J. Am. Chem. Soc.*, **83**, 4449 (1961).
7) K. Medziharadszki, V. Bruckner, M. Katjar, M. Löw, S. Bajusz, and L. Kisfaludy, *Acta. Chim. Acad. Sci. Hung.*, **30**, 105 (1962).
8) K. Medziharadszki and H.M. Schweiger, *Acta. Chim. Acad. Sci. Hung.*, **44**, 15 (1965).
9) C.A. Dekker, S.P. Taylor Jr., and J.S. Fruton, *J. Biol. Chem.*, **180**, 155 (1949).

in the presence of acetic acid. It is known experimentally that an acidic medium is indeed favourable for catalytic removal of the benzyloxycarbonyl group. However, successful application of this procedure in methionine-peptides has not been achieved. Alternatively debenzyloxycarbonylation procedures, such as hydrogen bromide in glacial acetic acid[10,11] or sodium in liquid ammonia[12,13] are known to cause some undesired side reaction to the methionine residue. These situations give rise to serious disadvantage for the synthesis of peptides containing methionine if the benzyloxycarbonyl protecting group is adopted for elongation of the peptide chain.

We wish now to report the detailed account of our observations that by addition of boron trifluoride etherate, instead of cyclohexylamine, the benzyloxycarbonyl group of methionine could quantitatively be removed catalytically over a palladium catalyst. Such hydrogenation in an acidic medium was used successfully for the syntheses of a number of methionine containing peptides.

Hydrogenation was carried out in anhydrous conditions. Hydrogen gas was washed, prior to its use, with a solution of pyrogallol in order to remove oxygen and subsequently dried by sulfuric acid. A palladium catalyst was prepared according to Zelinsky, *et al.*[14] and dried *in vacuo* over phosphorous pentoxide before use. To a solution of N^{α}–benzyloxycarbonylmethionine in anhydrous methanol, a palladium catalyst (approximately one third of the sample) and freshly distilled boron trifluoride etherate (5 equimoles) were added. Oxygen–free, dry hydrogen was bubbled through the solution which was stirred at 40° for 7 hr until the evolution of carbon dioxide ceased. During this period, complete disappearance of the starting material was detected by paper and thin–layer chromatographies. The catalyst was removed by filtration and the filtrate was treated with Amberlite IRA–400 (acetate cycle). A mixture of methionine and methionine methyl ester was obtained. Both were characterized by mixed melting point with the authentic samples and by comparison of their infrared (IR) spectra.

Similar reduction proceeded also in the system of ethanol–boron trifluoride etherate, where methionine and methionine ethyl ester were obtained. In order to avoid the esterification of the free carboxyl group of methionine, the solvent was replaced with *t*–butanol or glacial acetic acid. In these procedures, only methionine was regenerated, however, much prolonged hydrogenation and addition of fresh boron trifluoride etherate during the reaction was required.

The above result indicated that debenzyloxycarbonylation by catalytic hydrogenation proceeded more effectively in an anhydrous solution of methanol or ethanol rather than *t*–butanol or glacial acetic acid containing boron trifluoride etherate. It is noted that boron trifluoride etherate in methanol[15,16] itself did not cleave the benzyloxycarbonyl group of methionine and furthermore, catalytic hydrogenation of N^{α}–benzyloxycarbonylmethionine in the system of platinum oxide–boron trifluoride etherate–methanol was unsuccessful.

Catalytic reduction of N^{α}–benzyloxycarbonylmethionine was also proceeded in the system of boron trichloride or boron tribromide in methanol, where methionine methyl ester was a major product. These halides are rapidly decomposed by exposure to the air, so that handling was rather difficult. In order to examine the effect of the acidity in the reaction medium, boron halides in methanol were substituted by *p*–toluene sulfonic acid or trifluoro-

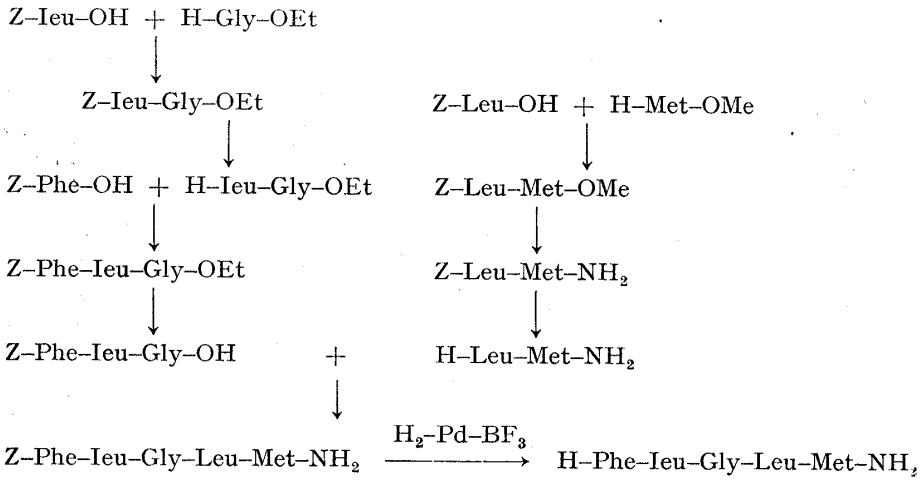
10) B. Iselin, *Helv. Chim. Acta*, **44**, 61 (1961).
11) St. Guttmann and R.A. Boissonnas, *Helv. Chim. Acta*, **41**, 1852 (1958); *ibid.*, **42**, 1257 (1959).
12) R.H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).
13) M. Bodanszky, M.A. Ondetti, "Peptide Synthesis," Inter–Sciences Publishers, New York, 1966, p. 60.
14) N. Zelinsky and N. Glinka, *Chem. Ber.*, **44**, 2305 (1911).
15) J. Passivirta and S. Brownstein, *J. Am. Chem. Soc.*, **87**, 3593 (1965).
16) H.S. Booth, D.R. Martin, "Boron Trifluoride and its derivatives," John Weiley and Sons, Co. Inc., New York, 1949, p. 63.

acetic acid. However, no reduction was observed in both cases. Only partial hydrogenation was observed when *p*-toluene sulfonic acid was used in glacial acetic acid.

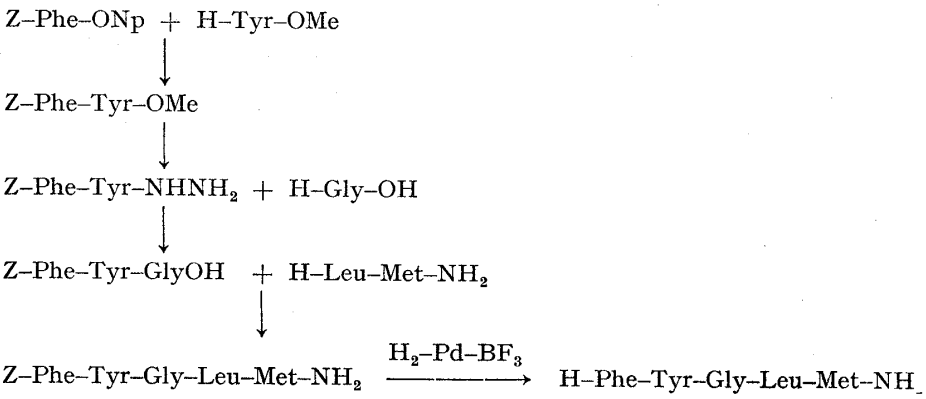
From these experimental results, it can be seen that the acidity of the reaction media is not the main cause of this debenzyloxycarbonylation. It could be assumed that boron halides either shield the sulfur atom in methionine or interact with the palladium catalyst to overcome the poisoning action of the sulfur atom of methionine.

Although methylation of the free carboxyl group as mentioned above is one of the side reaction and limitation of this hydrogenation procedure in the system of methanol–boron trifluoride etherate, we have examined its applicability to the synthesis of methionine peptides.

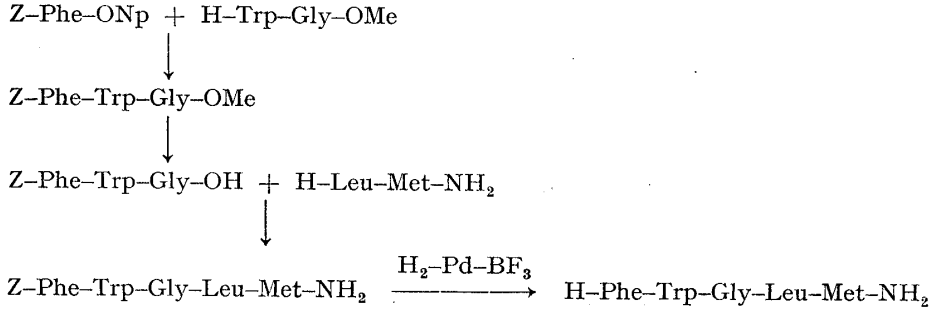
(a) Synthetic route to the C-terminal pentapeptide of eledoisin.



(b) Synthetic route to the C-ternimal pentapeptide of physalaemin.



(c) Synthetic route to an analogous pentapeptide related to eledoisin.



Z=benzyloxycarbonyl, ONp=*p*-nitrophenyl ester

Chart 1

For example, the C-terminal pentapeptides of eledoisin[17,18] and physalaemin[19,20] phenylalanylisoleucylglycylleucylmethionine amide[21–24] and phenylalanyltyrosylglycylleucylmethionine amide[20,25] were prepared. An analogous pentapeptide, phenylalanyltryptophylglycylleucylmethionine amide was also synthesized.

The synthetic schemes of these three peptides are illustrated in Chart 1. N^{α}-Benzyloxycarbonylleucine was condensed with methionine methyl ester by the dicyclohexylcarbodiimide procedure[26–28] and the resulting N^{α}-benzyloxycarbonylleucylmethionine methyl ester was treated with methanolic ammonia to form the corresponding amide, N^{α}-benzyloxycarbonylleucylmethionine amide. This amide was hydrogenated over a palladium catalyst in the system of boron trifluoride etherate and methanol as stated above. Evolution of carbon dioxide soon occurred indicating that the hydrogenation proceeded very smoothly. The solution was then treated with Amberlite IRA-400 (acetate cycle) and the product was characterized as its acetate. This dipeptide acetate was converted to its hydrochloride[28] and used for the subsequent coupling reactions.

The N-protected tripeptide related to eledoisin, N^{α}-benzyloxycarbonylphenylalanylisoleucylglycine ethyl ester was prepared according to Bernardi, *et al.*[29] and Sakakibara, *et al.*[30] and subsequently saponified to give N^{α}-benzyloxycarbonylphenylalanylisoleucylglycine, which was allowed to react with leucylmethionine amide hydrochloride by N-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward reagent)[31] in the presence of triethylamine. The resulting pentapeptide amide, N^{α}-benzyloxycarbonylphenylalanylisoleucylglycylleucylmethionine amide, was again hydrogenated under similar conditions as stated above to give phenylalanylisoleucylglycylleucylmethionine amide in quantitative yield. Homogeneity of the product, isolated as its acetate, was established by both paper and thin-layer chromatographies. Digestion of the product with leucine aminopeptidase (LAP)[32] gave the constituent amino acids in the ratios predicted by theory demonstrating that the removal of the N-terminal protecting benzyloxycarbonyl group of the peptide containing the methionine residue was satisfactorily removed by this hydrogenation procedure.

For the synthesis of the C-terminal pentapeptide of physalaemin, N^{α}-benzyloxycarbonylphenylalanyltyrosylglycine was prepared by the reaction of N^{α}-benzyloxycarbonylphenylalanyltyrosine azide and free glycine. This protected tripeptide was allowed to react with leucylmethionine amide by the Woodward reagent to give N^{α}-benzyloxycarbonylphenylalanyltyrosylglycylleucylmethionine amide. The product was subsequently hydrogenated to give phenylalanyltyrosylglycylleucylmethionine amide in quantitative yield. It is worthwhile to note that the benzyloxycarbonyl group was used extensively for these syntheses.

17) V. Erspamer and A. Anastasi, *Experientia*, **18**, 58 (1962).
18) A. Anastasi and V. Erspamer, *Arch. Biochem. Biophys.*, **101**, 56 (1963).
19) V. Erspamer, A. Anastasi, G. Bertaccini, and J.M. Cei, *Experientia*, **20**, 489 (1964).
20) L. Bernardi, G. Bosisio, O. Goffredo, and R. de Castiglione, *Experientia*, **20**, 490 (1964).
21) R. de Castiglione, *Gazz. Chim. Ital.*, **95**, 185 (1965).
22) L. Bernardi, G. Bosisio, F. Chillemi, G. de Caro, R. de Castiglione, V. Erspamer, A. Glaesser, and O. Goffredo, *Experientia*, **20**, 306 (1964).
23) B. Camerino, G. de Caro, R.A. Boissonnas, Ed. Sandrin, and E. Stümer, *Experientia*, **19**, 339 (1963).
24) K. Lübke, R. Hempel, and E. Schröder, *Experientia*, **21**, 70 (1965).
25) L. Bernardi, G. Bosisio, F. Chillemi, G. de Caro, R. de Castiglione, V. Erspamer, and O. Goffredo, *Experientia*, **22**, 29 (1966); F. Chillemi, *Gazz. Chim. Ital.*, **95**, 402 (1965).
26) J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
27) H.G. Khorana, *Chem. Rev.*, **53**, 145 (1953).
28) F. Kurzer and K.D.-Zadeh, *Chem. Rev.*, **67**, 107 (1967).
29) L. Bernardi, G. Bosisio, R. de Castiglione, O. Goffredo, and F. Chillemi, *Gazz. Chim. Ital.*, **94**, 853 (1964).
30) S. Sakakibara and M. Fujino, *Bull. Chem. Soc. Japan*, **39**, 947 (1966).
31) R.B. Woodward, R.A. Olofson, and H. Mayer, *J. Am. Chem. Soc.*, **83**, 1010 (1961).
32) Partially purified (through a second ammonium sulfate fractionation) LAP was prepared according to the method of D.H. Spackmann, E.L. Smith, and D.M. Brown, *J. Biol. Chem.*, **212**, 255 (1955).

Previously these peptides were prepared only using the acid labile *t*-butoxycarbonyl[33-35] or *t*-amyloxycarbonyl[30] group to protect the amino group.

An analogous peptide, phenylalanyltryptophylglycylleucylmethionine amide was similarly prepared by hydrogenation of the product resulted from the coupling reaction of N^{α}-benzyloxycarbonylphenylalanyltryptophylglycine and leucylmethionine amide with the Woodward reagent. The tripeptide was prepared by saponification of the corresponding methyl ester, N^{α}-benzyloxycarbonylphenylalanyltryptophylglycine methyl ester, derived from the reaction of N^{α}-benzyloxycarbonylphenylalanine *p*-nitrophenyl ester[36] and tryptophylglycine methyl ester.[37] Hydrolysate of the synthetic pentapeptide by LAP contained tryptophan in the ratio predicted by theory besides the other constituent amino acids. This result demonstrated clearly that the acid sensitive tryptophan survived intact under the acidic hydrogenation medium of boron trifluoride etherate.

Next, reduction of N^{α}-benzyloxycarbonyl-N^{G}-nitroarginylmethionine methyl ester was examined. It is known that the removal of the N^{G}-nitro group by catalytic hydrogenation itself required much longer time than the N^{α}-benzyloxycarbonyl group.[38] Arginylmethionine methyl ester was obtained after similar reduction at 40° for 14 hr. Recently, we have described the synthesis of arginylmethionine[39] by adopting the hydrogen fluoride method devised by Sakakibara, *et al.*[40] and the stannous chloride method by Noguchi, *et al.*[41] We recorded here another method for the preparation of the arginylmethionine derivative. The efficiency of boron trifluoride etherate in methanol for catalytic hydrogenation of methionine peptides seems to be demonstrated by the above experiment.

Reductive cleavage of the N^{α}-benzyloxycarbonyl group of S-benzylcysteine was unsuccessful by this procedure, though the reason is as yet unclear. Medziharadszky, *et al.*[8] reported non-effectiveness of cyclohexylamine to this compound also. With certain limitations as mentioned above, the experimental results described herein seem to demonstrate that the debenzyloxycarbonylation by catalytic hydrogenation is still useful for the preparation of peptides containing methionine.

Experimental

The general experimental methods are essentially the same as described in the Part IV[42] of this series. Rf^1 values refer to the system of *n*-BuOH, AcOH, H_2O (4:1:5) on paper and Rf^2 values refer to the system of *n*-BuOH, AcOH, Pyridine, H_2O (4:1:1:2) on silica gel (Kieselgel G, Merck) thin-layer chromatography.

Catalytic Hydrogenation of N^{α}-Benzyloxycarbonylmethionine——a) In the presence of BF_3 etherate in MeOH. To a solution of N^{α}-benzyloxycarbonylmethionine (1.41 g) in anhydrous MeOH (50 ml), BF_3 etherate (3.1 ml) and a Pd catalyst (approximately 0.5 g, dried over P_2O_5 in an evacuated desiccator) were added. Dry H_2 was bubbled into the solution, which was stirred at 40° for 7 hr. During this period, evolution of CO_2 had ceased. On paper chromatography, two spots with Rf^1 0.57 and 0.33 were detected by ninhydrin and methionine tests while Rf^1 0.90 of the starting material disappeared. The catalyst was removed by filtration, the filtrate, after treating with Amberlite IRA-400 (acetate cycle, approximately 20 g) for 2 hr, was evaporated and the residue was dissolved in ice-cold 3% ammonia, which was extracted quickly with ice-cold AcOEt. Conc. HCl (0.5 ml) was added to the AcOEt extract, which was dried over Na_2SO_4 and then evaporated. The residue was recrystallized from MeOH and ether; yield 0.29 g (31%), mp 145—147°, $[\alpha]_D^{20}$ +26.5° (c=0.5, H_2O). This hydrochloride was identical with methionine methyl ester hydrochloride (lit.[9]

33) F.C. McKay and N.F. Albertson, *J. Am. Chem. Soc.*, **79**, 4686 (1957).
34) G.W. Anderson and A.C. McGregor, *J. Am. Chem. Soc.*, **79**, 6180 (1957).
35) L.A. Carpino, *J. Am. Chem. Soc.*, **79**, 4427 (1957).
36) M. Bodanszky, M.A. Ondetti, C.A. Birkhimer, and P.L. Thomas, *J. Am. Chem. Soc.*, **86**, 4452 (1964).
37) K. Hofmann and S. Lande, *J. Am. Chem. Soc.*, **83**, 2286 (1961).
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39) H. Yajima, Y. Kinomura, T. Oshima, and Y. Okada, *Chem. Pharm. Bull.* (Tokyo), **15**, 1922 (1967).
40) S. Sakakibara and Y. Shimonishi, *Bull. Chem. Soc. Japan*, **38**, 1412 (1965).
41) T. Hayakawa, Y. Fujiwara, and J. Noguchi, *Bull. Chem. Soc. Japan*, **40**, 1205 (1967).
42) H. Yajima and K. Kubo, *Chem. Pharm. Bull.* (Tokyo), **13**, 759 (1965).

mp 151°, lit.[43] mp 145—146°, $[\alpha]_D^{19}$ +26.8° in H_2O) in mixed mp and in comparison of their IR spectra (KBr).

The alkaline solution separated above was neutralized with AcOH and then evaporated. The residue, after lyophilization, was recrystallized from H_2O and EtOH; yield 0.36 g (53%), mp 275°, decomp., $[M]_D^{21}$ +32.3° (c=0.4, 5 N HCl). This compound was identical with the authentic sample of methionine (lit.[44] mp 281°, decomp., $[M]_D$ +34.6° in 5 N HCl) in mixed mp and in comparison of their IR spectra (KBr). Similar reduction was carried out in an anhydrous EtOH solution at 40° for 7 hr. Two ninhydrin positive spots with Rf^1 0.33 (corresponding to methionine) and Rf^1 0.71 (corresponding to methionine ethyl ester[46]) were detected by paper chromatography. A trace of the starting material was detected by thin-layer chromatography. When N^{α}-benzyloxycarbonylmethionine (0.5 g) was kept in MeOH (10 ml) containing BF_3 etherate (0.8 ml) overnight, no ninhydrin positive material was detected by both paper and thin-layer chromatographies.

b) In the presence of BF_3 etherate in glacial AcOH. To a solution of N^{α}-benzyloxycarbonylmethionine (1.41 g) in glacial AcOH (distilled over triacetylboric acid,[45] 60 ml), BF_3 etherate (2 ml) and a Pd catalyst was added. Hydrogenation was continued at 40° for 7 hr. Some of the starting material still remained unhydrogenated. BF_3 etherate (1 ml) was added and H_2 was passed through the solution for an additional 14 hr. After the catalyst was removed by filtration, the filtrate was evaporated and the residue was dissolved in H_2O (100 ml). The solution was treated with Amberlite IRA-400 (acetate cycle, approximately 20 g) for 2 hr and then the solution was filtered. The filtrate was evaporated. Addition of EtOH to the residue gave a crystalline product, which was recrystallized from H_2O and EtOH; yield 0.68 g (91%), mp 270°, decomp., $[M]_D^{22}$ +25.3° (c=0.1, 5 N HCl), Rf^1 0.40. Identity of this product with methionine was confirmed by mixed mp and by comparison with their IR spectra (KBr).

c) In the presence of BF_3 etherate in t-BuOH. N^{α}-Benzyloxycarbonylmethionine (0.50 g) in t-BuOH (30 ml) was hydrogenated over a Pd catalyst in the presence of BF_3 etherate (1.1 ml) at 40° for 7 hr. Examination of the solution by paper chromatography revealed that approximately one half of the starting materials still remained unhydrogenated.

d) In the presence of BCl_3 or BBr_3. To a solution of N^{α}-benzyloxycarbonylmethionine (0.5 g) in MeOH (25 ml), N_2 gas was bubbled. After the air of the flask was replaced with N_2, a Pd catalyst (approximately 0.2 g) and BCl_3 (0.7 ml) or BBr_3 (1.5 ml) was added. Hydrogenation was carried out at 40° for 8 hr. Paper chromatographic examination of the solutions showed both the presence of two ninhydrin positive spots, Rf^1 0.33 (faint spot) and Rf^1 0.57 (heavy spot corresponding to methionine methyl ester).

e) In the presence of p-toluene sulfonic acid or trifluoroacetic acid. H_2 was bubbled to a solution of N^{α}-benzyloxycarbonylmethionine (0.5 g) in MeOH (25 ml) in the presence of a Pd catalyst and p-toluene sulfonic acid (0.8 g) or trifluoroacetic acid (0.15 ml) at 40° for 7 hr. No reduction was observed.

f) Over a PtO_2 catalyst in methanolic BF_3 etherate. A PtO_2 catalyst (0.10 g) and BF_3 etherate (1.1 ml) were added to a solution of N^{α}-benzyloxycarbonylmethionine (0.50 g) in anhydrous MeOH (25 ml). Hydrogenation was continued at 40° for 10 hr. Most of the starting material was recovered unchanged.

g) Over a Pd catalyst in the presence of p-toluene sulfonic acid in glacial AcOH. p-Toluene sulfonic acid (0.86 g) was added to a solution of N^{α}-benzyloxycarbonylmethionine (0.50 g) in glacial AcOH (30 ml). Hydrogenation was continued over a Pd catalyst at 40° for 17 hr. Paper chromatographic examination of the aliquot revealed that approximately 50% of the starting material was hydrogenated.

h) Over a Pd catalyst in the presence of cyclohexylamine. Cyclohexylamine[8] (0.83 ml) was added to a solution of N^{α}-benzyloxycarbonylmethionine (0.50 g) in MeOH (5 ml). In the presence of a Pd catalyst, H_2 was bubbled into the solution at 20° for 7 hr. The solution was examined by paper chromatography. Trace amount of a ninhydrin positive spot was detected.

N^{α}-Benzyloxycarbonylleucylmethionine Methyl Ester——To a solution of N^{α}-benzyloxycarbonylleucine (3.18 g) and methionine methyl ester (prepared from 2.40 g of the hydrochloride and 1.9 ml of triethylamine) in DMF (15 ml) dicyclohexylcarbodiimide[46] (DCC, 2.89 g) was added under cooling. The solution was stirred at room temperature overnight and then filtered. The filtrate was evaporated and the residue was dissolved in AcOEt, which was washed successively with 1 N HCl, 5% NH_4OH and H_2O, dried over Na_2SO_4 and then evaporated. The resulting oily product was crystallized from petroleum ether (bp 30—50°) and recrystallized from AcOEt and petroleum ether; yield 3.48 g (71%), mp 66—67°, $[\alpha]_D^{19}$ −41.9° (c=1.0, MeOH). *Anal.* Calcd. for $C_{20}H_{30}O_5N_2S$: C, 58.5; H, 7.4; N, 6.8. Found: C, 58.8; H, 7.4; N, 6.8.

N^{α}-Benzyloxycarbonylleucylmethionine Amide——Into a flask containing a solution of N^{α}-benzyloxycarbonylleucylmethionine methyl ester (2.05 g) in MeOH (20 ml), liquid NH_3 (approximately 20 ml) was collected under cooling with dry ice-acetone. With a tight stopper, the flask was kept at room temperature for 4 days. The bulk of the solvent was then removed *in vacuo* and the residue was recrystallized from MeOH;

43) M. Brenner and R.W. Pfister, *Helv. Chim. Acta*, **34**, 2085 (1951).
44) J.P. Greenstein, M. Winitz, "Chemistry of Amino Acids," Vol. 3, John Wiley, New York, 1961, p. 2125.
45) A. Pictet and A. Geleznoff, *Chem. Ber.*, **36**, 2219 (1903).
46) D. Fles and A.M. Prpic, *Croat. Chem. Acta*, **29**, 79 (1957).

yield 1.80 g (86%), mp 207—208°, $[\alpha]_D^{24}$ −41.7° (c=0.7, MeOH). *Anal.* Calcd. for $C_{19}H_{29}O_4N_3S$: C, 57.7; H, 7.4; N, 10.6. Found: C, 57.9; H, 7.6; N, 10.5.

Leucylmethionine Amide Acetate Monohydrate——N^{α}-Benzyloxycarbonylleucylmethionine amide (0.20 g) in MeOH (20 ml) containing BF_3 etherate (0.19 ml) was hydrogenated over a Pd catalyst under anhydrous condition. A slow stream of H_2 was passed through the solution with stirring at 40° for 8 hr. After filtration, the solution was treated with dry powder of Amberlite IRA-400 (acetate cycle, approximately 10.0 g) at 40° for 2 hr. The resin was removed by filtration, the filtrate was evaporated and the residue was lyophilized to give a fluffy powder; yield 0.14 g (84%), $[\alpha]_D^{18}$ +2.8° (c=0.5, H_2O), $[\alpha]_D^{20}$ +9.0° (c=0.6, 0.1 N **HCl**), Rf^1 0.68, Rf^2 0.60, single spot positive to ninhydrin and methionine tests, amino acid ratios in a LAP digest $Leu_{1.00}$ $Met_{1.07}$ (average recovery 83%). (lit.[28]) hydrochloride, mp 191—194°, $[\alpha]_D^{22}$ +10.2° in H_2O). *Anal.* Calcd. for $C_{11}H_{23}O_2N_3S \cdot CH_3COOH \cdot H_2O$: C, 46.6; H, 8.7; N, 12.5. Found: C, 46.9; H, 8.8; N, 12.9.

N^{α}-Benzyloxycarbonylphenylalanylisoleucylglycine Ethyl Ester——The title compound was prepared according to Bernardi, *et al.*[29] and Sakakibara, *et al.*[30] mp 178—180°, $[\alpha]_D^{22}$ −23.5° (c=0.8, AcOH). (lit.[29] mp 175—176°, lit.[30] mp 179.5—180.5°, $[\alpha]_D^{24}$ −21.0° in AcOH). *Anal.* Calcd. for $C_{27}H_{35}O_6N_3$: C, 65.2; H, 7.1; N, 8.5. Found: C, 64.9; H, 6.9; N, 8.3.

N^{α}-Benzyloxycarbonylphenylalanylisoleucylglycine——To a solution of N^{α}-benzyloxycarbonylphenylalanylisoleucylglycine ethyl ester (4.83 g) in DMF (20 ml), a mixture of 1 N NaOH (20 ml) and MeOH (20 ml) was added dropwise. After the mixture was stirred at room temperature for 1 hr, the pH of the solution was adjusted to 6 with AcOH and the solvent was evaporated *in vacuo*. The residue was dissolved in hot 5% NH_4OH, which, after filtration, was acidified with 1 N HCl. The resulting crystalline mass was collected and recrystallized from MeOH; yield 4.08 g (87%), mp 199—202°, $[\alpha]_D^{24}$ −18.5° (c=0.8, AcOH). *Anal.* Calcd. for $C_{25}H_{31}O_6N_3$: C, 63.9; H, 6.7; N, 9.0. Found: C, 64.4; H, 6.8; N, 9.0.

N^{α}-Benzyloxycarbonylphenylalanylisoleucylglycylleucylmethionine Amide——N-Ethyl-5-phenylisoxazolium-3′-sulfonate[31] (1.0 g) was added to a solution of N^{α}-benzyloxycarbonylphenylalanylisoleucylglycine (0.94 g) and triethylamine (0.28 ml) in DMF (10 ml). The solution was stirred at room temperature for 30 min. When the reagent was dissolved completely, a solution of leucylmethionine amide (prepared from 0.58 g of the hydrochloride with 0.28 ml of triethylamine) in DMF (5 ml) was added. The solution was continuously stirred at room temperature overnight. After evaporation of the solvent, H_2O was added to the residue. The resulting solid was washed batchwise with 3% NH_4OH, 0.5 N HCl and H_2O, dried *in vacuo* and finally recrystallized twice from MeOH; yield 0.76 g (54%), mp 222—224°, $[\alpha]_D^{22}$ −21.3° (c=1.0, DMF), amino acid ratios in an acid hydrolysate $Phe_{0.93}Ieu_{1.01}Gly_{1.00}Leu_{0.97}Met_{0.76}$ (average recovery 98%). *Anal.* Calcd. for $C_{36}H_{52}O_7N_6S$: C, 60.7; H, 7.4; N, 11.8. Found: C, 60.5; H, 7.1; N, 11.5.

Phenylalanylisoleucylglycylleucylmethionine Amide Acetate Hemihydrate——N^{α}-Benzyloxycarbonylphenylalanylisoleucylglycylleucylmethionine amide (0.35 g) in anhydrous MeOH (60 ml) containing BF_3 etherate (0.14 ml) was hydrogenated over a Pd catalyst at 40° for 8 hr. The catalyst was removed by filtration and the filtrate was treated with Amberlite IRA-400 (acetate cycle, approximately 10 g) for 3 hr. The resin was removed by filtration, the filtrate was evaporated and the residue was triturated with AcOEt to give a solid, which was recrystallized from MeOH and AcOEt; yield 0.28 g (89%), mp 188—192°, $[\alpha]_D^{23}$ −30.6° (c=0.6, DMF), $[\alpha]_D^{19}$ −30.0° (c=0.6, 95% AcOH), Rf^1 0.77, Rf^2 0.66, single spot positive to ninhydrin and methionine tests, amino acid ratios in an acid hydrolysate $Phe_{0.96}Ieu_{1.03}Gly_{1.04}Leu_{1.00}Met_{0.86}$ (average recovery 98%), amino acid ratios in a LAP digest $Phe_{0.99}Ieu_{1.06}Gly_{1.00}Leu_{1.02}Met_{1.04}$ (average recovery 88%), (lit.[23] acetate, mp 170°, decomp., $[\alpha]_D^{22}$ −14° in 95% AcOH. lit.[21] hydrochloride, mp 230°, decomp., $[\alpha]_D^{21}$ −20° in 95% AcOH). *Anal.* Calcd. for $C_{28}H_{46}O_5N_6S \cdot CH_3COOH \cdot \frac{1}{2}H_2O$: C, 55.6; H, 7.9; N, 13.0. Found: C, 55.3; H, 7.9; N, 13.3.

N^{α}-Benzyloxycarbonylphenylalanyltyrosine Methyl Ester——N^{α}-Benzyloxycarbonylphenylalanine p-nitrophenyl ester[36] (7.50 g) was added to a solution of tyrosine methyl ester (prepared from 5.0 g of the hydrochloride and 3.0 ml of triethylamine) in DMF (30 ml). After stirring at room temperature overnight, the solution was filtered and the filtrate was evaporated. The residue was dissolved in AcOEt, which after washing with 1 N HCl, 5% $NaHCO_3$ and H_2O, was dried over Na_2SO_4 and then evaporated. The residue was treated with ether to form a solid which was recrystallized from AcOEt and ether; yield 5.80 g (63%), mp 139—141° (lit.[47] 137—139°), $[\alpha]_D^{32}$ −14.7° (c=1.0, MeOH). *Anal.* Calcd. for $C_{27}H_{28}O_6N_2$: C, 68.1; H, 5.9; N, 5.9. Found: C, 67.9; H, 6.2; N, 6.0.

N^{α}-Benzyloxycarbonylphenylalanyltyrosine Hydrazide——N^{α}-Benzyloxycarbonylphenylalanyltyrosine methyl ester (5.0 g) was dissolved in EtOH (20 ml) and 80% hydrazine hydrate (1.9 ml) was added and the solution was refluxed for 1.5 hr. The solution was cooled to room temperature and the resulting solid was collected by filtration and washed with EtOH; yield 4.58 g (92%), mp 219—221°, $[\alpha]_D^{33}$ −14.6° (c=1.0, AcOH), *Anal.* Calcd. for $C_{26}H_{28}O_5N_4$: C, 65.5; H, 5.9; N, 11.8. Found: C, 65.3; H, 5.8; N, 11.6.

N^{α}-Benzyloxycarbonylphenylalanyltyrosylglycine Hemihydrate——The entire operation was carried out in a cold room at 4°. To a solution of N^{α}-benzyloxycarbonylphenylalanyltyrosine hydrazide (1.70 g) in a mixture of AcOH (20 ml) and 1 N HCl (7.2 ml), $NaNO_2$ (0.36 g) in H_2O (2 ml) was added. After standing

47) H.J. Panneman, A.F. Marx, and J.F. Arens, *Rec. Trav. Chim.*, **78**, 478 (1959).

for 5 min, the solution was neutralized with 50% K_2CO_3 and the resulting powder was collected by filtration. This solid azide was combined to a solution of glycine (0.75 g) in 50% DMF (34 ml) containing triethylamine (1.4 ml). The solution was stirred at 4° for 24 hr and then the solvent was evaporated *in vacuo*. The residue was dissolved in 5% NH_4OH, which after washing with AcOEt, was acidified with 1 N HCl. The resulting solid was collected and recrystallized from MeOH and AcOEt; yield, 1.0 g (55%), mp 159—165°, $[\alpha]_D^{33}$ −25.5° (c=1.0, MeOH). *Anal.* Calcd. for $C_{28}H_{29}O_7N_3 \cdot \frac{1}{2}H_2O$: C, 63.6; H, 5.7; N, 8.0. Found: C, 63.7; H, 6.0; N, 7.7.

N$^{\alpha}$-Benzyloxycarbonylphenylalanyltyrosylglycylleucylmethionine Amide Hemihydrate——To a solution of N$^{\alpha}$-benzyloxycarbonylphenylalanyltyrosylglycine (0.52 g) and triethylamine (0.14 ml) in DMF (5 ml), N-ethyl-5-phenylisoxazolium-3′-sulfonate (0.25 g) was added. The solution was stirred for 30 min and then leucylmethionine amide (prepared from 0.30 g of the hydrochloride with 0.14 ml of triethylamine) in DMF (2 ml) was combined. Stirring was continued at room temperature for 24 hr and the solvent was evaporated. The residue was washed with three portions of 5% NH_4OH, two portions of 0.5 N HCl and finally H_2O. The resulting powder was recrystallized from MeOH and AcOEt; yield 0.41 g(54%), mp 218—221°, $[\alpha]_D^{33}$ −39.2° (c=0.5, DMF). *Anal.* Calcd. for $C_{39}H_{50}O_8N_6S \cdot \frac{1}{2}H_2O$: C, 60.7; H, 6.7; N, 10.9. Found: C, 60.8; H, 6.3; N, 10.3.

Phenylalanyltyrosylglycylleucylmethionine Amide Acetate——N$^{\alpha}$-Benzyloxycarbonylphenylalanyltyrosylglycylleucylmethionine amide (0.19 g) in anhydrous MeOH (40 ml) containing BF_3 etherate (0.14 ml) was hydrogenated over a Pd catalyst at 40° for 7 hr. After filtration, the solution was treated with Amberlite IRA-400 (acetate cycle, approximately 10 g) for 1 hr. The solution was filtered, the filtrate was evaporated and the resulting powder was recrystallized from MeOH and AcOEt; yield 0.15 g (87%), mp 126°,decomp., $[\alpha]_D^{34}$ −37.9° (c=0,3 DMF), Rf^1 0.73, Rf^2 0.69, single spot positive to ninhydrin, Pauly and methionine tests. (lit.[20]) hydrochloride, mp 120—122°, $[\alpha]_D^{20}$ −66° in DMF, $[\alpha]_D^{20}$ −11.0° in 95% AcOH. lit.[25]) mp 157—160°, $[\alpha]_D^{20}$ −17.0° in AcOH). *Anal.* Calcd. for $C_{31}H_{44}O_6N_6S \cdot CH_3COOH$: C, 57.5; H, 7.0; N, 12.2. Found: C, 58.3; H, 7.2; N, 11.9.

N$^{\alpha}$-Benzyloxycarbonylphenylalanyltryptophylglycine Methyl Ester——To a solution of tryptophylglycine methyl ester[37]) (prepared from 9.35 g of the hydrochloride and 5.6 ml of triethylamine) in DMF (50 ml), N$^{\alpha}$-benzyloxycarbonylphenylalanine *p*-nitrophenyl ester (12.61 g) and imidazole[48,49])(20.42 g) were added. The mixture was stirred at room temperature overnight. The solution was filtered and the filtrate was evaporated to give a solid, which was washed batchwise with 5% NH_4OH, 2 N HCl and H_2O and precipitated from DMF with AcOEt; yield 15.0 g (90%), mp 197—200°, $[\alpha]_D^{18}$ −7.5° (c=1.2, AcOH). *Anal.* Calcd. for $C_{31}H_{32}O_6N_4$: C, 66.9; H, 5.8; N, 10.1. Found: C, 67.0; H, 6.0; N, 9.8.

N$^{\alpha}$-Benzyloxycarbonylphenylalanyltryptophylglycine——To a solution of N$^{\alpha}$-benzyloxycarbonylphenylalanyltryptophylglycine methyl ester (5.42 g) in DMF (8 ml), a mixture of 1 N NaOH (10 ml) and MeOH (10 ml) was added dropwise and the solution was stirred at room temperature for 1 hr. After the pH of the solution was brought to 6 with AcOH, the solvent was evaporated *in vacuo* and the residue was acidified with 1 N HCl. The resulting solid was collected and recrystallized from MeOH; yield 4.66 g (88%), mp 207—210°, $[\alpha]_D^{17}$ −5.0° (c=0.7, AcOH). *Anal.* Calcd. for $C_{30}H_{30}O_6N_4$: C, 66.4; H, 5.6; N, 10.3. Found: C, 66.6; H, 5.8; N, 10.1.

N$^{\alpha}$-Benzyloxycarbonylphenylalanyltryptophylglycylleucylmethionine Amide——To a solution of N$^{\alpha}$-benzyloxycarbonylphenylalanyltryptophylglycine (0.54 g) in DMF (5 ml), triethylamine (0.14 ml) was added followed by N-ethyl-5-phenylisoxazolium-3′-sulfonate (0.25 g). The solution was stirred at room temperature for 30 min. When the reagent dissolved, a solution of leucylmethionine amide (prepared from 0.30 g of the hydrochloride with 0.14 ml of triethylamine) in DMF (2.5 ml) was combined. The solution was stirred at room temperature overnight and the solvent was evaporated. The resulting solid was washed batchwise with three portions of 3% NH_4OH, 0.5 N HCl and H_2O, and recrystallized twice from MeOH; yield 0.42 g (54%), mp 197—199°, $[\alpha]_D^{21}$ −30.6° (c=0.4, DMF). *Anal.* Calcd. for $C_{41}H_{51}O_7N_7S$: C, 62.7; H, 6.5; N, 12.5. Found: C, 62.6; H, 6.5; N, 12.5.

Phenylalanyltryptophylglycylleucylmethionine Amide Acetate——N$^{\alpha}$-Benzyloxycarbonylphenylalanyltryptophylglycylleucylmethionine amide (0.35 g) in anhydrous MeOH (50 ml) was hydrogenated over a Pd catalyst in the presence of BF_3 etherate (0.14 ml). The catalyst was removed by filtration and the filtrate was treated with Amberlite IRA-400 (acetate cycle, approximately 10 g) for 3 hr. The resin was removed by filtration and the filtrate was evaporated. The residue was triturated with AcOEt to form a solid, which was recrystallized from MeOH and AcOEt; yield 0.26 g (82%), mp 112—115°, $[\alpha]_D^{22}$ −39.3° (c=0.3, DMF), Rf^1 0.88, Rf^2 0.75, single spot positive to ninhydrin, Ehrlich and methionine tests, amino acid ratios in an acid hydrolysate $Phe_{1.00}Gly_{1.05}Leu_{1.03}Met_{0.85}$ (Trp was destroyed, average recovery 92%), amino acid ratios in a LAP digest $Phe_{1.00}Trp_{1.00}Gly_{1.02}Leu_{1.03}Met_{0.90}$ (average recovery 82%). *Anal.* Calcd. for $C_{33}H_{45}O_5N_7S \cdot CH_3COOH$: C, 59.1; H, 6.9; N, 13.8. Found: C, 59.4; H, 7.0; N, 14.0.

Arginylmethionine Methyl Ester Dihydrochloride Tetrahydrate——N$^{\alpha}$-Benzyloxycarbonyl-N^{G}-nitroarginylmethionine methyl ester[39]) (0.25 g) in anhydrous MeOH (20 ml) containing BF_3 etherate (0.32 ml) was

48) Th. Wieland and K. Vogeler, *Angew. Chem.*, **74**, 904 (1962).
49) R.H. Mazur, *J. Org. Chem.*, **28**, 2498 (1963).

hydrogenated over a Pd catalyst at 40° for 14 hr, when the absorbancy at 270 mμ in the solution disappeared. Examination of the reaction mixture by paper chromatography revealed the presence of a single spot positive to ninhydrin and methionine tests with Rf^1 0.40 and no starting material was detected. The solution, after filtration, was treated with Amberlite IRA-400 (acetate cycle, approximately 7 g) under N_2 for 2 hr. The resin was removed by filtration and the filtrate was evaporated. The residue was dissolved in a small amount of H_2O, and the solution, after addition of 1 N HCl (3 ml), was lyophilized to give a fluffy powder; yield 0.16 g (80%), Rf^1 0.40, Rf^2 0.38, single spot positive to ninhydrin and methionine tests, amino acid ratios in an acid hydrolysate $Arg_{1.00}Met_{0.84}$ (average recovery 90%), amino acid ratios in a LAP digest $Arg_{1.00}$ $Met_{0.88}$ (average recovery 87%). $[\alpha]_D^{21}$ −7.6° (c=0.6, H_2O). *Anal.* Calcd. for $C_{12}H_{25}O_3N_5S \cdot 2HCl \cdot 4H_2O$: C, 31.0; H, 7.5; N, 15.1. Found: C, 30.4; H, 7.2; N, 15.9.

Attempt to Hydrogenate N^{α}-Benzyloxycarbonyl-S-benzylcysteine——In the presence of a Pd or PtO_2 catalyst, H_2 was bubbled to a solution of N^{α}-benzyloxycarbonyl-S-benzylcysteine (0.50 g) in anhydrous MeOH (40 ml) containing BF_3 etherate (0.47 ml) at 40° for 9 hr. Sizable amount of the ninhydrin positive material was not detected on paper chromatogram of the mixture in both cases.

Bioassay——The relative hypotensive activities of three pentapeptides related to eledoisin and physalaemin described above were compared with that of bradykinin (Sandoz) in rabbit: Phe-Ieu-Gly-Leu-Met-NH_2 (1/20), Phe-Tyr-Gly-Leu-Met-NH_2 (1/25) and Phe-Trp-Gly-Leu-Met-NH_2 (1/100).

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