at a pH higher than 5.7. However, as the pH is dropped below 5.7, there is an abrupt rise in the melting temperature and a plateau is reached between pH 3.8 and 4.4. As seen in Figure 2b, this is the region in which approximately one proton has been added for every two cytosine bases. Lowering the pH further results in the addition of more protons, and these can only be added by opening up the pairing seen in Figure 1. This is accompanied by a destabilization of the helix as shown by a drop in the melting temperature. When the ionic strength of the solution is raised to 1 *M* NaCl there is a shift in the melting temperature. The helix does not begin to form until pH 5.4 and, in addition, the melting temperature is decreased. The poly C helix is thus destabilized by the addition of added electrolyte in sharp contrast to the enhanced stability of DNA and other polynucleotides. This difference is readily explained by the fact that the helical form of poly C with the hydrogen bonding shown in Figure 1 is stabilized somewhat by forming an inner salt. The positive charge centrally located on the pyrimidines partially neutralizes the two negative charges on the ribose phosphate chains on the outside of the helix. The molecule is stabilized by this electrostatic interaction. Raising the ionic strength reduces the magnitude of the interaction and this results in a destabilization of the molecule as shown by the lower melting temperature. The same electrolyte effect is seen in the helical form of polyadenylic acid[14] which forms at acid pH. In that structure the proton is also attached to the centrally located purine bases and it neutralizes the charge on the externally located phosphate groups.[15]

Several types of experimental investigations have been described above which suggest that the poly C molecule has the same form in the solution as that which

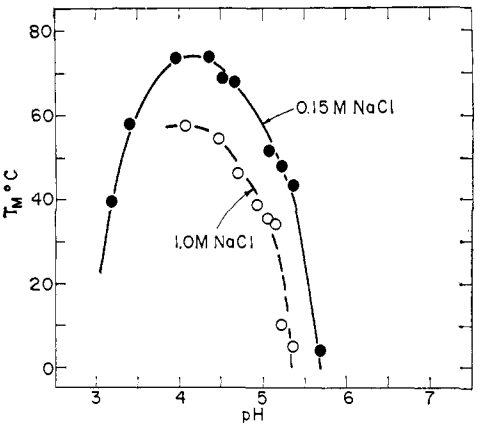

Figure 8. The melting temperature of poly C as a function of pH and ionic strength. In 0.15 *M* NaCl no melting is observed above pH 5.7 where the molecule is single stranded. Increasing ionic strength decreases the melting temperature down to pH 4.

was observed by X-ray diffraction studies of fibers in the solid state.[1] The titration experiments have shown that the single p*K* which is observed in cytidine or cytidylic acid at pH 4.3 has been modified in poly C so that half of the protons are titrated in a cooperative manner at pH 5.7, while the other half are titrated cooperatively at pH 3.0. In the region between pH 5.7 and 4, vibrations from both protonated and nonprotonated cytosine rings are seen in the infrared spectrum which also imply that the polymer is half protonated. Further evidence for helix formation at pH 5.7 is seen from the changes in the ultraviolet absorption spectra. Finally, thermal denaturation experiments are also consistent with the interpretation that the helical form of poly C is held together by pyrimidine rings which are only half protonated.

Acknowledgment. This work was supported by grants from the National Institutes of Health, U. S. Public Health Service, and from the National Science Foundation.

(14) P. O. P. Ts'o, G. K. Helmkamp, and C. Sander, *Proc. Natl. Acad. Sci. U. S.*, **48**, 686 (1962).
(15) A. Rich, D. R. Davies, F. H. C. Crick, and J. D. Watson, *J. Mol. Biol.*, **3**, 71 (1961).

Studies on Peptides. II. Synthesis and Physiological Properties of D-Histidyl-D-phenylalanyl-D-arginyl-D-tryptophylglycine, an Optical Antipode of an Active Fragment of α-Melanocyte-Stimulating Hormone[1,2]

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Synthesis of the pentapeptide D-*histidyl*-D-*phenylalanyl*-D-*arginyl*-D-*tryptophylglycine, an optical antipode of an active fragment of α-melanocyte-stimulating hormone, is described. This synthetic peptide has been shown to inhibit the action of* L-*histidyl*-L-*phenylalanyl*-L-*arginyl*-L-*tryptophylglycine in frog melanocyte, in vivo and in vitro.*

(1) This work has been supported in part by a grant from the Ministry of Education, Japan.
(2) Preliminary report of the biological observations described herein has appeared in *Biochim. Biophys. Acta*, **90**, 201 (1964). The authors wish to express their appreciation to Dr. K. Hano and Mr. M. Koida, University of Osaka, for the biological assays which were performed according to the method of K. Shizume, A. B. Lerner, and T. B. Fitzpatrick, *Endocrinology*, **54**, 553 (1954).

It is a well-documented phenomenon that heating pituitary extracts in an alkaline solution causes prolongation of melanocyte-stimulating activity as well as potentiation of its action to frog skin in an *in vivo* system.[3-5] Among the hormones produced by the mammalian pituitary glands, adrenocorticotropic hormone (ACTH)[6] and two types of melanocyte-stimulating hormones[6] (MSH), α-MSH and β-MSH, are known to be the main components capable of bringing about darkening of the skin.[7] Very little is known about the chemical or physicochemical changes in the molecules of MSH and ACTH treated with sodium hydroxide which would account for the observed biological changes.

In 1963, Lee, *et al.*,[8,9] observed that treatment of homogeneous porcine α-MSH with 0.1 *N* sodium hydroxide at 100° for 10 min. resulted in prolongation of its action on frog skin melanocyte *in vitro*. Potentiation of melanocyte-stimulating activity by sodium hydroxide treated α-MSH was not observed by these authors. They concluded that this procedure resulted in racemization within the intact peptide chain which in turn caused the modification in biological properties.

Though the modified MSH activity of alkali-treated ACTH was mentioned briefly by Shepherd, *et al.*,[10] Engel,[11] and Lee, *et al.*,[8] recently Pickering and Li[12] confirmed that alkali treatment of sheep ACTH potentiated and prolonged the intrinsic MSH potency of ACTH with a loss of adrenal-stimulating and lipolytic activity. Racemization of several amino acids and a limited cleavage of the second peptide bond from the N-terminus were reported by these authors.

Li's group[12] also observed racemization in alkali-treated bovine β-MSH and suggested that it was responsible for the prolongation effect of this hormone.

Attention should be focused on the fact that the melanocyte-stimulating activity of alkali-treated α- and β-MSH is of the same magnitude as that of the untreated materials, and MSH potency of alkali-treated ACTH is higher than that of the native hormone. In all cases racemization was noted especially at the amino acid residues in the heptapeptide sequence, methionylglutamylhistidylphenylalanylarginyltryptophylglycine, a common structural feature of these hormones.

The structure of α-MSH[13] was determined with an extremely small amount of the hormone, and information pertaining to the stereochemical nature of its constituent amino acids was not available. Based on the premise that only a molecule possessing the all-L-configuration would possess maximum biological activity, Hofmann, *et al.*,[14] undertook the synthesis of α-MSH and its related peptides. But later it was found that some peptides containing D-amino acid residue were as active biologically as the corresponding all-L-peptide; for example, L-histidyl-L-phenylalanyl-D-arginyl-L-tryptophylglycine[15] and L-histidyl-D-phenylalanyl-L-arginyl-L-tryptophylglycine[16,17] exhibited activities equivalent to that of the synthetic pentapeptide, L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine (all-L-isomer).[15,18-21] This amino acid sequence corresponds to positions 6 to 10 in α-MSH, and the all-L-pentapeptide is the smallest peptide fragment derived from the α-MSH molecule which possesses biological activity.

These observations have cast some doubt that the L-configuration is a structural requirement for MSH activity[8] or that biological receptor sites exhibit a high degree of stereospecificity.[12] In view of these conflicting findings, evaluation of the physiological properties of the structural antipode of the all-L-isomer D-histidyl-D-phenylalanyl-D-arginyl-D-tryptophylglycine (I) would be of interest.

For synthesis of I, the commercial preparations of D-histidine, D-phenylalanine, and D-tryptophan were used.[22] D-Histidine was converted into carbobenzoxy-D-histidine hydrazide following the method of Holley and Sondheimer.[23] The rotation value of the hydrazide, $+36.2°$ in 1 *N* hydrochloric acid, was measured for comparison with the corresponding L-isomer. D-Phenylalanine and D-tryptophan were converted into carbobenzoxy derivatives by the standard procedure. For the preparation of D-arginine, acetyl-DL-arginine was exposed to the action of acylase[24] at pH 8 according to the procedure described by Greenstein, *et al.*,[25] and Nadai.[26] The resulting acetyl-D-arginine was deacetylated by acid. The physical properties of the resulting D-arginine hydrochloride were identical with those described in the literature.[25,26] D-Arginine hydrochloride was converted into carbobenzoxynitro-D-arginine according to the procedure reported by Gibian, *et al.*,[27] and Hofmann, *et al.*[28]

(3) F. W. Landgrebe and H. Waring, *Quart. J. Exptl. Physiol.*, **31**, 31 (1941); F. W. Landgrebe, E. Reid, and H. Waring, *ibid.*, **32**, 121 (1943); F. W. Landgrebe and G. M. Mitchell, *ibid.*, **39**, 11 (1954).

(4) L. T. Hogben and C. Gordon, *J. Exptl. Biol.*, 7, 286 (1930).

(5) C. H. Li, *Advan. Protein Chem.*, **12**, 269 (1957); *Lab. Invest.*, **8**, 574 (1959).

(6) Review articles for ACTH, see K. Hofmann, *Ann. Rev. Biochem.*, **31**, 213 (1962), and for MSH, see A. B. Lerner and T. H. Lee, *Vitamins Hormones*, **20**, 337 (1963).

(7) Lipotropin, C. H. Li, *Nature*, **201**, 924 (1964), peptide (L'), Y. Birk and C. H. Li, *J. Biol. Chem.*, **239**, 1048 (1964), and Astwood peptide I, Hofmann and Serdarevich, unpublished, in K. Hofmann and P. G. Katsoyannis, *Proteins*, 1, 53 (1963), were reported to possess MSH activity.

(8) T. H. Lee, A. B. Lerner, and V. B. Janusch, *Ann. N. Y. Acad. Sci.*, **100**, 658 (1963).

(9) T. H. Lee and V. B. Janusch, *J. Biol. Chem.*, **238**, 2012 (1963).

(10) R. G. Shepherd, S. D. Willson, K. S. Howard, P. H. Bell, D. S. Davis, S. B. Davis, E. A. Eigner, and N. E. Shakespeare, *J. Am. Chem. Soc.*, **78**, 5067 (1958).

(11) F. L. Engel, *Vitamins Hormones*, **19**, 189 (1961).

(12) B. T. Pickering and C. H. Li, *Arch. Biochem. Biophys.*, **104**, 119 (1964); I. I. Geschwind and C. H. Li, *ibid.*, **106**, 200 (1964).

(13) J. I. Harris and A. B. Lerner, *Nature*, **179**, 1346 (1957).

(14) K. Hofmann, H. Yajima, and E. T. Schwartz, *J. Am. Chem. Soc.*, **82**, 3732 (1960).

(15) K. Hofmann and S. Lande, *ibid.*, **83**, 2286 (1961).

(16) E. Schnabel and C. H. Li, *ibid.*, **82**, 4576 (1960).

(17) C. H. Li, E. Schnabel, and D. Chung, *J. Am. Chem. Soc.*, **82**, 2062 (1960).

(18) K. Hofmann, M. E. Woolner, G. Spühler, and E. T. Schwartz, *ibid.*, **80**, 1486 (1958).

(19) K. Hofmann, M. E. Woolner, H. Yajima, G. Spühler, T. A. Thompson, and E. T. Schwartz, *ibid.*, **80**, 6458 (1958).

(20) K. Hofmann, T. A. Thompson, M. E. Woolner, G. Spühler, H. Yajima, J. D. Cipera, and E. T. Schwartz, *ibid.*, **82**, 3721 (1960).

(21) R. Schwyzer and C. H. Li, *Nature*, **182**, 1669 (1958).

(22) D-Histidine was purchased from Nutritional Biochemicals Corporation. D-Phenylalanine and D-tryptophan were purchased from Tanabe Pharmaceutical Co.

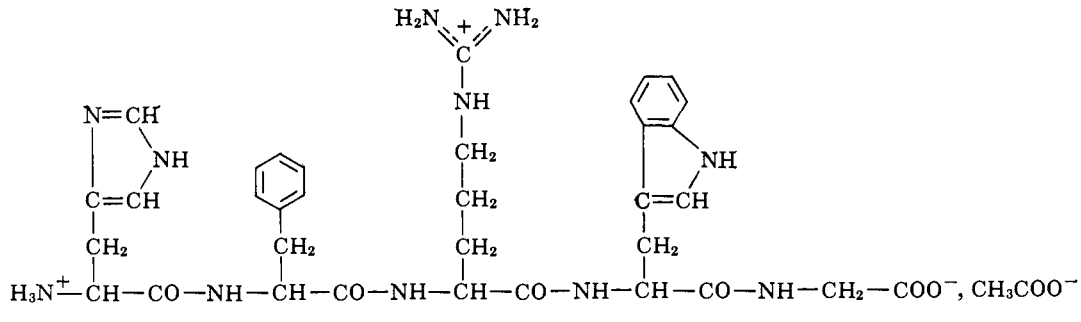
(23) R. W. Holley and E. Sondheimer, *J. Am. Chem. Soc.*, **76**, 1326 (1954).

(24) Acylase prepared from Takadiastase was used. The authors express their appreciation to Sankyo Co. for a generous supply of this enzyme.

(25) S. M. Birbaum, M. Winitz, and J. P. Greenstein, *Arch. Biochem. Biophys.*, **60**, 496 (1956).

(26) Y. Nadai, *J. Biochem.* (Tokyo), **45**, 687 (1958).

(27) H. Gibian and E. Schröder, *Ann.*, **642**, 145 (1961).



I-acetate

For the preparation of this pentapeptide sequence, the stepwise elongation approach[29] starting from C-terminal glycine methyl ester was employed. Carbobenzoxy-D-tryptophan was condensed with glycine methyl ester by the dicyclohexylcarbodiimide procedure[30] to give carbobenzoxy-D-tryptophylglycine methyl ester, which was hydrogenated over a palladium catalyst. The resulting dipeptide ester acetate was converted into its hydrochloride and characterized. Carbobenzoxynitro-D-arginine was then condensed with the dipeptide ester *via* the mixed anhydride procedure[31] to give carbobenzoxynitro-D-arginyl-D-tryptophylglycine methyl ester, which was exposed to the action of hydrogen bromide in acetic acid.[32] The resulting decarbobenzoxylated tripeptide ester was allowed to react with the mixed anhydride of carbobenzoxy-D-phenylalanine[33] to form the protected tetrapeptide ester, carbobenzoxy-D-phenylalanylnitro-D-arginyl-D-tryptophylglycine methyl ester. It was decarbobenzoxyloted to the corresponding tetrapeptide ester, D-phenylalanylnitro-D-arginyl-D-tryptophylglycine methyl ester, by the action of hydrogen bromide in acetic acid. The action of hydrogen bromide on a tryptophan-containing peptide usually gives a pink-colored material, but the reaction under vigorously anhydrous conditions minimized this difficulty.

Carbobenzoxy-D-histidine hydrazide was converted into its azide, which was allowed to react with the above tetrapeptide ester to form the protected pentapeptide ester, carbobenzoxy-D-histidyl-D-phenylalanylnitro-D-arginyl-D-tryptophylglycine methyl ester. Saponification of this protected pentapeptide ester by sodium hydroxide followed by hydrogenolysis over a palladium catalyst gave the crude pentapeptide, D-histidyl-D-phenylalanyl-D-arginyl-D-tryptophylglycine. A preliminary test indicated that some histidine was released by the action of partially purified leucine aminopeptidase (LAP).[34] The product was exposed to the action of LAP in preparative scale. The hydrolysate was applied to a carboxymethylcellulose (CMC)[35] column and the compound was eluted with pyridine acetate buffer (0.1 *M*) at pH 5.0. The lyophilized material was further treated with trypsin[36] in order to remove a very small amount of contamination of the peptide containing L-arginine, and the pentapeptide was purified on a CMC column. Lyophilization of the final product gave a white powder which revealed a single spot by Pauly, Sakaguchi, Ehrlich, and ninhydrin tests after chromatography on paper. The amino acid ratios in acid hydrolysate were identical with that predicted by theory.

In order to ascertain the configuration of the constituent amino acids, an acid hydrolysate of the synthetic product was exposed to the action of D-amino acid oxidase[37] and subsequently subjected to quantitative amino acid analysis. Extensive disappearance of phenylalanine was observed. Since D-amino acid oxidase does not react quantitatively with D-histidine and D-arginine,[38] the acid hydrolysate was exposed to the action of L-amino acid oxidase.[39] After this treatment, the recovery of the constituent amino acids was almost the same as that of the original acid hydrolysate.

LAP treatment did not release any histidine. The peptide was incubated with α-chymotrypsin[40] and trypsin,[36] respectively. The resulting solutions were examined by paper chromatography, and in both cases no other spot besides the original spot of pentapeptide was detected by ninhydrin test.

Acid hydrolysis does not permit one to ascertain the configuration of tryptophan, but D-tryptophylglycine (1.15 μmoles) which was derived from carbobenzoxy-D-tryptophylglycine methyl ester by alkaline treatment followed by hydrogenolysis was found to release 0.03 μmole of tryptophan and glycine by the action of LAP.

The experimental evidence cited appears to justify the conclusion that the synthetic pentapeptide possesses a high degree of homogeneity and stereochemical homogeneity of the constituent amino acid with the

(28) K. Hofmann, W. D. Peckham, and A. Rheiner, *J. Am. Chem. Soc.*, **78**, 238 (1956).
(29) R. Geiger, K. Sturm, and W. Siedel, *Ber.*, **96**, 1080 (1963).
(30) J. C. Sheehan and G. P. Hess, *ibid.*, **77**, 1067 (1941). (1955).
(31) J. R. Vaughan, Jr., and R. L. Osato, *ibid.*, **73**, 3547 (1951).
(32) G. W. Anderson, J. Blodinger, and A. D. Welcher, *ibid.*, **74**, 5309 (1952).
(33) C. S. Smith and A. E. Brown, *ibid.*, **63**, 2605 (1941).
(34) Partially purified (through second ammonium sulfate fractionation) LAP was prepared according to the method of D. H. Spackman, E. L. Smith, and D. M. Brown, *J. Biol. Chem.*, **212**, 255 (1955). Contamination of some chymotryptic activity was found in this preparation as stated by these authors.
(35) E. A. Peterson and H. A. Sober, *J. Am. Chem. Soc.*, **78**, 751 (1956). The carboxymethylcellulose (Cellex-CM) was obtained from the BioRad Corporation, Richmond, Calif.
(36) Sigma Chemical Co., Lot T62B-232.
(37) Hog kidney preparation. The authors express their appreciation to Dr. T. Suzuki, The Protein Institute, University of Osaka, for a generous supply of this enzyme.
(38) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1961, p. 1782.
(39) Snake venom preparation. The authors express their appreciation to Dr. T. Suzuki, The Protein Institute, University of Osaka, for a generous supply of this enzyme.
(40) Worthington No. H29809.

exception of D-tryptophan which could not be established in the pentapeptide moiety.

The rotation value of I, +12.0° in 1 *N* hydrochloric acid, indicates that I is an optical antipode of the all-L-isomer, whose rotation value, −11.8°[15] or −11.7°,[29] was reported in the literature.

Throughout this synthesis, the physical constants of all of the synthetic intermediates were compared with those of the corresponding L-isomers which appear in the literature. As expected, every rotation figure of the synthetic intermediates was equal but opposite in direction to those of the respective L-isomers. Furthermore we have demonstrated the usefulness of the enzymatic technique in the preparative scale for the present peptide synthesis.

In a preliminary communication[2] we reported that this peptide showed no darkening effect on the isolated frog skin but had the ability to lighten the color of a predarkened specimen by the all-L-isomer or by the synthetic α-MSH,[14] though it failed to lighten the color of the skin predarkened by caffeine. When an equal amount of the L-isomer (33 μg./ml.) was mixed with I at the beginning of the assay, no darkening was observed. When I was injected into the thigh of a frog which was pre-injected with the L-isomer and darkened, the lightening of the color was observed around this area. The color-lightening potency is one-millionth that of melatonin.[41]

The above experiment suggests that the all-D-pentapeptide (I) acts as an inhibitor of the all-L compound and furthermore implies that caffeine acts on melanocyte in a different manner from that of α-MSH or the all-L-pentapeptide fragment. The synthetic peptide does not possess any lipolytic activity.[42]

Since the above observation was made by testing only a fragment of α-MSH, it might be insufficient evidence to answer conclusively the question whether L-configuration is a structural requirement for α-MSH activity. However we offer here the first attempt to examine the physiological properties of the structural antipode in peptide hormone research.

Experimental[43]

Carbobenzoxy-D-histidine Hydrazide. D-Histidine hydrochloride (4.4 g.)[22] was converted into its methyl ester by passing the dry hydrogen chloride gas into methanolic solution of the amino acid with heating. The resulting methyl ester (4.8 g.) was converted into carbobenzoxy-D-histidine methyl ester essentially in the manner described in preparation of the corresponding L-isomer by Holley and Sondheimer.[23] The oily product was dissolved in ethanol (10 ml.), and 80% hydrazine hydrate (2.5 ml.) was added. The crystalline product formed on standing at room temperature was collected and recrystallized from ethanol; yield 4.1 g. (67.5%), m.p. 172–173°, $[\alpha]^{30}D$ +36.8° (*c* 2.0, 1 *N* hydrochloric acid). The rotation of the L-isomer was $[\alpha]^{30}D$ −35.7° (*c* 2.0, 1 *N* hydrochloric acid); lit.[23] m.p. 172–173°.

Anal. Calcd. for $C_{14}H_{17}N_5O_3$: C, 55.4; H, 5.7; N, 23.1. Found: C, 55.2; H, 5.9; N, 23.0.

D-Arginine Hydrochloride.[25,26] Acetyl-DL-arginine, m.p. 100–103° (lit.[25] m.p. 108–110°), prepared from acetyl-L-arginine according to Greenstein, *et al.*,[25] was incubated at pH 8.0 with acylase prepared from Takadiastase[24] according to Nadai.[26] The enzyme was denaturated by heating and the filtrate was condensed to give crystalline acetyl-D-arginine, m.p. 265–268°, $[\alpha]^{20}D$ +6.6° (*c* 2.0, 1 *N* hydrochloric acid), lit.[25] m.p. 270°, $[\alpha]^{25}D$ +7.0° in 1 *N* hydrochloric acid. According to the procedure of Greenstein, *et al.*,[25] it was deacetylated by heating with 2 *N* hydrochloric acid to give D-arginine hydrochloride; m.p. 220–223°, $[\alpha]^{20}D$ −21.9° (*c* 2.0, 5 *N* hydrochloric acid); lit.[25] $[\alpha]^{25}D$ −23.4° in 5 *N* hydrochloric acid; lit.[26] m.p. 220°, $[\alpha]^{25}D$ −22.0° in 5 *N* hydrochloric acid.

Carbobenzoxynitro-D-arginine.[27] D-Arginine was nitrated according to Gibian, *et al.*,[27] m.p. 254–255°, $[\alpha]^{22}D$ −24.7° (*c* 0.6, 2 *N* hydrochloric acid); lit.[27] m.p. 253–254°, $[\alpha]^{22}D$ −22.6° in 2 *N* hydrochloric acid. Nitro-D-arginine was converted into carbobenzoxynitro-D-arginine by the standard procedure; m.p. 134–136°, $[\alpha]^{24}D$ +2.5° (*c* 2.0, methanol); lit.[27] m.p. 132–134°, $[\alpha]^{23}D$ +2.8° in methanol.

Anal. Calcd. for $C_{14}H_{19}N_5O_6$: C, 47.6; H, 5.4; N, 19.8. Found: C, 47.8; H, 5.7; N, 19.6.

Carbobenzoxy-D-phenylalanine.[33] D-Phenylalanine[22] (8.26 g.) was converted into the carbobenzoxy derivative by the standard procedure; yield 14.15 g. (95.3%), m.p. 84–86.5°, $[\alpha]^{29}D$ −6.0° (*c* 0.75, glacial acetic acid); lit.[33] m.p. 88–89°, $[\alpha]^{24}D$ −4.6° in glacial acetic acid.

Carbobenzoxy-D-tryptophan. D-Tryptophan[22] (14.49 g.) was carbobenzoxylated with carbobenzoxy chloride (12.6 ml.) and 1 *N* sodium hydroxide (160 ml.). The product was recrystallized from ethyl acetate and ether; yield 22.1 g. (92%), m.p. 127–129°, $[\alpha]^{24}D$ +5.0° (*c* 0.8, methanol) (L-isomer: lit.[44] m.p. 126°).

Anal. Calcd. for $C_{19}H_{18}N_2O_4$: C, 67.4; H, 5.4; N, 8.3. Found: C, 67.6; H, 5.4; N, 8.2.

Carbobenzoxy-D-tryptophylglycine Methyl Ester. The compound was prepared essentially in the manner described by Hofmann, *et al.*,[18] in the synthesis of the corresponding L-isomer. Glycine methyl ester hydrochloride (6.29 g.) was dissolved in a mixture of methanol (25 ml.) and dimethylformamide (25 ml.). After addition of triethylamine (7.0 ml.) the methanol was concentrated to sirup at 35° under reduced pressure. Dimethylformamide (20 ml.) was added and the solution was mixed with a dioxane solution (20 ml.) of carbobenzoxy-D-tryptophan (13.53 g.). To this mixture cooled to 0° N,N′-dicyclohexylcarbodiimide[30] (10.32 g.) was added portionwise with rapid stirring over a period of 15 min. The reaction mixture was stirred at

(41) A. B. Lerner, J. D. Case, and R. V. Heinzelman, *J. Am. Chem. Soc.*, 81, 6084 (1959). We are indebted to Dr. S. Lande, School of Medicine, Yale University, for the determination of this potency.

(42) We are indebted to Drs. A. Tanaka and H. Otsuka of Shionogi Research Laboratory, Shionogi & Co., Ltd., for these assays.

(43) Doubly distilled water was employed. The organic solvents were freshly distilled. The melting points are uncorrected. Rotations were determined in a Rex photoelectric polarimeter, Model NEP-2 (6302309). The amino acid composition of the acid and enzymatic hydrolysates was determined with a Hitachi amino acid analyzer, Model KLA-2, according to the method of S. Moore, D. H. Spackman, and W. H. Stein, *Anal. Chem.*, 30, 1185 (1958). Toyo filter paper No. 51 was used for paper chromatography. R_f^1 values refer to the Partridge system (S. M. Partridge, *Biochem. J.*, 42, 238 (1948)); R_f^2 values refer to the 2-butanol–ammonia system (J. F. Roland and A. M. Gross, *Anal. Chem.*, 26, 502 (1954)) and are expressed as a multiple of distance traveled by L-phenylalanine under identical conditions. Unless stated otherwise, solvents were evaporated *in vacuo* at a bath temperature of 40–50° in a rotatory evaporator. For column chromatography, Toyo fraction collector Model SF-200-A was used.

(44) E. L. Smith, *J. Biol. Chem.*, 175, 39 (1948).

room temperature overnight. N,N′-Dicyclohexylurea was removed by filtration and the filtrate was concentrated. The oily residue was dissolved in ethyl acetate. The solution was washed with 10% sodium carbonate, 2 *N* hydrochloric acid, and water successively, and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was crystallized from methanol; yield 14.46 g. (70.6%), m.p. 158–159°, $[\alpha]^{24}D$ +11.4° (*c* 2.0, glacial acetic acid) (L-isomer: lit.[18] m.p. 156–158°, $[\alpha]^{27}D$ −11.0° in glacial acetic acid).

Anal. Calcd. for $C_{22}H_{23}N_3O_5$: C, 64.5; H, 5.7; N, 10.3. Found: C, 64.6; H, 5.8; N, 10.3.

Methyl D-*Tryptophylglycinate Hydrochloride Monohydrate.* Carbobenzoxy-D-tryptophylglycine methyl ester (2.46 g.) was hydrogenated in the usual manner over a palladium catalyst in 90% methanol (26 ml.) containing glacial acetic acid (1.75 ml.). The catalyst was removed by filtration and the clear solution was concentrated *in vacuo*. The resulting oily product was dissolved in ice cold 1 *N* hydrochloric acid (6 ml.), and this solution after filtration was lyophilized. The product was dried over potassium hydroxide pellets *in vacuo;* yield 1.59 g. (82.8%), $[\alpha]^{29}D$ −54.7° (*c* 0.8, 1 *N* hydrochloric acid), R_f^1 0.7, single, sharp, ninhydrin- and Ehrlich-positive spot (L-isomer: lit.[15] $[\alpha]^{28}D$ +48.6° in 1 *N* hydrochloric acid, R_f^1 0.7).

Anal. Calcd. for $C_{14}H_{17}N_3O_3 \cdot HCl \cdot H_2O$: C, 51.0; H, 6.1; N, 12.7. Found: C, 51.1; H, 6.0; N, 12.9.

*Carbobenzoxy-*D-*tryptophylglycine.* Carbobenzoxy-D-tryptophylglycine methyl ester (1.0 g.) was treated with 1 *N* sodium hydroxide (3 ml.) in methanol (25 ml.) at room temperature for 1 hr. The bulk of the methanol was removed *in vacuo* (bath temperature 20°). The residue was dissolved in water (10 ml.). The aqueous solution was washed with ethyl acetate and then acidified with 2 *N* hydrochloric acid. The resulting material was extracted with ethyl acetate, and the ethyl acetate extract was washed with a saturated solution of sodium chloride, dried over anhydrous sodium sulfate, and the solvent evaporated. The product was recrystallized from ethyl acetate by addition of petroleum ether; yield 0.95 g. (98.5%), m.p. 158–161°, $[\alpha]^{24}D$ +19.8° (*c* 1.0, methanol) (L-isomer: lit.[18] m.p. 158–159°).

Anal. Calcd. for $C_{21}H_{21}N_3O_5$: C, 63.8; H, 5.4; N, 10.6. Found: C, 63.8; H, 5.6; N, 10.9.

D-*Tryptophylglycine Monohydrate.* Carbobenzoxy-D-tryptophylglycine (0.5 g.) was hydrogenated over a palladium catalyst in 90% methanol (15 ml.) containing glacial acetic acid (1 ml.). The resulting free dipeptide was crystallized from water by addition of ethanol and recrystallized from water; yield 0.29 g. (85%), m.p. 164–165°, $[\alpha]^{24}D$ −93.0° (*c* 1.76, water), R_f^1 0.50, R_f^2 0.76 (L-isomer: lit.[18] m.p. 176–178°, $[\alpha]^{26}D$ +81.7° in water). LAP released Try (0.03 μmole) and Gly (0.03 μmole) from the peptide (1.15 μmoles). This result indicated that contamination of L-tryptophylglycine was 2.6%. *Anal.* Calcd. for $C_{13}H_{15}N_3O_3 \cdot H_2O$: C, 55.9; H, 6.1; N, 15.0. Found: C, 55.9; H, 6.4; N, 14.8.

*Carbobenzoxynitro-*D-*arginyl-*D-*tryptophylglycine Methyl Ester.* Ethyl chloroformate (0.26 ml.) was added to a cold solution of carbobenzoxynitro-D-arginine (0.97 g.) in anhydrous dioxane (12 ml.) containing tri-*n*-butylamine (0.65 ml.). The mixture was stirred at 0° for 30 min. This solution was added with stirring to a cold solution of D-tryptophylglycine methyl ester (prepared from 0.86 g. of the hydrochloride with 0.39 ml. of triethylamine). The reaction mixture was stirred for 20 min. under cooling in an ice bath, allowed to reach room temperature, and kept at that temperature for 2 hr. with stirring. The solvent was evaporated, the resulted oily product was dissolved in ethyl acetate (*ca.* 150 ml.), and the solution was washed successively with water, 5% ammonium hydroxide, 2 *N* hydrochloric acid, and a saturated solution of sodium chloride, and dried over sodium sulfate. After evaporation of the solvent, the oily residue was dissolved in methanol which was concentrated to a small volume. Ethyl acetate (*ca.* 50 ml.) was added to remove small amounts of undissolved material. The filtrate was then concentrated to dryness and the residue was solidified on treatment with ethanol. The product was recrystallized from ethanol; yield 0.81 g. (48.2%), m.p. 122–125°, $[\alpha]^{27}D$ +25.0° (*c* 0.93, methanol) (L-isomer: lit.[29] m.p. 126–128°, $[\alpha]^{22}D$ −25.8° in methanol). *Anal.* Calcd. for $C_{28}H_{34}N_8O_8$: C, 55.1; H, 5.6; N, 18.4. Found: C, 54.9; H, 5.9; N, 18.4.

*Nitro-*D-*arginyl-*D-*tryptophylglycine Methyl Ester Dihydrobromide Trihydrate.* Carbobenzoxynitro-D-arginyl-D-tryptophylglycine methyl ester (0.50 g.) was dissolved in anhydrous glacial acetic acid (4 ml.) and 7.6 *N* hydrogen bromide in glacial acetic acid (2 ml.) was added at room temperature. After 1 hr. the product was precipitated by addition of anhydrous ether, collected by filtration, and washed with three portions of anhydrous ether and dried over potassium hydroxide pellets and phosphorus pentoxide *in vacuo;* yield 0.50 g. (89.2%), $[\alpha]^{24}D$ −18.4° (*c* 0.8, methanol), R_f^1 0.64, R_f^2 1.4, single ninhydrin- and Ehrlich-positive spot. *Anal.* Calcd. for $C_{20}H_{28}N_8O_6 \cdot 2HBr \cdot 3H_2O$: C, 34.7; H, 5.2; N, 16.2. Found: C, 34.5; H, 5.0; N, 16.5.

*Carbobenzoxy-*D-*phenylalanylnitro-*D-*arginyl-*D-*tryptophylglycine Methyl Ester Monohydrate.* A mixed anhydride was prepared in the usual manner from carbobenzoxy-D-phenylalanine (0.30 g.) in ice-cold dioxane (5 ml.) with tri-*n*-butylamine (0.24 ml.) and ethyl chloroformate (0.1 ml.). This solution was added to an ice-cold solution of nitro-D-arginyl-D-tryptophylglycine methyl ester (prepared from 0.51 g. of the hydrobromide with 0.22 ml. of triethylamine in 5 ml. of dimethylformamide). The mixture was kept in an ice bath for 2 hr. after which the solvent was removed *in vacuo*. The residue was dissolved in ethyl acetate and the solution was washed successively with 5% ammonium hydroxide, 2 *N* hydrochloric acid, and a saturated solution of sodium chloride, and dried over anhydrous sodium sulfate. The product which was precipitated during evaporation of the solvent was collected by filtration. For purification it was dissolved in methanol and condensed to a small volume to give an oily residue. It was dissolved in ethyl acetate. On storage of the solution a white solid material was obtained; yield 0.48 g. (84.1%), m.p. 145–148°, $[\alpha]^{20}D$ +20.5° (*c* 0.61, methanol), R_f^1 0.95, R_f^2 1.9 (L-isomer: lit.[15] m.p. 145–148°, $[\alpha]^{25}D$ −18.8° in dimethylformamide, R_f^2 1.9; lit.[29] m.p. 148–150°, $[\alpha]^{22}D$ −18.2° in methanol). *Anal.* Calcd. for $C_{37}H_{43}N_9O_9 \cdot H_2O$: C,

57.3; H, 5.8; N, 16.3. Found: C, 57.0; H, 5.9; N, 16.0.

D-*Phenylalanylnitro*-D-*arginyl*-D-*tryptophylglycine Methyl Ester Hydrobromide.* The above protected tetrapeptide methyl ester (1.25 g.) was treated with 2.5 *N* hydrogen bromide in acetic acid (15 ml.) at room temperature for 1 hr. The product was then precipitated by addition of anhydrous ether, collected by filtration, washed with anhydrous ether, and dried *in vacuo* over potassium hydroxide pellets and phosphorus pentoxide; yield 1.22 g. (99.5%), $[\alpha]^{20}D$ −5.6° (*c* 0.61, methanol), R_f^1 0.72, R_f^2 1.8, single ninhydrin- and Ehrlich-positive spot.

Carbobenzoxy-D-*histidyl*-D-*phenylalanylnitro*-D-*arginyl*-D-*tryptophylglycine Methyl Ester Dihydrate.* An ice-cold ethyl acetate solution (30 ml.) containing the azide of carbobenzoxy-D-histidine (prepared from 0.88 g. of the corresponding hydrazide) was added to an ice-cold solution of D-phenylalanylnitro-D-arginyl D-tryptophylglycine methyl ester hydrobromide (1.42 g.) and triethylamine (0.5 ml.) in dimethylformamide (15 ml.). The mixture was stirred at 4° for 20 hr. and an ethyl acetate solution containing additional azide (prepared from 0.44 g. of the hydrazide) was added. The reaction was allowed to proceed for 20 hr. and the product was precipitated by addition of water, collected by filtration, and washed with water. For purification the product was reprecipitated from dimethylformamide by addition of water; yield 1.28 g. (78.2%), m.p. 178–180°, $[\alpha]^{19}D$ +26.9° (*c* 0.6, dimethylformamide), R_f^1 0.87, R_f^2 1.9, ninhydrin-negative, Pauly- and Ehrlich-positive spot (L-isomer: lit.[29] m.p. 176–178°, $[\alpha]^{22}D$ −27.9° in dimethylformamide). *Anal.* Calcd. for $C_{43}H_{50}N_{12}O_{10}\cdot 2H_2O$: C, 55.5; H, 5.8; N, 18.1. Found: C, 55.1; H, 6.2; N, 18.2.

Carbobenzoxy-D-*histidyl*-D-*phenylalanylnitro*-D-*arginyl*-D-*tryptophylglycine* (2.5 *Hydrate*). Carbobenzoxy-D-histidyl-D-phenylalanylnitro-D-arginyl-D-tryptophylglycine methyl ester (0.68 g.) was treated with 1 *N* sodium hydroxide (1.47 ml.) in a mixture of methanol (7 ml.) and dimethylformamide (1 ml.) at room temperature for 1 hr. The solution after filtration was acidified to pH 3 with 1 *N* hydrochloric acid under ice cooling. The precipitate was collected by filtration and recrystallized from 50% acetic acid; yield 0.49 g. (71.8%), m.p. 199–201°, $[\alpha]^{18}D$ +25.4° (*c* 0.65, dimethylformamide), R_f^1 0.83, R_f^2 1.7, ninhydrin-negative, single Pauly- and Ehrlich-positive spot (L-isomer: lit.[15] m.p. 246–249°, $[\alpha]^{26}D$ −29.2° in dimethylformamide, R_f^2 1.6; lit.[20] m.p. 241–242° $[\alpha]^{25}D$ −29.6° in dimethylformamide; lit.[29] m.p. 226–228°, $[\alpha]^{22}D$ −26.8° in dimethylformamide). *Anal.* Calcd. for $C_{42}H_{48}N_{12}O_{10}\cdot 2.5H_2O$: C, 54.5; H, 5.8; N, 18.2. Found: C, 54.3; H, 6.0; N, 18.4.

D-*Histidyl*-D-*phenylalanyl*-D-*arginyl*-D-*tryptophylglycine Monoacetate Pentahydrate.* Carbobenzoxy-D-histidyl-D-phenylalanylnitro-D-arginyl-D-tryptophylglycine (0.17 g.) was dissolved in 90% (v./v.) aqueous acetic acid (40 ml.) and hydrogenated over a palladium catalyst for 20 hr. Fresh catalyst was added after 8 hr. of hydrogenation. The catalyst was removed by filtration, the solution was decolorized with charcoal, and the filtrate was lyophilized; yield 0.13 g. In preliminary test, this product (1.0 μmole) released histidine (0.02 μmole) by the action of LAP.[34] The product was dissolved in water (20 ml.), 0.01 *M* magnesium chloride (3.0 ml.) and 0.5 *M* Tris buffer (1.0 ml.) were added, and the pH of the solution was adjusted to 8.0. To this solution pre-incubated LAP[34] solution (2.7 ml.) was added and the solution was incubated at 37° for 18 hr. The solution was heated at 100° for 1 min. and the denatured enzyme was removed by filtration. The solution was diluted with water (100 ml.) and applied to a carboxymethylcellulose (CMC)[35] column (2.8 × 15 cm.) which was eluted successively with pyridine acetate buffer (pH 5.0) 0.05 *M* (550 ml.) and 0.1 *M* (700 ml.). The individual fractions (10 ml.) were collected at a flow rate of 3–4 ml./min., and the absorbancy at 280 mμ was determined for each fraction. The desired product was present in the 0.1 *M* eluates (tubes 69–130) which were pooled and the solvent removed first by evaporation to a small volume and then by lyophilization.

The product was again dissolved in 0.2 *M* phosphate buffer (20 ml.) at pH 7.2 and incubated with crystalline trypsin[36] (0.05 mg.) at 37° for 18 hr. The solution was diluted with water and again applied to a CMC column and the material was eluted as described above. Lyophilization gave a fluffy white powder; yield 0.11 g., $[\alpha]^{18}D$ +12.0° (*c* 1.0, 1 *N* hydrochloric acid), R_f^1 0.45, R_f^2 1.1, sharp ninhydrin-, Pauly-, Sakaguchi-, and Ehrlich-positive spot (L-isomer: lit.[15] $[\alpha]^{26}D$ −11.8° in 1 *N* hydrochloric acid, R_f^1 0.50, R_f^2 0.74; lit.[18] $[\alpha]^{27}D$ −10.0° in 1 *N* hydrochloric acid, R_f^1 0.51, R_f^2 = phe; lit.[29] $[\alpha]^{22}D$ −11.7° in 1 *N* hydrochloric acid); amino acid ratios in acid hydrolysate $His_{1.02}$-$Phe_{0.98}Arg_{1.00}Gly_{1.02}$ (average recovery 92%). *Anal.* Calcd. for $C_{34}H_{43}N_{11}O_6\cdot CH_3CO_2H\cdot 5H_2O$: C, 50.8; H, 6.7; N, 18.1. Found: C, 50.8; H, 6.9; N, 18.5.

Enzymatic Analysis. LAP digest was performed according to the procedure of Hofmann and Yajima.[45] The pentapeptide (1.5 μmoles) was incubated with the activated enzyme (0.2 ml.) at 37° for 18 hr. After removing the denaturated enzyme the solution was examined by paper chromatography and by the Moore–Stein analysis. No histidine and glycine were detected.

Chymotryptic and tryptic digest of this peptide were performed in a phosphate buffer of pH 7.2 with an enzyme–substrate ratio of 1/50 (w./w.) at 37° for 18 hr. In both cases no extra spot beside original pentapeptide was detected on a paper chromatogram in both Partridge and 2-butanol–ammonia systems by ninhydrin test.

Aliquots of an acid hydrolysate of the pentapeptide (2.01 μmoles) were exposed to the action of D-amino acid oxidase[37] (5.03 mg.) in 0.1 *M* pyrophosphate buffer at pH 8.2 and L-amino acid oxidase[39] (2.0 mg.) in 0.1 *M* Tris buffer at pH 7.4, respectively. Amino acid ratios in the acid, D-amino acid oxidase, and L-amino acid oxidase hydrolysates were $His_{1.00,0.82,0.93}$-$Phe_{0.96,0.43,0.93}Arg_{0.98,0.78,0.92}Gly_{1.00(92\%),1.00(85\%),1.00(88\%)}$ (calculation based on glycine recovery).

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(45) K. Hofmann and H. Yajima, *J. Am. Chem. Soc.*, **83**, 2289 (1961).