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64. **Haruaki Yajima, Kazuo Kubo, and Yasuhiko Kinomura** : Studies on Peptides. XIII.[*1,*2] Contribution of the Arginine Residue in L-Histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine to Its Melanotropic Activity.

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Synthesis and melanotropic activity (expressed as MSH U/g.) of three pentapeptides, L-histidyl-L-phenylalanyl-L-ornithyl-L-tryptophylglycine (practically inactive), L-histidyl-L-phenylalanyl-L-lysyl-L-tryptophylglycine (1×10^4) and L-histidyl-L-phenylalanyl-D-arginyl-L-tryptophylglycine (practically inactive) were reported and the physiological role of the arginine residue in L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine (I), an active fragment of α-melanocyte-stimulating hormone (α-MSH) was discussed. In addition, MSH activity of D-histidyl-L-phenylalanyl-D-arginyl-L-tryptophylglycine (practically inactive) and L-histidyl-D-phenylalanyl-L-arginyl-D-tryptophylglycine (1×10^6) was recorded and stereochemical nature of I was discussed in relation to the *in vitro* MSH activity.

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During the course of our systematic studies on stereoisomeric pentapeptides related to L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine (I), an active fragment of α-melanocyte-stimulating hormone (α-MSH), we have discussed the stereochemical nature of the histidine, phenylalanine and tryptophan residues in relation to the malanotropic activity of I.1~6) Now, the effect on biological activity of altering the L-arginyl residue was examined.

Previously replacement of the arginine moiety of I to the corresponding ornithine residue was conducted by Li, Schnabel and Chung in 1960.7) They observed that their synthetic L-histidyl-L-phenylalanyl-L-ornithyl-L-tryptophylglycine (II) exhibited the activity equivalent to 2.9×10^4 MSH U/g. However, later Bodanszky, *et al.*8) employing the ornithine derivative (II) as an intermediate in the synthesis of I, reported that their synthetic peptide was inactive.8,9) With consideration of these contrasting results between these two groups of investigators, we have now tried to examine the physiological role of the arginine residue, including its conformational effect for the melanotropic activity of this fragment. In later aspects, biological information about the stereochemical nature of the arginine residue was, at present, offered only by Hofmann, *et al.*10,11) who recorded that their L-histidyl-L-phenylalanyl-D-arginyl-L-tryptophylglycine (III) isolated from tryptic hydrolysate of the pentapeptide containing racemized arginine residue, was as active as I.

[*1] A preliminary communication of this paper : Biochim. Biophys. Acta, **127**, 545 (1966).

[*2] Part XII. H. Yajima, Y. Okada, Y. Kinomura, E. Seto : This Bulletin, **15**, 270 (1967).

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1) K. Hano, M. Koida, K. Kubo, H. Yajima : Biochim. Biophys. Acta, **90**, 201 (1964).
2) H. Yajima, K. Kubo : J. Am. Chem. Soc., **87**, 2039 (1965).
3) *Idem* : Biochim. Biophys. Acta, **97**, 596 (1965).
4) *Idem* : This Bulletin, **13**, 759 (1965).
5) H. Yajima, K. Kubo, Y. Okada : *Ibid.*, **13**, 1326 (1965).
6) H. Yajima, K. Kubo, T. Oshima : *Ibid.*, **14**, 775 (1966).
7) C. H. Li, E. Schnabel, D. Chung : J. Am. Chem. Soc., **82**, 2062 (1960).
8) M. Bodanszky, M. A. Ondetti, C. A. Birkhimer, P. L. Thomas : *Ibid.*, **86**, 4452 (1964).
9) M. Bodanszky, M. A. Ondetti, B. Rubin, J. J. Piala, J. Fried, J. T. Sheehan, C. A. Birkhimer : Nature, **194**, 482 (1962).
10) K. Hofmann : Ann. Rev. Biochem., **31**, 213 (1963).
11) K. Hofmann, S. Lande : J. Am. Chem. Soc., **83**, 2286 (1961).

We have synthesized II by an alternate method to the above authors and found that it exhibited practically no activity in frog skins from *Rana pipiens*.[12] Our synthetic D-arginine isomer (III) was completely devoid of the *in vitro* MSH activity. Based on these experimental results and the observations on the stereoisomeric pentapeptides of I which we have recently synthesized, a role of the arginine residue in this active fragment of α-MSH will be discussd and explanation about a possible relationship between stereochemical nature of this pentapeptide (I) and its biological function will be included in this paper.

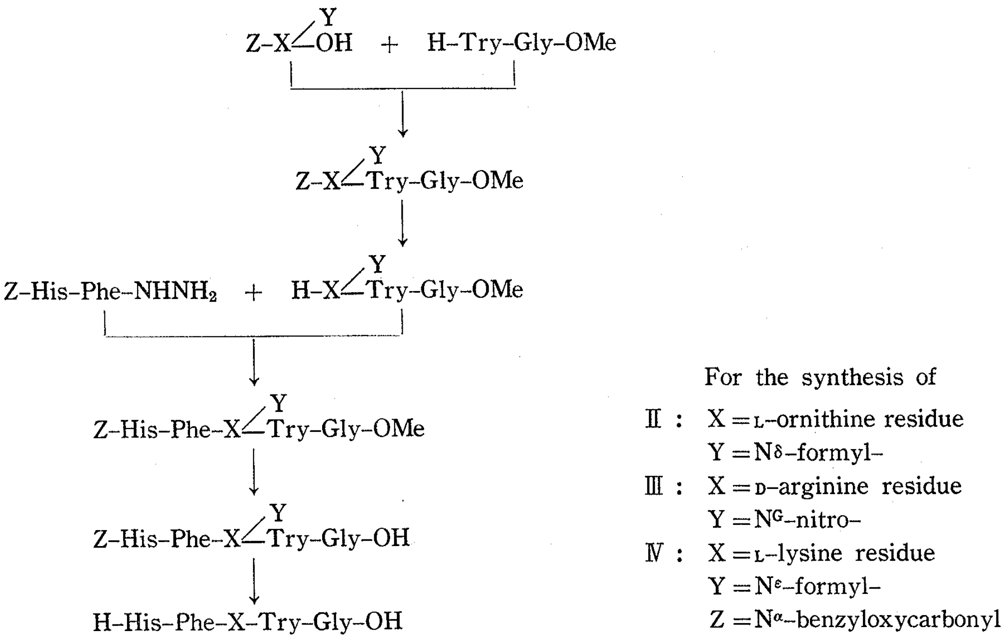

Chart 1. Synthetic Scheme of L-Arginine-substituted Pentapeptides

The synthetic scheme of II is illustrated in Chart 1, the formyl group was adopted for protection of the δ-amino function of ornithine.[13] N^{α}-Benzyloxycarbonyl-N^{δ}-formyl-L-ornithine was prepared according to the direction of Hofmann, *et al.*[14] in the preparation of the corresponding L-lysine derivative. It was condensed with L-tryptophylglycine methyl ester[11] by the dicyclohexylcarbodiimide procedure[15] to form N^{α}-benzyloxycarbonyl-N^{δ}-formyl-L-ornithyl-L-tryptophylglycine methyl ester, which was then hydrogenated over a palladium catalyst. The resulting tripeptide ester was allowed to react with N^{α}-benzyloxycarbonyl-L-histidyl-L-phenylalanine azide[16,17] to yield the protected pentapeptide ester, N^{α}-benzyloxycarbonyl-L-histidyl-L-phenylalanyl-N^{δ}-formyl-L-ornithyl-L-tryptophylglycine methyl ester, which was then saponified by sodium hydroxide. The resulting N^{α}-benzyloxycarbonyl-L-histidyl-L-phenylalanyl-N^{δ}-formyl-L-ornithyl-L-tryptophylglycine was hydrogenated and subsequently treated with $0.5N$ hydrochloric acid to remove the formyl group. The crude pentapeptide (II) thus obtained was purified by ion exchange chromatography on carboxymethylcellulose

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[12] The *in vitro* MSH assays were performed according to the method of K. Shizume, A.B. Lerner, T.B. Fitzpatrick: Endocrinol., **54**, 553 (1954).

[13] K. Okawa, S. Hase: Bull. Chem. Soc. Japan, **36**, 754 (1963).

[14] K. Hofmann, E. Stütz, G. Spühler, H. Yajima, E.T. Schwartz: J. Am. Chem. Soc., **82**, 3727 (1960).

[15] J.C. Sheehan, G.P. Hess: J. Am. Chem. Soc., **77**, 1067 (1955).

[16] H. Yajima, K. Kawasaki, M. Koida, S. Lande: This Bulletin, **14**, 884 (1966).

[17] K. Hofmann, H. Kappeler, A.E. Furlenmeier, M.E. Woolner, E.T. Schwartz, T.A. Thompson: J. Am. Chem. Soc., **79**, 1641 (1957).

(CMC).[18] Pyridine acetate buffer served to elute the desired compound. The purified sample (Ⅱ) was demonstrated to be homogeneous by paper chromatography in two different solvent systems and amino acid analysis of acid and leucine aminopeptidase (LAP)[19] hydrolysates.

Pigmentation produced by our preparation (Ⅱ) was not observed even at about ten times higher doses (0.14 mg./ml.) than that of I which usually exhibits an observable response. This result seems to be agreement with the result of Bodanszky, *et al.*,[8] however lightening effect of Ⅱ was not observed in our assay.

In order to examine the differnce in physiological properties between L-ornithine- and L-lysine-containing pentapeptides, we have further synthesized the L-lysine isomer, L-histidyl-L-phenylalanyl-L-lysyl-L-tryptophylglycine (Ⅳ) by the same procedure. The MSH activity of 1×10^4 MSH U/g. was found for this preparation. From the above observations, it appears that the L-arginine residue can be substituted with L-lysine, a much less basic moiety, with almost complete retention of activity, while a similar substitution with L-ornithine yields an inactive peptide.

For the synthesis of the D-arginine isomer (Ⅲ), N^{α}-benzyloxycarbonyl-N^{G}-nitro-D-arginine[2] was coupled with L-tryptophylglycine methyl ester by dicyclohexylcarcobodiimide to form N^{α}-benzyloxycarbonyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine methyl ester. The N^{α}-benzyloxycarbonyl group was cleaved by the action of hydrogen bromide in acetic acid[20] and the resulting tripeptide ester hydrobromide was allowed to react with N^{α}-benzyloxycarbonyl-L-histidyl-L-phenylalanine azide as described above in the preparation of Ⅱ to form N^{α}-benzyloxycarbonyl-L-histidyl-L-phenylalanyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine methyl ester. Saponificatication followed by hydrogenation of this protected peptide ester gave a crude pentapeptide (Ⅲ) which was purified on a column of CMC. The purified product exhibited a single spot on paper chromatography in two different solvent systems. An acid hydrolysate gave the constituent amino acids in nearly equal molar ratios except for tryptophan which was destroyed during the hydrolysis. Examination of the tryptic hydrolysate[*4] of this peptide by paper chromatography revealed no extra spot besides the original pentapeptide. The above tests seem to jusify the conclusion that our synthetic D-arginine pentapeptide (Ⅲ) is homogeneous and possesses the well defined D-arginyl bond within the sequence.

This synthetic peptide (Ⅲ) exhibited no melanotropic activity in our laboratory even at high dose levels, but rather a weak inhibitory action to α-MSH. This result is in contrast to the observation on the product isolated from the racemic mixture.[11] No well founded explanation can be offered for these discrepancies. Our synthesis was duplicated and the above assay results were confirmed.

Previously we have observed that the D-histidine isomer, D-histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine[4] (Ⅴ) exhibited no MSH activity while D-phenylalanyl and D-tryptophyl isomer, L-histidyl-D-phenylalanyl-L-arginyl-L-tryptophylglycine[6] (Ⅵ) and L-histidyl-L-phenylalanyl-L-arginyl-D-tryptophylglycine[4] (Ⅶ) exhibited the activity equivalent to 1×10^6 and 1×10^5 MSH U/g. respectively. Within these frameworks certain trends can be seen in the relationship between structure and activity. The *in vitro* assay exhibits a stereospecificity with regard to the ionizable residues of the pentapetide;

[*4] Trypsin was purchased from Shigma Chemicals Co., Lot T62-B-232.

18) E. A. Peterson, H. A. Sober: J. Am. Chem. Soc., **78**, 751 (1956). Commercial preparation (Cellex-CM) was purchased from Bio. Rad Laboratories.

19) Partially purified LAP was prepared according to the method of D. H. Spackman, E. L. Smith, D. M. Brown: J. Biol. Chem., **212**, 255 (1955). Digestion was performed according to the method of K. Hofmann, H. Yajima: J. Am. Chem. Soc., **83**, 2289 (1961).

20) G. W. Anderson, J. Blodinger, A. D. Welcher: J. Am. Chem. Soc., **74**, 5309 (1952).

the basic histidyl and arginyl residues. On the other hand, peptides containing the D-isomers of the non-ionized aromatic residues, phenylalanine or tryptophan are more active than the all-L-analogue (I) as shown in Chart 2.

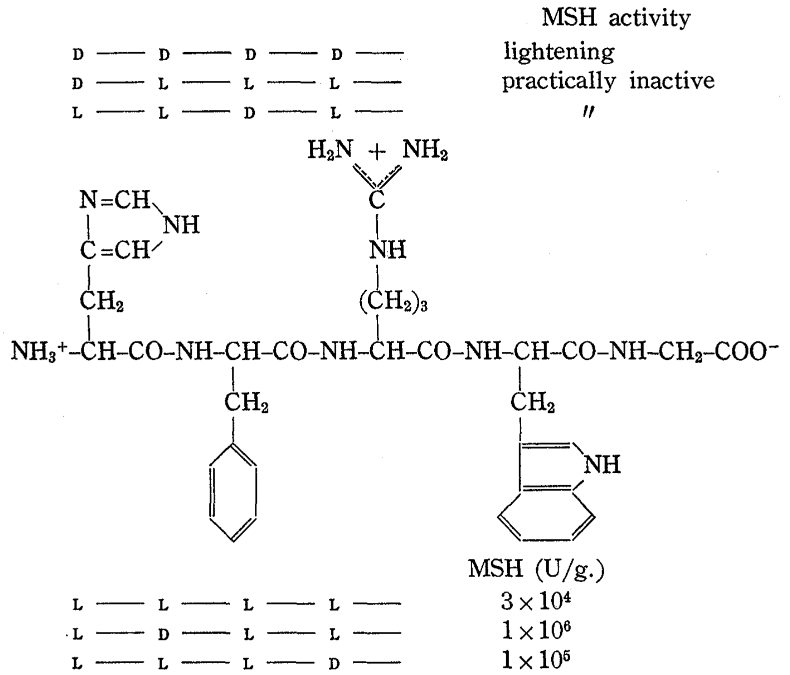

Chart 2. Relationship between Stereochemical Nature of the Pentapeptide and Melanocyte-stimulating Hormone Activity

We have now been able to evaluate the MSH activity of each peptide substituted by single D-amino acid moiety in this pentapeptide fragment. However the contribution of the given amino acid toward biological activity may be affected by the stereochemical natuue of neighboring residues. We have then synthesized two pentapeptides, D-histidyl-L-phenylalanyl-D-arginyl-L-tryptophylglycine (VIII) and L-histidyl-D-phenylalanyl-L-arginyl-D-tryptophylglycine (IX) according to the manner described earlier in the preparation of the enantiomer of I[2)] by the stepwise elongation method of the peptide chain, where necessary amino acid isomers were used. These two compounds were assayed *in vitro.* It was found that the compound (VIII) exhibited no activity at the dose (0.2 mg./ml.) which is about ten times higher than that of I produces the normal darkening effect. The stereoisomer (IX) which possesses the configuration of L-D-L-D-glycine exhibited the activiy of 1×10^6 MSH U/g. as listed in Table I.

Table I. Melanocyte-stimulating Hormone Activities of the Synthetic Pentapeptides

Pentapeptide	Configuration	MSH (U/g.)
His-Phe-Orn-Try-Gly	L-L-L-L-	practically inactive
His-Phe-Lys-Try-Gly	L-L-L-L-	1×10^4
His-Phe-Arg-Try-Gly	L-L-D-L-	practically inactive
His-Phe-Arg-Try-Gly	D-L-D-L-	//
His-Phe-Arg-Try-Gly	L-D-L-D-	1×10^6

The L-configuration of the two basic groups, histidine and arginine residues are, as now observed, important for MSH activity. However, previously we have observed that our synthetic two D-phenylalanine-containing pentapeptides, D-histidyl-D-phenylalanyl-L-arginyl-L-tryptophylglycine[6] (X) and L-histidyl-D-phenylalanyl-D-arginyl-L-tryptophylglycine[5] (XI) exhibited 5.5×10^4 and 2.1×10^4 MSH U/g. of the activity respectively. It was shown that the presence of D-phenylalanyl residue can overcome the detrimental effect of adjacent D-histidyl or D-arginyl moiety. These results may have a connection with the fact that the D-phenylalanine isomer (VI) exhibited a considerably higher activity than I. Summarizing these results, it became obvious that the histidine and arginine residues are under influences of the other conformational effects mainly by the phenylalanine residue as far as the *in vitro* MSH activity is concerned.

As for the observations that VI, VII, and IX possessing either one or both of D-phenylalanine and D-tryptophan residue exhibited much higher activity than I, mechanism of such potentiated activity is not known. However it may be concluded that the amino acid residues which are non-stereospecific for the activity are present within this pentapeptide sequence. These results seem to support the view expressed by Pickering and Li[21] that the receptor site does not exhibit high degree of stereospecificity as far as melanotropic activity is concerned and furthermore may support the explanation of the phenomenon already known; the survival of the MSH activity in alkali-treated α-,[22,23] β-MSH[21] and adrenocorticotropic hormone[24] which are racemized.

Experimental

Analytical procedures and general experimental methods employed in these experiments are essentially the same as described in the Part IV[4] of this series. The following abbreviations were used for amino acids: His=histidine, Phe=phenylalanine, Arg=arginine, Try=tryptophan, Gly=glycine, Orn=ornithine, and Lys=lysine. Rf^1 values refer to the system of Partridge.[25] Rf^2 values refer to the system of *sec*-butanol-3% ammonia[26] and are expressed as multiples of the distance traveled by a phenylalanine marker.

i. Synthesis of L-Histidyl-L-phenylalanyl-L-ornithyl-L-tryptophylglycine (II)

N^{δ}-Formyl-L-ornithine——The titled compound was prepared according to the procedure described in the preparation of the corresponding N^{ε}-formyl-L-lysine[14] through the ornithine copper complex. m.p. 212~214°, $[\alpha]_D^{18}$ $+1.5°$($c=1.0$, H_2O), Rf^1 0.25, Rf^2 0.27. (lit.[13] m.p. 217°, $[\alpha]_D^{15}$ $+2.8°$ in H_2O). *Anal.* Calcd. for $C_6H_{12}O_3N_2$: C, 45.0; H, 7.6; N, 17.5. Found: C, 44.9; H, 7.7; N, 17.4.

N^{α}-Benzyloxycarbonyl-N^{δ}-formyl-L-ornithine——N^{δ}-Formyl-L-ornithine was benzyloxycarbonylated by the standard procedure; m.p. 109~111°, $[\alpha]_D^{20}$ $-2.9°$($c=1.0$, MeOH). *Anal.* Calcd. for $C_{14}H_{18}O_5N_2$: C, 57.1; H, 6.2; N, 9.5. Found: C, 57.3; H, 6.4; N, 9.6.

N^{α}-Benzyloxycarbonyl-N^{δ}-formyl-L-ornithyl-L-tryptophylglycine Methyl Ester——N^{α}-Benzyloxycarbonyl-N^{δ}-formyl-L-ornithine (2.94 g.) was mixed with a solution of L-tryptophylglycine methyl ester[11] (prepared from 3.30 g. of the hydrochloride with 1.4 ml. of triethylamine) in dimethylformamide (15 ml.). After addition of dicyclohexylcarbodiimide (2.2 g.), the mixture was stirred at room temperature for 18 hr. Dicyclohexylurea formed during the reaction was removed by filtration, the filtrate was condensed *in vacuo* and the residue was dissolved in AcOEt which was washed successively with 5% Na_2CO_3, 1*N* HCl and H_2O. The organic phase was dried over Na_2SO_4 and evaporated to give a solid which was recrystallized from MeOH; yield 3.34 g. (61%), m.p. 144~146°, $[\alpha]_D^{22}$ $+4.2°$($c=0.4$, MeOH). *Anal.* Calcd. for $C_{28}H_{33}O_7N_5$: C, 61.0; H, 6.0; N, 12.7. Found: C, 60.8; H, 6.5; N, 12.7.

N^{δ}-Formyl-L-ornithyl-L-tryptophylglycine Methyl Ester Acetate Monohydrate——N^{α}-Benzyloxycarbonyl-N^{δ}-formyl-L-ornithyl-L-tryptophylglycine methyl ester (2.50 g.) in a mixture of MeOH (30 ml.) and 10% AcOH (5 ml.) was hydrogenated over a Pd catalyst. The catalyst was removed by filtration and the filtrate was condensed *in vacuo*. The residue was lyophilized to give a fluffy powder; yield 2.10 g. (97%), $[\alpha]_D^{22}$ $-6.3°$($c=0.6$, MeOH). Rf^1 0.60. *Anal.* Calcd. for $C_{20}H_{27}O_5N_5\cdot CH_3COOH\cdot H_2O$: C, 53.3; H, 6.7; N, 14.1. Found: C, 53.9; H, 6.9; N, 13.9.

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21) B. T. Pickering, C. H. Li: Arch. Biochem. Biophys., **104**, 119 (1964).
22) T. H. Lee, A. B. Lerner, V. B.-Janusch: Ann. N. Y. Acad. Sci., **100**, 658 (1963).
23) T. H. Lee, V. B.-Janusch: J. Biol. Chem., **238**, 2012 (1963).
24) I. I. Geschwind, C. H. Li: Arch. Biochem. Biophys., **106**, 200 (1964).
25) S. M. Partridge: Biochem. J., **42**, 238 (1948).
26) J. F. Roland, A. M. Gross: Ann. Chem., **26**, 502 (1954).

N^{α}-Benzyloxycarbonyl-L-histdiyl-L-phenylalanyl-N^{δ}-formyl-L-ornithyl-L-tryptophylglycine Monohydrate——N^{δ}-Formyl-L-ornithyl-L-tryptophylglycine methyl ester acetate (1.80 g.) was dissolved in dimethylformamide (15 ml.) and triethylamine (0.6 ml.) was added. To this ice-cold solution was added the solid N^{α}-benzyloxycarbonyl-L-histidyl-L-phenylalanine azide (prepared from 2.0 g. of the corresponding hydrazide).[16,17] The mixture was kept at 4° for 24 hr. The solvent was evaporated and the residue was dissolved in a mixture of MeOH (30 ml.) and dimethylformamide (10 ml.), and 1*N* NaOH (8.0 ml.) was added. The solution was stirred at room temperature for 30 min. The solution was neutralized with AcOH, condensed *in vacuo* and then acidified with ice-cold 1*N* HCl. The resulting precipitate was collected by filtration, washed with hot MeOH, and re-precipitated from dimethylformamide with H_2O; yield 1.80 g. (58%), m.p. 231°(decomp.), $[\alpha]^{24}_{D}$ $-22.3°$(c=0.2, dimethylformamide). Rf^1 0.80, ninhydrin negative and single Ehrlich positive spot. *Anal.* Calcd. for $C_{42}H_{47}O_9N_9 \cdot H_2O$. C, 60.1; H, 5.9; N, 15.0. Found: C, 59.9; H, 6.6; N, 14.8.

L-Histidyl-L-phenylalanyl-L-ornithyl-L-tryptophylglycine Acetate 1½ Hydrate (II)——N^{α}-Benzyloxycarbonyl-L-histidyl-L-phenylalanyl-N^{δ}-formyl-L-ornithyl-L-tryptophylglycine (1.80 g.) in 70% AcOH (30 ml.) was hydrogenated over a Pd catalyst. The catalyst was removed by filtration and the filtrate was condensed *in vacuo*. The residue was dissolved in 0.5*N* HCl (50 ml.). The solution was heated in a boiling water-bath for 30 min., then cooled, neutralized with 1*N* NaOH and finally diluted with H_2O (300 ml.). This solution was applied to a column of CMC (3×25 cm.) which was then eluted successively with the following pyridine acetate buffers (pH 5.0): 0.025*M* (1000 ml.), 0.05*M* (1000 ml.), 0.1*M* (500 ml.) and 0.15*M* (1500 ml.). Individual fractions (18 ml. each) were collected with a flow rate of 3 to 4 ml./min. and absorbancy at 280 mμ was determined in each fraction. The desired fraction which was present in the 0.15*M* eluate was pooled and the solvent was evaporated *in vacuo*. The residue was lyophilized to give a white fluffy powder; yield 1.44 g. (88%), $[\alpha]^{22}_{D}$ $-20.4°$(c=0.3, 1*N* HCl). (lit.[7] $[\alpha]^{26}_{D}$ $-7.8°$ in H_2O, lit.[8] $[\alpha]^{20}_{D}$ $-13°$ in H_2O). Rf^1 0.43, Rf^2 0.88. Amino acid ratios in the acid hydrolysate $His_{1.04}Phe_{1.00}Orn_{1.00}Gly_{0.98}$ (Try was destroyed, average recovery 96%), amino acid ratios in the LAP digest $His_{1.04}Phe_{1.02}Orn_{1.00}Try_{1.00}Gly_{0.98}$ (average recovery 87%). *Anal.* Calcd. for $C_{33}H_{41}O_6N_9 \cdot CH_3COOH \cdot 1{\cdot}5H_2O$: C, 56.3; H, 6.5; N, 16.9. Found: C, 56.4; H, 6.7; N, 16.5.

ii. Synthesis of L-Histidyl-L-phenylalanyl-L-lysyl-L-tryptophylglycine (IV)

N^{α}-Benzyloxycarbonyl-N^{ε}-formyl-L-lysyl-L-tryptophylglycine Methyl Ester——The titled compound was prepared from N^{α}-benzyloxycarbonyl-N^{ε}-formyl-L-lysine[14] (3.08 g.) and L-tryptophylglycine methyl ester hydrochloride[11] (3.30 g.) according to the manner described in the preparation of the corresponding ornithine derivatives; yield 4.30 g. (76%), m.p. 172~174°, $[\alpha]^{18}_{D}$ $-19.4°$(c=0.6, MeOH). *Anal.* Calcd. for $C_{29}H_{35}O_7N_5$: C, 61.6; H, 6.2; N, 12.4. Found: C, 61.7; H, 6.5; N, 12.4.

N^{ε}-Formyl-L-lysyl-L-tryptophylglycine Methyl Ester Acetate——N^{α}-Benzyloxycarbonyl-N^{ε}-formyl-L-lysyl-L-tryptophylglycine methyl ester (2.81 g.) was hydrogenated as described in the preparation of the corresponding ornithine derivative; yield 2.50 g. (97%), $[\alpha]^{20}_{D}$ $+5.2°$(c=1.0, MeOH), Rf^1 0.58. *Anal.* Calcd. for $C_{21}H_{29}O_5N_5 \cdot CH_3COOH$: C, 53.2; H, 6.8; N, 13.5. Found: C, 52.9; H, 7.0; N, 13.5.

N^{α}-Benzyloxycarbonyl-L-histidyl-L-phenylalanyl-N^{ε}-formyl-L-lysyl-L-tryptophylglycine——N^{ε}-Formyl-L-lysyl-L-tryptophylglycine methyl ester acetate (2.40 g.) was allowed to react with N^{α}-benzyloxycarbonyl-L-histidyl-L-phenylalanine azide (prepared from 2.20 g. of the hydrazide) according to the procedure described in the preparation of the corresponding ornithine derivative. The solvent was removed *in vacuo* and the residue was treated with 1*N* NaOH (8.0 ml.) at room temperature for 30 min. The product was isolated as described in the purification of the corresponding ornithine isomer; yield 3.06 g. (84%), m.p. 202~204°, $[\alpha]^{18}_{D}$ $-30.9°$(c=0.5, dimethylformamide). *Anal.* Calcd. for $C_{43}H_{49}O_9N_9 \cdot 0.5H_2O$: C, 61.1; H, 6.0; N, 14.9. Found: C, 61.0; H, 6.4; N, 14.8.

L-Histidyl-L-phenylalanyl-L-lysyl-L-tryptophylglycine Acetate Monohydrate (IV)——N^{α}-Benzyloxycarbonyl-L-histidyl-L-phenylalanyl-N^{ε}-formyl-L-lysyl-L-tryptophylglycine (3.06 g.) in 40% AcOH (80 ml.) was hydrogenated over a Pd catalyst. The catalyst was removed by filtration and the filtrate was evaporated *in vacuo*. The residue was treated with 0.5*N* HCl (60 ml.) in a boiling water-bath for 30 min. The solution, after neutralization with 1*N* NaOH, was applied to a CMC column (3×30 cm.). The compound was eluted with 0.15*M* pyridine acetate buffer at pH 5.0 and lyophilized to give a white fluffy powder; yield 2.36 g. (82%), $[\alpha]^{17}_{D}$ $-10.3°$(c=0.8, 1*N* HCl), Rf^1 0.42, Rf^2 0.89. Amino acid ratios in the acid hydrolysate $His_{1.00}Phe_{1.00}Lys_{0.99}Gly_{1.00}$ (Try was destroyed, average recovery 90%), amino acid ratios in the LAP digest $His_{1.00}Phe_{1.04}Lys_{0.99}Try_{1.01}Gly_{0.99}$ (average recovery 90%). *Anal.* Calcd. for $C_{34}H_{43}O_6N_9 \cdot CH_3COOH \cdot H_2O$: C, 58.9; H, 6.5; N, 17.2. Found: C, 58.6; H, 6.8; N, 17.0.

iii. Synthesis of L-Histidyl-L-phenylalanyl-D-arginyl-L-tryptophylglycine (III)

N^{α}-Benzyloxycarbonyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine Methyl Ester——The titled compound was prepared from N^{α}-benzyloxycarbonyl-N^{G}-nitro-D-arginine[2] (3.45 g.), L-tryptophylglycine methyl ester hydrochloride (3.30 g.) and dicyclohexylcarbodiimide (2.20 g.) as described in the preparation of the corresponding ornithine derivative. The product was purified by precipitation from MeOH with ether; yield 4.69 g. (73%), m.p. 92°, $[\alpha]^{20}_{D}$ $-22.4°$(c=0.8, MeOH). *Anal.* Calcd. for $C_{28}H_{34}O_8N_8$: C, 55.1; H, 5.6; N, 18.4. Found: C, 55.3; H, 5.9; N, 18.1.

N^{α}-Benzyloxycarbonyl-L-histidyl-L-phenylalanyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine Dihydrate——N^{α}-Benzyloxycarbonyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine methyl ester (1.30 g.) in glacial AcOH (5.0 ml.) was treated with 4.7*N* HBr in glacial AcOH (7.0 ml.) at room temperature for 30 min. The product precipitated by addition of anhydrous ether was dried over KOH pellets and P_2O_5; yield 1.32 g. (96%). This hydrobromide (1.28 g.) was treated with triethylamine (0.55 ml.), subsequently condensed with N^{α}-benzyloxycarbonyl-L-histidyl-L-phenylalanine azide (prepared from 1.35 g. of the hydrazide). Reaction, subsequent saponification and purification procedure were essentially the same as described in the preparation of the protected ornithine pentapeptide; yield 1.28 g. (72%), m.p. 235° (decomp.), $[\alpha]_D^{22}$ $-32.2°$ (c=0.7, dimethylformamide). *Anal.* Calcd. for $C_{42}H_{48}O_{10}N_{12}\cdot 2H_2O$: C, 55.0; H, 5.7; N, 18.3. Found: C, 54.2; H, 6.1; N, 17.9.

L-Histidyl-L-phenylalanyl-D-arginyl-L-tryptophylglycine Acetate Trihydrate (III)——N^{α}-Benzyloxycarbonyl-L-histidyl-L-phenylalanyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine (1.10 g.) was hydrogenated over a Pd catalyst in 50% AcOH (50 ml.). The catalyst was removed by filtration and the filtrate was condensed *in vacuo*. The residue was dissolved in H_2O and the solution was applied to a CMC column (3×35 cm.). The desired fraction which was present in 0.1*M* pyridine acetate buffer (pH 5.0) was pooled and the solvent was removed first by evaporation and finally by lyophilization to give a fluffy powder; yield 0.77 g. (75%), $[\alpha]_D^{20}$ $+24.7°$ (c=0.8, 1*N* HCl), Rf^1 0.42, Rf^2 1.06. Amino acid ratios in the acid hydrolysate $His_{1.00}Phe_{1.02}Arg_{0.99}Gly_{1.00}$ (Try was destroyed, average recovery 97%). Treatment of the peptide with trypsin in 0.01*M* phosphate buffer at pH 7.2 (enzyme substrate ratio 1/50) followed by ninhydrin test on paper chromatogram of the hydrolysate in the system of Partridge revealed that no cleavage of the peptide bond occurred. *Anal.* Calcd. for $C_{34}H_{43}O_6N_{11}\cdot CH_3COOH\cdot 3H_2O$: C, 53.0; H, 6.5; N, 18.9. Found: C, 52.9; H, 7.1; N, 19.2.

iv. Synthesis of D-Histidyl-L-phenylalanyl-D-arginyl-L-tryptophylglycine (VIII) and L-Histidyl-D-phenylalanyl-L-arginyl-D-tryptophylglycine (IX)

These compounds were prepared by the stepwise elongation method as previously described in the preparation of all-D-pentapeptide, namely D-histidyl-D-phenylalanyl-D-arginyl-D-tryptophylglycine.[2] Here, physical constants and analytical data of the synthetic intermediates and the final products were recorded.

N^{α}-Benzyloxycarbonyl-L-phenylalanyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine Methyl Ester Monohydrate——m.p. 110°, $[\alpha]_D^{17}$ $-14.0°$ (c=0.9, dimethylformamide), Rf^1 0.91 *Anal.* Calcd. for $C_{37}H_{43}O_9N_9\cdot H_2O$: C, 57.3; H, 5.8; N, 16.3. Found: C, 57.9; H, 6.2; N, 15.6.

N^{α}-Benzyloxycarbonyl-D-histidyl-L-phenylalanyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine Dihydrate——m.p. 165~170°, $[\alpha]_D^{19}$ $-7.4°$ (c=1.0, dimethylformamide), Rf^1 0.81. *Anal.* Calcd. for $C_{42}H_{48}O_{10}N_{12}\cdot 2H_2O$: C, 55.0; H, 5.7; N, 18.3. Found: C, 54.8; H, 6.5; N, 18.4.

D-Histidyl-L-phenylalanyl-D-arginyl-L-tryptophylglycine Acetate Tetrahydrate (VIII)——$[\alpha]_D^{17}$ $-20.8°$ (c=0.8, 1*N* HCl), Rf^1 0.50, Rf^2 1.0. Amino acid ratios in the acid hydrolysate $His_{1.06}Phe_{1.00}Arg_{1.00}Gly_{1.00}$ (Try was destroyed, average recovery 90%). *Anal.* Calcd. for $C_{34}H_{43}O_6N_{11}\cdot CH_3COOH\cdot 4H_2O$: C, 51.8; H, 6.7; N, 18.5. Found: C, 51.5; H, 7.3; N, 18.3.

N^{α}-Benzyloxycarbonyl-D-phenylalanyl-N^{G}-nitro-L-arginyl-D-tryptophylglycine Methyl Ester Monohydrate——m.p. 134°, $[\alpha]_D^{21}$ $+5.2°$ (c=1.2, dimethylformamide), Rf^1 0.88. *Anal.* Calcd. for $C_{37}H_{43}O_9N_9\cdot H_2O$: C, 57.3; H, 5.8; N, 16.3. Found: C, 58.1; H, 6.2; N, 15.9.

N^{α}-Benzyloxycarbonyl-L-histidyl-D-phenylalanyl-N^{G}-nitro-L-arginyl-D-tryptophylglycine Dihydrate——m.p. 170~175°, $[\alpha]_D^{23}$ $+4.9°$ (c=1.0, dimethylformamide), Rf^1 0.89. *Anal.* Calcd. for $C_{42}H_{48}O_{10}N_{12}\cdot 2H_2O$: C, 55.0; H, 5.7; N, 18.3. Found: C, 55.5; H, 6.5; N, 17.5.

L-Histidyl-D-phenylalanyl-L-arginyl-D-tryptophylglycine Acetate Tetrahydrate (IX)——$[\alpha]_D^{22}$ $+26.9°$ (c=0.7, 1*N* HCl), Rf^1 0.59, Rf^2 1.0. Amino acid ratios in the acid hydrolysate $His_{1.03}Phe_{1.00}Arg_{0.94}Gly_{1.00}$ (Try was destroyed, average recovery 90%). *Anal.* Calcd. for $C_{34}H_{43}O_6H_{11}\cdot CH_3COOH\cdot 4H_2O$: C, 51.8; H, 6.7; N, 18.5. Found: C, 51.4; H, 7.1; N, 18.0.

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