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170. Haruaki Yajima, Kazuo Kubo, and Yoshio Okada : Studies on Peptides. VI.*1 Synthesis of Three Stereoisomeric Pentapeptides of Histidylphenylalanylarginyltryptophylglycine Possessing D-Phenylalanyl-D-arginyl Moiety within the Sequence and Their Melanocyte-stimulating Activities *in vitro*.

(*Faculty of Pharmaceutical Sciences, Kyoto University**2)

In 1963, Lee, *et al.*[1] reported that treatment of α-melanocyte-stimulating hormone (α-MSH) with dilute alkali resulted in racemization within the intact peptide chain, and this in turn brought about the modification in biological properties, such as an increased time of darkening (prolongation) or a delayed action (retardation) to the frog melanocyte *in vitro*. Such modified MSH activities can be considered as the result of the changes in the optical properties of individual amino acid residues or their combinations. The fact that alkali-treated α-MSH possessed the same magnitude of biological activity as that of the untreated hormone suggests that there is a subtle relationship between stereochemical properties of this peptide and its biological function. We felt that the systematic replacement of amino acid residues in an active fragment of α-MSH, L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine (I), with the respective D-amino acid residue would be one of the approaches which permit us to evaluate the physiological properties of this peptide hormone.

We have recently synthesized three pentapeptide isomers, D-histidyl-D-phenylalanyl-D-arginyl-D-tryptophylglycine (all-D-isomer),[2,3] D-histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine (D-His-isomer)[4,5] and L-histidyl-L-phenylalanyl-L-arginyl-D-tryptophylglycine (D-Try-isomer)[4,5] and have observed that the all-D-isomer and the D-His-isomer are both inert while the D-Try-isomer exhibits the activity of 1×10^5 MSH U/g. We have also noted specific inhibitory actions of the all-D-isomer and the D-His-isomer to the MSH active peptides such as I or α-MSH itself. Therefore the L-histidine residue seems to play an important role for the MSH activity of I. Thus we have offered the evidence for the first time that peptide containing D-amino acid residue inhibits the action of the corresponding peptide of L-configuration in the series of investigations related to α-MSH.

We wish to record here the syntheses and the physiological properties of three pentapeptide isomers, L-histidyl-D-phenylalanyl-D-arginyl-L-tryptophylglycine (II), D-histidyl-D-phenylalanyl-D-arginyl-L-tryptophylglycine (III) and L-histidyl-D-phenylalanyl-D-arginyl-D-tryptophylglycine (IV).

A key intermediate in the synthesis of these isomers is N^{α}-benzyloxycarbonyl-D-phenylalanyl-N^{G}-nitro-D-arginine. This was prepared by direct coupling of N^{α}-benzyloxycarbonyl-D-phenylalanine[6] with the triethylammonium salt of N^{G}-nitro-D-arginine[3] *via* the mixed anhydride procedure[7] in nearly 85% yield. The corresponding L-form

*1 Part V : Biochim. et Biophys. Acta, **107**, 141 (1965).
*2 Sakyo-ku, Kyoto (矢島治明, 久保一雄, 岡田芳男).
1) T. H. Lee, V. B.-Janusch : J. Biol. Chem., **238**, 2012 (1963).
2) Part I. K. Hano, M. Koida, K. Kubo, H. Yajima : Biochim. et Biophys. Acta, **90**, 201 (1964).
3) Part II. H. Yajima, K. Kubo : J. Am. Chem. Soc., **87**, 2039 (1965).
4) Part III. H. Yajima, K. Kubo : Biochim. et Biophys. Acta, **97**, 596 (1965).
5) Part IV. H. Yajima, K. Kubo : This Bulletin, **13**, 759 (1965).
6) C. S. Smith, A. E. Brown : J. Am. Chem. Soc., **63**, 2605 (1941).
7) J. R. Vaughan, Jr., R. L. Osato : *Ibid.*, **73**, 3547 (1951).

of the protected dipeptide was prepared by Hofmann, *et al.*[8] by saponification of N^{α}-benzyloxycarbonyl-L-phenylalanyl-N^{G}-nitro-L-arginine methyl ester. Reaction of a mixed anhydride of N^{α}-benzyloxycarbonyl-D-phenylalanyl-N^{G}-nitro-D-arginine with L-tryptophylglycine methyl ester[9] or with D-tryptophylglycine methyl ester[3] gave N^{α}-benzyloxycarbonyl-D-phenylalanyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine methyl ester and N^{α}-benzyloxycarbonyl-D-phenylalanyl-N^{G}-nitro-D-arginyl-D-tryptophylglycine methyl ester respectively. This two by two coupling reaction has been demonstrated successfully by Hofmann, *et al.*[9] in the preparation of the protected tetrapeptide of L-form without a detectable amount of racemization.

The protected tetrapeptide ester, N^{α}-benzyloxycarbonyl-D-phenylalanyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine methyl ester was treated with sodium hydroxide followed by hydrogen bromide to give D-phenylalanyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine. Introduction either N^{α}-benzyloxycarbonyl-L-histidine or N^{α}-benzyloxycarbonyl-D-histidine to this tetrapeptide *via* the azide procedure[10] gave N^{α}-benzyloxycarbonyl-L-histidyl-D-phenylalanyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine and N^{α}-benzyloxycarbonyl-D-histidyl-D-phenylalanyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine respectively. After hydrogenation, the products, L-histidyl-D-phenylalanyl-D-arginyl-L-tryptophylglycine (II) and D-histidyl-D-phenylalanyl-D-arginyl-L-tryptophylglycine (III) were purified on a carboxymethylcellulose (CMC)[11] column using pyridine acetate buffer as an eluent. The synthetic pentapeptides (II) and (III) showed identical Rf value on paper chromatogram in two different solvent systems. Acid hydrolyses of these products (II) and (III) gave the amimo acid ratios (minus tryptophan) predicted by theory. Leucine aminopeptidase (LAP)[12] released less amount of histidine from our preparation of II than that predicted by theory. Smith, *et al.*[13] observed the similar behavior of LAP to peptides containing L-D bond. Histidine released from our pentapeptide (III) by the action of LAP was neglisible.

For the preparation of IV, N^{α}-benzyloxycarbonyl-D-phenylalanyl-N^{G}-nitro-D-arginyl-D-tryptophylglycine methyl ester, which was identical with the product obtained previously during the course of the synthesis of the all-D-isomer[3] by the stepwise elongation method, was saponified and then hydrogenated to give a free tetrapeptide, D-phenylalanyl-D-arginyl-D-tryptophylglycine. The tetrapeptide was reacted with N^{α}-benzyloxycarbonyl-L-histidine azide[14] to form N^{α}-benzyloxycarbonyl-L-histidyl-D-phenylalanyl-D-arginyl-D-tryptophylglycine. The resulting protected pentapeptide was hydrogenated to give IV which was purified as described in the preparation of II. The pentapeptide (IV) thus obtained showed a single spot on paper chromatogram in two different solvent systems. Acid hydrolysis of this synthetic pentapeptide (IV) gave an equimole of the constituent amino acids except tryptophan which was destroyed during the acid hydrolysis. LAP released less amount of histidine from our preparation of IV than that predicted by theory as in the case of II.

The pentapeptides (II), (III). and (IV) were exposed to the action of α-chymotrypsin*3 and trypsin*4 in phosphate buffer at pH 7.2. The resulting solutions were examined by paper chromatography in two different solvent systems. In all cases, no extra

*3 α-Chymotrypsin was purchased from Worthington Biochem. Corp. No. H29809.
*4 Trypsin was purchased from Sigma Chem. Lot T62 B-232.

8) K. Hofmann, W. D. Peckham, A. Rheiner : J. Am. Chem. Soc., **78**, 238 (1956).
9) K. Hofmann, S. Lande : *Ibid.*, **83**, 2286 (1961).
10) T. Curtius : J. prakt. Chem., **70**, 57 (1902); Ber., **35**, 3226 (1902).
11) E. A. Peterson, H. A. Sober : J. Am. Chem. Soc., **78**, 751 (1956).
12) Partially purified (through a second ammonium sulfate fractionation) LAP was prepared according to the method of D. H. Spackman, E. L. Smith, D. M. Brown : J. Biol. Chem., **212**, 255 (1955).
13) E. L. Smith, D. H. Spackman, W. J. Polglase : J. Biol. Chem., **199**, 801 (1952).
14) R. W. Holley, E. Sondheimer : J. Am. Chem. Soc., **76**, 1326 (1954).

spot besides the original spot of the pentapeptide was detected. Thus we conclude that our synthetic pentapeptides possess the well defined D-phenylalanyl-D-arginyl bond in the sequence. Although some release of glycine from II and III might be expected in the case of α-chymotryptic digestion, ninhydrin test on the paper chromatography did not show any detectable amount of glycine.

The malanocyte-expanding activity of these synthetic pentapeptides was examined using the frog-skin from *Rana pipiens.*[15] It was found that II, which possesses the configuration of L-D-D-L-Gly, exhibited the activity of 2.1×10^4 MSH U/g. It has been reported that L-histidyl-D-phenylalanyl-L-arginyl-L-tryptophylglycine[16] and L-histidyl-L-phenylalanyl-D-arginyl-L-tryptophylglycine[8] both possess MSH activity. This indicates that replacement of either or both phenylalanine and arginine residues in the pentapeptide by the corresponding D-isomers does not effect the level of activity of I significantly. Experiment *in vitro* demonstrated that our peptide (II) did not exhibit the effect of retardation or prolongation of MSH activity.

The pentapeptides (III) and (IV) did not possess MSH activity, but a rather weak inhibitory action toward α-MSH activity was noted (1 mg. of these compounds was equivalent to 10^{-6} mg. of melatonin[17]).

Hofmann, *et al.*[18] suggested that both histidine and tryptophan in α-MSH or the pentapeptide (I) are essential for MSH activity. Compounds (III) and (IV) possess L-configuration only at one of these two points within the pentapeptide sequence. The present results indicate that single L-configuration of histidine or tryptophan residue does not contribute to MSH activity of this pentapeptide when the rest of the residues takes D-configuration.

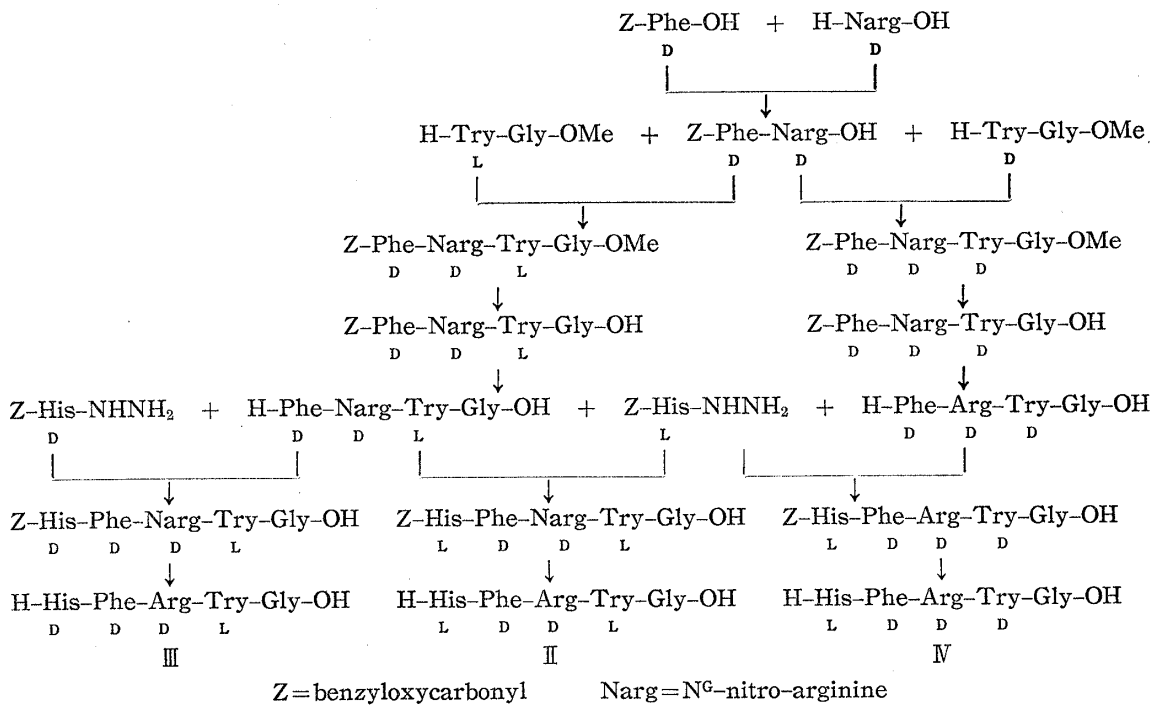

Chart 1. Synthetic Scheme of the Stereoisomeric Pentapeptides (II), (III), and (IV)

15) The MSH assays were performed by Dr. S. Lande, School of Medicine, Yale University, U. S. A. according to the method of K. Shizume, A. B. Lerner, T. B. Fitzpatrick : Endocrinol., **54**, 553 (1954).
16) E. Schnabel, C. H. Li : J. Am. Chem. Soc., **82**, 4576 (1960).
17) A. B. Lerner, J. D. Case, Y. Takahashi : J. Biol. Chem., **235**, 1992 (1960).
18) K. Hofmann, H. Yajima : "Recent Prog. in Hormone Res.," Ed. by G. Pincus, **18**, 41 (1962), Academic Press, New York, N. Y.

Experimental

The melting points were uncorrected. Rotations were determined in a Rex Photoelectric Polarimeter Model NEP-2. The amino acid compositions of the acid and enzymatic hydrolysates were determined with a Hitachi Amino Acid Analyzer, Model KLA-2 according to the method of Moore, *et al.*[19] The following abbreviations for the constituent amino acids, His=histidine, Phe=phenylalanine, Arg=arginine, Try=tryptophan, and Gly=glycine were used. Toyo filter paper No. 51 was used for paper chromatography. Rf^1 values refer to the Partridge system[20]; Rf^2 values refer to the *sec*-BuOH–NH_4OH system[21] and were expressed as a multiple of distance traveled by phenylalanine under identical conditions. Unless stated otherwise, solvents were evaporated *in vacuo* at a bath temperature of 40~50° in a rotatory evaporator.

N^{α}-Benzyloxycarbonyl-D-phenylalanyl-N^{G}-nitro-D-arginine——A mixed anhydride was prepared in the usual manner from N^{α}-benzyloxycarbonyl-D-phenylalanine[6] (5.99 g.) in dry dioxane (25 ml.) with triethylamine (2.8 ml.) and ethyl chloroformate (1.9 ml.). This solution was added slowly with stirring to a chilled solution of N^{G}-nitro-D-arginine[3] (4.38 g.) and triethylamine (2.8 ml.) in H_2O (60 ml.). The mixture was stirred in an ice-bath for 30 min. and at room temperature for 2 hr. The solvent was evaporated. The residue was acidified with 2*N* HCl and was extracted with three 100 ml. portions of AcOEt. The AcOEt extracts were washed successively with 2*N* HCl and H_2O, and were dried over anhydrous Na_2SO_4. Evaporation of the solvent gave an oily residue which crystallized when ether was added. The crystalline mass was washed with ether and recrystallized from EtOH; yield 8.70 g. (87%), m.p. 176~180°. $[\alpha]_D^{20}$ −3.0° (c=0.9, pyridine). (L-isomer: lit.[8] m.p. 185~186°, $[\alpha]_D^{27}$ +1.5°, pyridine). *Anal.* Calcd. for $C_{23}H_{28}O_7N_6$: C, 55.2; H, 5.6; N, 16.8. Found: C, 55.0; H, 5.9; N, 16.5.

N^{α}-Benzyloxycarbonyl-D-phenylalanyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine Methyl Ester——Ethyl chloroformate (0.5 ml.) was added to a cold solution (−10°) of N^{α}-benzyloxycarbonyl-D-pheylalanyl-N^{G}-nitro-D-arginine (2.50 g.) in tetrahydrofuran (20 ml.) containing triethylamine (0.7 ml.) and the mixture was stirred at −10° for 15 min. under anhydrous conditions. This solution was added at −10° to a dimethylformamide (DMF) solution (10 ml.) containing L-tryptophylglycine methyl ester (prepared from 1.65 g. of the hydrochloride[9] and 0.7 ml. of triethylamine). The reaction mixture was stirred for 30 min. at −10°, was allowed to reach room temperature and was kept at that temperature for 1 hr. with stirring. The solvents were evaporated, the resulting oil was dissolved in AcOEt and the solution was successively washed with 5% NH_4OH, 1*N* HCl, and H_2O. Evaporation of the solvent gave a solid material (3.99 g.) which was recrystallized from MeOH by addition of AcOEt; yield 2.39 g. (63%), m.p. 202° (decomp.), $[\alpha]_D^{23}$ −7.0° (c=0.8, DMF). *Anal.* Calcd. for $C_{37}H_{43}O_9N_9$: C, 58.7; H, 5.7; N, 16.6. Found: C, 58.7; H, 6.0; N, 16.5.

N^{α}-Benzyloxycarbonyl-D-phenylalanyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine——N^{α}-Benzyloxycarbonyl-D-phenylalanyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine methyl ester (2.20 g.) was dissolved in MeOH (10 ml.) and 1*N* NaOH (5 ml.) was added. The solution was kept at room temperature for 40 min. and then neutralized with AcOH. MeOH was removed *in vacuo* and the residue was acidified with 1*N* HCl to congo red. The resulting precipitate was collected and recrystallized from MeOH; yield 2.0 g. (91%), m.p. 227° (decomp.), $[\alpha]_D^{20}$ −1.9° (c=0.5, DMF), Rf^1 0.93 ninhydrin negative and single Ehrlich positive spot. *Anal.* Calcd. for $C_{36}H_{41}O_9N_9 \cdot 2H_2O$: C, 55.5; H, 5.8; N, 16.2. Found: C, 55.8; H, 5.9; N, 16.5.

D-Phenylalanyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine Hydrobromide——A 4.7*N* glacial AcOH solution of HBr (5 ml.) was added to a solution of N^{α}-benzyloxycarbonyl-D-phenylalanyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine (1.20 g.) in glacial AcOH (14 ml.). The mixture was stirred at room temperature for 90 min. Dry ether was added and the resulting precipitate was collected by filtration and dried over KOH pellets and P_2O_5 *in vacuo*; yield 1.29 g. (100%), $[\alpha]_D^{33}$ −5.9° (c=0.5, MeOH), Rf^1 0.7, single ninhydrin and Ehrlich positive spot. *Anal.* Calcd. for $C_{28}H_{35}O_7N_9 \cdot 2HBr \cdot H_2O$: C, 42.6; H, 5.0; N, 16.0. Found: C, 42.8; H, 5.5; N, 15.6.

N^{α}-Benzyloxycarbonyl-L-histidyl-D-phenylalanyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine——An ice cold AcOEt solution (approximately 20 ml.) containing N^{α}-benzyloxycarbonyl-L-histidine azide (prepared from 0.46 g. of the hydrazide[14]) was added to an ice cold solution of D-phenylalanyl-N^{G}-nitro-D-arginyl-L-tryptophylglycine hydrobromide (0.80 g.) and triethylamine (3.1 ml.) in DMF (22 ml.). The mixture was kept at 4° for 24 hr. when an AcOEt solution containing additional azide (prepared from 0.23 g. of the hydrazide) was added. The mixture was kept at 4° for an additional 24 hr. The solvent was condensed to one third of the volume. The product precipitated by addition of H_2O was collected by filtration and recrystallized from a mixture of DMF and H_2O (1:1, v/v); yield 0.70 g. (77%), m.p. 223° (decomp.),

19) S. Moore, D. H. Spackman, W. H. Stein: Anal. Chem., **30**, 1185 (1958).
20) S. M. Partridge: Biochem. J., **42**, 238 (1948).
21) J. F. Roland, A. M. Gross: Anal. Chem., **26**, 502 (1954).

$[\alpha]_D^{30}$ +13.3°(c=1.0, DMF), Rf^1 0.78, ninhydrin negative, single Pauly and Ehrlich positive spot. *Anal.* Calcd. for $C_{42}H_{48}O_{10}N_{12}\cdot 1.5H_2O$: C, 55.6; H, 5.7; N, 18.5. Found : C, 55.7; H, 6.0; N, 18.3.

L-Histidyl-D-phenylalanyl-D-arginyl-L-tryptophylglycine (II)——N^{α}-Benzyloxycarbonyl-L-histidyl-D-phenylalanyl-N^G-nitro-D-arginyl-L-tryptophylglycine (0.60 g.) was dissolved in 50% v/v aqueous AcOH (15 ml.) and was hydrogenated over a Pd catalyst.

The catalyst was removed from the clear solution which was evaporated to dryness. The residue was dissolved in H_2O (200 ml.) and the solution was applied to a CMC[11] column (3×20 cm.) which was eluted with the following pH 5.0 pyridine acetate buffers : 0.025*M* (400 ml.), 0.05*M* (900 ml.) and 0.1*M* (1500 ml.). Individual fractions of 15 ml. each were collected at a flow rate of 3 to 4 ml. per min. and the absorbancy of each fraction was determined at 280 mμ. The 0.1*M* eluates containing the desired product were pooled, concentrated to a small volume *in vacuo* and lyophilized; yield 0.34 g. (60%), $[\alpha]_D^{30}$ −11.2°(c=1.0, 1*N* HCl), Rf^1 0.49, Rf^2 1.02 single ninhydrin, Pauly, Sakaguchi and Ehrlich positive spot, amino acid ratios in acid hydrolysate $His_{0.96}\ Phe_{1.00}\ Arg_{0.93}\ Gly_{1.06}$ (average recovery 96%, Try destroyed). His released by the action of LAP[12] was 0.18 μmole from 6.05 μmoles of the product (II). α-Chymotryptic*3 and tryptic*4 digestion of this peptide were performed in a phosphate buffer at pH 7.2 with an enzyme-substrate ratio of 1/50 (w/w) at 37° for 18 hr. In both cases no extra spot besides the original pentapeptide was detected on a paper chromatogram in both partridge and *sec*-BuOH-NH_4OH system by ninhydrin test. *Anal.* Calcd. for $C_{34}H_{43}O_6N_{11}\cdot CH_3COOH\cdot 5H_2O$: C, 50.7; H, 6.7; N, 18.1. Found : C, 51.1; H, 7.2; N, 18.4.

N^{α}-Benzyloxycarbonyl-D-histidyl-D-phenylalanyl-N^G-nitro-D-arginyl-L-tryptophylglycine——This protected pentapeptide was prepared essentially in the same manner as described above in the preparation of the benzyloxycarbonyl derivative of (II) by using D-phenylalanyl-N^G-nitro-D-arginyl-L-tryptophylglycine (0.39 g.) and N^{α}-benzyloxycarbonyl-D-histidine hydrazide[3] (0.34 g.); yield 0.25 g. (54%), m.p. 215° (decomp.), $[\alpha]_D^{30}$ +12.5°(c=1.0, DMF), Rf^1 0.78, ninhydrin negative, single Pauly and Ehrlich positive spot. *Anal.* Calcd. for $C_{42}H_{48}O_{10}N_{12}\cdot 1.5H_2O$: C, 55.6; H, 5.7; N, 18.5. Found : C, 55.8; H, 5.9; N, 18.3.

D-Histidyl-D-phenylalanyl-D-arginyl-L-tryptophylglycine (III)——N^{α}-Benzyloxycarbonyl-D-histidyl-D-phenylalanyl-N^G-nitro-D-arginyl-L-tryptophylglycine (0.20 g.) was hydrogenated over a Pd catalyst and the product was purified as described in the preparation of II; yield 0.08 g. (40%), $[\alpha]_D^{30}$ −25.8°(c=0.3, 1*N* HCl), Rf^1 0.49, Rf^2 1.02, single ninhydrin, Pauly, Sakaguchi and Ehrlich positive spot. Amino acid ratios in acid hydrolysate $His_{1.00}\ Phe_{0.98}\ Arg_{0.99}\ Gly_{0.95}$ (average recovery 85%, Try destroyed). LAP[12] release 0.07 μmole of His from 3.25 μmoles of the product (III). Digestion of this peptide with α-chymotrypsin*3 and trypsin*4 and examination of these hydrolysates by paper chromatography were conducted exactly as described in the case of II. No extra spot besides the original peptide was detected by ninhydrin test on the paper chromatogram. *Anal.* Calcd. for $C_{34}H_{43}O_6N_{11}\cdot CH_3COOH\cdot 3H_2O$: C, 53.0; H, 6.6; N, 18.9. Found : C, 53.2; H, 6.8; N, 18.7.

N^{α}-Benzyloxycarboanyl-D-phenylalanyl-N^G-nitro-D-arginyl-D-tryptophylglycine Methyl Ester——This compound was prepared from N^{α}-benzyloxycarbonyl-D-phenylalanyl-N^G-nitro-D-arginine (2.43 g.) and D-tryptophylglycine methyl ester hydrochloride[3] (1.53 g.) by the mixed anhydride procedure essentially as described above in the preparation of N^{α}-benzyloxycarbonyl-D-phenylalanyl-N^G-nitro-D-arginyl-L-tryptophylglcine methyl ester; yield 1.97 g. (54%), m.p. 146~150°, $[\alpha]_D^{17}$ +14.8°(c=0.5, MeOH). (lit.[3] m.p. 145~148°, $[\alpha]_D^{20}$ +20.5°, MeOH). *Anal.* Calcd. for $C_{37}H_{43}O_9N_9$: C, 58.8; H, 5.7; N, 16.6. Found : C, 58.8; H, 6.0; N, 16.3.

N^{α}-Benzyloxycarbonyl-D-phenylalanyl-N^G-nitro-D-arginyl-D-tryptophylglycine—— N^{α}-Benzyloxycarbonyl-D-phenylalanyl-N^G-nitro-D-arginyl-D-tryptophylglycine methyl ester (1.01 g.) in MeOH (15 ml.) was treated with 1*N* NaOH (2.6 ml.) at room temperature for 60 min. The product was purified as described above in the preparation of N^{α}-benzyloxycarbonyl-D-phenylalanyl-N^G-nitro-D-arginyl-L-tryptophylglycine; yield 0.88 g. (89%), m.p. 205~209°, $[\alpha]_D^{35}$ +21.5°(c=1.0, DMF). *Anal.* Calcd. for $C_{36}H_{41}O_9N_9\cdot 0.5H_2O$: C, 57.4; H, 5.6; N, 16.7. found : C, 57.3; H, 5.6; N, 16.5.

D-Phenylalanyl-D-arginyl-D-tryptophylglycine——N^{α}-Benzyloxycarbonyl-D-phenylalanyl-N^G-nitro-D-arginyl-D-tryptophylglycine (0.86 g.) was hydrogenated over a Pd catalyst in a mixture of MeOH (20 ml.) and 50% (v/v) aqueous AcOH (10 ml.) for 15 hr. The catalyst was removed from the pink colored solution which was evaporated to dryness. The residue was lyophilized. The product was dissolved in H_2O (200 ml.) and applied to a CMC column (2×20 cm.) which was eluted with the following pH 5.0 pyridine acetate buffers : 0.025*M* (600 ml.) and 0.05*M* (900 ml.). Individual fractions (15 ml. each) were collected at a flow rate of 3 to 4 ml. per min., and the absorbancy at 280 mμ was determined for each fraction. The desired product was present in the 0.05*M* eluates (tubes 45~85) which were pooled, evaporated to a small volume and lyophilized; yield 0.46 g. (65%), $[\alpha]_D^{32}$ −3.7°(c=0.6, 1*N* HCl), Rf^1 0.58, Rf^2 1.1, single ninhydrin, Sakaguchi and Ehrlich positive spot. *Anal.* Calcd. for $C_{28}H_{36}O_5N_8\cdot CH_3COOH\cdot 2H_2O$: C, 54.5; H, 6.9; N, 16.9. Found : C, 54.0; H, 7.2; N, 17.3.

N^{α}-Benzyloxycarbonyl-L-histidyl-D-phenylalanyl-D-arginyl-D-tryptophylglycine——D-Phenylalanyl-D-arginyl-D-tryptophylglycine (0.16 g.) was reacted with N^{α}-benzyloxycarbonyl-L-histidine azide (prepared from 0.17 g. of the hydrazide[14]). The reaction and the purification procedure are essentially the same

as described in the preparation of the benzyloxycarbonyl derivative of II; yield 0.15 g. (69%), m.p. 218~220°, $[\alpha]_D^{35}$ +26.6°(c=1.0, 50% v/v aqueous AcOH). *Anal.* Calcd. for $C_{42}H_{49}O_8N_{11}\cdot 4H_2O$: C, 55.6; H, 6.3; N, 17.0. Found : C, 55.6; H, 6.5; N, 16.7.

L-Histidyl-D-phenylalanyl-D-arginyl-D-tryptophylglycine (IV)——N^{α}-Benzyloxycarbonyl-L-histidyl-D-phenylalanyl-D-arginyl-D-tryptophylglycine (0.80 g.) was hydrogenated over a Pd catalyst in 50% v/v aqueous AcOH until the evolution of CO_2 had ceased. The product was purified on a CMC column as described in the preparation of II; yield 0.74 g. (99%), $[\alpha]_D^{23}$ +30.2°(c=0.5, 1*N* HCl), Rf^1 0.49, Rf^2 1.0, single ninhydrin, Pauly, Sakaguchi and Ehrlich positive spot. Amino acid ratios in acid hydrolysate $His_{0.99}\,Phe_{1.02}\,Arg_{0.96}\,Gly_{1.00}$ (average recovery 89%, Try destroyed). LAP[12] release 0.09 μmole of His from 1.46 μmoles of IV. Digestion of this peptide with α-chymotrypsin*[3] and trypsin*[4] and examination of these hydrolysates by paper chromatography were conducted exactly as described in the case of II. No extra spot besides the original peptide was detected by ninhydrin test on the paper chromatogram. *Anal.* Calcd. for $C_{34}H_{43}O_6N_{11}\cdot CH_3COOH\cdot 3H_2O$: C, 53.0; H, 6.6; N, 18.9. Found : C, 52.5; H, 7.0; N, 19.3.

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Summary

The syntheses of three pentapeptide isomers, L-histidyl-D-phenylalanyl-D-arginyl-L-tryptophylglycine (II), D-histidyl-D-phenylalanyl-D-arginyl-L-tryptophylglycine (III) and L-histidyl-D-phenylalanyl-D-arginyl-D-tryptophylglycine (IV) were described. N^{α}-Benzyloxycarbonyl-D-phenylalanyl-N^{G}-nitro-D-arginine served as a key intermediate for the synthesis of these isomers. Melanocyte-stimulating activities of these compounds were examined using the frog-skin from *Rana pipiens*. It was found that II possessed the activity identical to that of L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine (I). The peptides (III) and (IV) were both inactive.

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