tained during pyrolysis (procedure A) and in addition a compound with an R_f value of 0.82. As this compound reacted not only with $FeCl_3$–$K_3[Fe(CN)_6]$ but also with diazotized N^1,N^1-diethylsulfanilamide (brownish-yellow spot), it cannot be a 3',5'-di-*t*-butyl analog of thyroxine. It is suspected to be 2,4,6-triiodophenol.[19]

Spontaneous Decomposition of the Quinol Ether VII (R_3 = I, R_4 = $CH_2COOC_2H_5$).—The following is an example for the spontaneous decomposition of solutions of the iodinated quinol ethers of the type VII, in the absence of an acidic catalyst.

The quinol ether VII (R_3 = I, R_4 = $CH_2COOC_2H_5$) (0.80 g., 1.2 mmoles) was dissolved at room temperature in 30 ml. of *n*-pentane. The resulting light-blue solution began to turn brownish-red after a few minutes. After standing 3 days at room temperature the reaction mixture was filtered to remove an amorphous precipitate that had formed (0.13 g.). The filtrate was titrated with 0.1 *N* sodium thiosulfate. This titration showed that 24% of the iodine of the quinol ether had been liberated as iodine. The pentane layer was concentrated, and the concentrate filtered to remove some more amorphous precipitate (0.18 g.). Attempts to crystallize the two batches of amorphous powder (total weight 0.31 g.), failed. The powder seems to consist of a mixture of polymerized substances. It was apparently formed by free radical-induced polymerization.

The filtrate, upon standing at −25°, deposited yellow crystals of bis-(4-oxo-1,3,5-tri-*t*-butyl-2,5-cyclohexadien-1-yl) peroxide,[14b] m.p. 144° dec.

BETHESDA, 14 MD.

[CONTRIBUTION FROM THE HORMONE RESEARCH LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY]

The Synthesis of L-Histidyl-L-phenylalanyl-L-ornithyl-L-tryptophyl-glycine and L-Histidyl-D-phenylalanyl-L-ornithyl-L-tryptophyl-glycine and their Melanocyte-stimulating Activity

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The synthesis of peptides L-histidyl-L-phenylalanyl-L-ornithyl-L-tryptophyl-glycine and L-histidyl-D-phenylalanyl-L-ornithyl-L-tryptophyl-glycine is described. These two synthetic peptides have been shown by bioassay to possess melanotropic activities identical to their L-phenylalanyl- and L-arginyl analogs.

In the course of the purification and isolation of adrenocorticotropins (ACTH) from pituitary glands of various species, it has been observed by a number of investigators that every adrenocortically-active fraction always possesses melanocyte-stimulating activity.[1] It is now well established that the melanocyte-stimulating activity found in the adrenocorticotropins is an intrinsic biologic property of the hormone.[1,2] When the chemical structure of the melanotropins (MSH)[3,4] is compared with that of the adrenocorticotropins,[1,5] it is evident that a heptapeptide sequence, L-methionyl-glutamyl-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycine, occurs in all preparations of these two hormones. Indeed, synthetic peptides[6,7,8] related to this heptapeptide have recently been shown to possess melanocyte-stimulating activity. One of the outstanding features of these natural and synthetic peptides is the effect on them of alkali-heat treatment. For example, when a solution of L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycine (1 mg. per ml.) in 0.1 *M* NaOH was kept in a boiling water-bath for 15 minutes, the activity which is responsible for darkening the skin of hypophysectomized frogs was greatly prolonged.[7,9] It was suspected from unpublished observations in this Laboratory that either the conversion of arginine to ornithine or the racemization of L-phenylalanine, or both, may be responsible for the "prolongation" effect of the alkali–heat treatment; consequently, the synthesis of L-histidyl-L-phenylalanyl-L-ornithyl-L-tryptophyl-glycine (I) and L-histidyl-D-phenylalanyl-L-ornithyl-L-tryptophyl-glycine (II) was undertaken.

The synthesis of I was accomplished in two different ways, which differed only in the blocking of the imidazo group of histidine. In one instance, the benzyl group was used to protect the imidazo group, whereas in the other the imidazo group remained free. Carbobenzoxy-L-histidine azide was coupled with L-phenylalanine methyl ester and converted to the carbobenzoxy-dipeptide hydrazide in the manner described by Hofmann, *et al.*[10] The azide was only slightly soluble in ethyl acetate and chloroform; therefore, after the amorphous material was filtered, it was suspended in an ethyl acetate solution of δ-carbobenzoxy-L-ornithine methyl ester[11] and kept in the refrigerator for 3 days with occasional shaking. The carbobenzoxy-L-histidyl-L-phenylalanyl-δ-carbobenzoxy-L-ornithine methyl ester was contaminated with some histidyl carbobenzoxy-L-histidyl-L-phenylalanine[10] and material formed by Curtius degradation, and could be purified only by recrystallization from a mixture of boiling water and ethyl acetate. The crystalline product was saponified with $Ba(OH)_2$, and carbobenzoxy-L-histidyl-L-phenylalanine-δ-carbobenzoxy-L-ornithine (III) was obtained in crystalline form. The condensation of III with L-tryptophyl-glycine benzyl ester (IV) was accomplished by means of the dicyclohexylcarbodiimide (DCCI) method.[12] The product V was

(1) C. H. Li, *Adv. Protein Chem.*, **11**, 101 (1956).

(2) C. H. Li, P. Fønss-Bech, I. I. Geschwind, T. Hayashida, G. Hungerford, A. J. Lostroh, W. R. Lyons, H. D. Moon, W. O. Reinhardt and M. Sideman, *J. Expt. Med.*, **105**, 335 (1957).

(3) C. H. Li, *Adv. Protein Chem.*, **12**, 269 (1957).

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

(5) C. H. Li, J. S. Dixon and D. Chung, THIS JOURNAL, **80**, 2587 (1958).

(6) K. Hofmann, T. A. Thompson and E. T. Schwartz, *ibid.*, **79**, 6087 (1957).

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

(8) K. Hofmann, M. E. Woolner, H. Yajima, G. Spiokler, T. A. Thompson and E. T. Schwartz, THIS JOURNAL, **80**, 6458 (1958).

(9) C. H. Li, *Laboratory Investigation*, **8**, 574 (1959).

(10) K. Hofmann, H. Kappeler, A. E. Furlenmeier, M. E. Wooner, E. T. Schwartz and T. A. Thompson, THIS JOURNAL, **79**, 1641 (1957).

(11) R. L. Synge, *Biochem. J.*, **42**, 99 (1948).

(12) J. C. Sheehan and G. P. Hess, THIS JOURNAL, **77**, 1067 (1955).

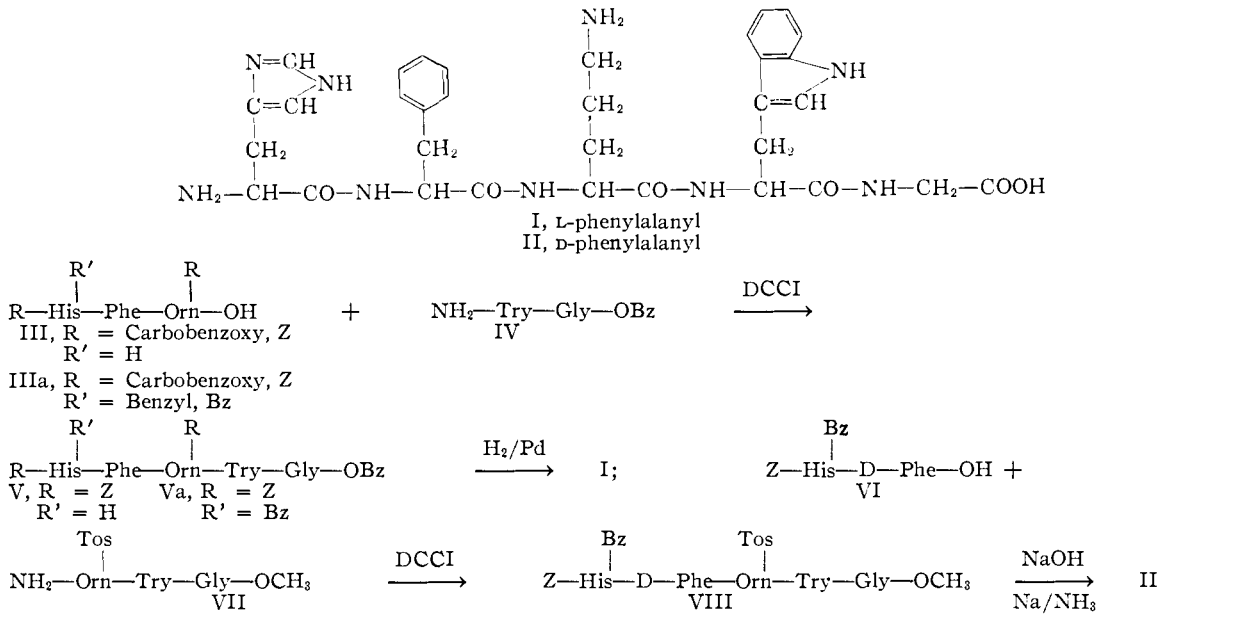

Fig. 1.—Outline of the synthesis of L-histidyl-L-phenylalanyl-L-ornithyl-L-tryptophyl-glycine and L-histidyl-D-phenylalanyl-L-ornithyl-L-tryptophyl-glycine.

crystallized from methanol with a yield of 59%. Compound IV was obtained by decarbobenzoxylation with HBr in glacial acetic acid[13] of the corresponding carbobenzoxy-dipeptide ester, which was synthesized *via* the cyanomethyl ester of carbobenzoxy-L-tryptophan.[14]

For the synthesis of peptide I by the second method, benzyl-L-histidine was employed instead of free histidine. The carbobenzoxy-im-benzyl-L-histidine[15] derivative was coupled by the DCCI method with L-phenylalanine methyl ester. The resulting crystalline carbobenzoxy-im-benzyl-L-histidyl-L-phenylalanine methyl ester was saponified with $Ba(OH)_2$; the crystalline Ba salt was filtered off and found to be insoluble in water but readily soluble in methanol. The free acid was obtained by the addition of an equivalent of HCl; it had a melting point of 127–130°; the material was chromatographically pure when developed on paper (R_f SBA = 0.45)[16] by the chlorine method.[17] It was then coupled with δ-carbobenzoxy-L-ornithine methyl ester by the DCCI method. The solution containing the blocked tripeptide in ethyl acetate could be extracted with HCl to remove the unreacted ester, since the hydrochloride was insoluble in the aqueous phase. After saponification with 1 *N* NaOH the resulting acid was allowed to react with IV according to the DCCI method; the product was Va.

In the synthesis of both V and Va, all protecting groups could be cleaved by catalytic hydrogenation, although the im-benzyl group came off very slowly. In the synthesis in which benzylhistidine was used, the product was purer and hence no further purification by countercurrent distribution was necessary. Both samples manifested the same chromatographic, electrophoretic and optical behavior. The diacetate salts of I may be converted to the free base by being passed through an XE-64 resin column.

Peptide II was synthesized in a stepwise manner from the C-terminus as follows: L-tryptophyl-glycine methyl ester[7] was coupled with carbobenzoxy-δ-tosyl-L-ornithine by means of the DCCI method. The protected tripeptide ester was then decarbobenzoxylated by catalytic hydrogenation to VII and subsequently coupled with carbobenzoxy-im-benzyl-L-histidyl-D-phenylalanine (VI) by means of the DCCI method to produce VIII. The blocked peptide VIII was first saponified, and then reduced with sodium–liquid ammonia[18]; in this manner, the peptide was freed from all protecting groups. After purification of the product on an XE-64 resin column, peptide II was obtained in a lyophilized form as the dihydrate (see Fig. 1 for an outline of the synthesis).

Pentapeptides I and II were subjected to amino acid analyses[19]; in the former, L-His-L-Phe-L-Orn-L-Try-Gly, the amino acids were present in a molar ratio of 1.0/1.1/1.0/1.0/0.9, and in the latter, L-His-D-Phe-L-Orn-L-Try-Gly, in a molar ratio of 0.8/1.1/1.1/0.8/0.8. Chymotryptic and tryptic digestion of I and II released fragments which were expected from the known specificity of these proteolytic enzymes. Hydrolysis of I with chymotrypsin yielded L-His-L-Phe, L-Orn-L-Try and glycine, whereas II gave glycine and L-His-D-Phe-L-Orn-L-Try. Tryptic digestion of I and II revealed only traces of L-Try-Gly, indicating that the L-Orn-L-Try bond is only slightly cleaved by the enzyme.

(13) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).

(14) R. Schwyzer, M. Feurer, B. Iselin and H. Kagi, *Helv. Chim. Acta*, **38**, 80 (1955).

(15) B. G. Overell and V. Petrow, *J. Chem. Soc.*, 232 (1955).

(16) The following abbreviations will be used to designate the paper chromatographic solvent systems: SBA = 2-butanol–10% ammonia, 85:15; BAW = *n*-butyl alcohol–acetic acid–water, 4:1:1.

(17) H. Zahn and E. Rexroth, *Z. anal. Chem.*, **148**, 181 (1955).

(18) V. du Vigneaud and O. K. Behrens, *J. Biol. Chem.*, **117**, 27 (1937).

(19) A. L. Levy, *Nature*, **174**, 126 (1954).

The action of leucine aminopeptidase[20] (LAP) on I and II has also been studied. In peptide I, digestion amounting to approximately 50% occurred, and the amino acids liberated appeared in the following molar ratio: His/Phe/Orn/Try/Gly = 1.0/1.0/0.55/0.55/0.57. Apparently, the Orn-Try bond is the slowest to be cleaved and hence becomes the rate-determining step in the digestion. In peptide II, only histidine was liberated in any appreciable quantity (28%); it is evident that the D-phenylalanine prevents any further hydrolysis by LAP.

The melanocyte-stimulating activity of I and II was estimated by the isolated frog skin method[21]; the results of these assays, presented in Table I, indicate that I and II possess the almost identical specific activity of 2.6–2.9 × 10^4 units/g., a value which is comparable to that previously reported for L-His-L-Phe-L-Arg-L-Try-Gly.[7,8] The biological activity of I and II was also investigated *in vivo* in hypophysectomized frogs; both peptides exerted a good darkening effect at a dose of 50 μg. Without further treatment, II exhibited the characteristic "prolongation" of the darkening effect,[3,9] which was absent from I. If, however, a solution (1 mg./ml.) of peptide I in 0.1 *M* NaOH was kept at 100° for 15 minutes and injected into hypophysectomized frogs,[22] this characteristic "prolongation" effect was now observed in I. On the other hand, when peptide II was subjected to similar treatment, not only did the "prolongation" effect disappear, but also the melanocyte-stimulating activity as assayed in the hypophysectomized frog was abolished. These data provide some interesting conclusions concerning the relation of chemical structure to biological activity in melanotropically active peptides. It is evident that the replacement of arginine by ornithine in the pentapeptide does not alter its melanocyte-stimulating activity. Although no difference in specific activity was observed between I and II, substitution of L-phenylalanine by D-phenylalanine brings about the characteristic "prolongation" of the darkening effect on the frog skin. The fact that alkali–heat treatment destroys the melanocyte-stimulating activity of II implies that neither the racemization of L-phenylalanine nor the conversion of arginine to ornithine in the synthetic pentapeptide, L-His-L-Phe-L-Arg-L-Try-Gly,[7] or in the melanotropins[3] or the adrenocorticotropins,[1] can explain completely the "prolongation" phenomenon noted after mild treatment with NaOH.

TABLE I

MELANOCYTE-STIMULATING ACTIVITY OF SYNTHETIC PEPTIDES

Peptides	No. of assays[a]	Activity
L-His-L-Phe-L-Arg-L-Try-Gly[b]	3	3.1 × 10^4
L-His-L-Phe-L-Orn-L-Try-Gly	3	2.9 × 10^4
L-His-D-Phe-L-Orn-L-Try-Gly	2	2.6 × 10^4

[a] Each assay was performed on 4 to 6 pieces of frog skin. [b] See ref. 7.

(20) R. L. Hill and E. L. Smith, *J. Biol. Chem.*, **228**, 577 (1957).

(21) K. Shizume, A. B. Lerner and T. B. Fitzpatrick, *Endocrinol.*, **54**, 553 (1954).

(22) L. T. Hogben and D. Slome, *Proc. Roy. Soc.* (*London*), **B108**, 10 (1931).

Experimental

Carbobenzoxy-L-histidyl-L-phenylalanyl-δ-carbobenzoxy-L-ornithine Methyl Ester.—Carbobenzoxy-L-histidyl-L-phenylalanine hydrazide,[10] 5 g., was dissolved in a mixture of 10 ml. of concd. HCl and 120 ml. of H_2O. The solution was cooled to 0°, and a saturated solution of 0.3 g. of $NaNO_2$ was added dropwise with vigorous stirring. After 10 minutes 250 ml. of ethyl acetate and 150 ml. of chloroform were added and the *p*H of the aqueous phase was adjusted to 9.5 with a 50% solution of potassium carbonate. The azide dissolved readily in the organic phase, but after a short time an amorphous precipitate formed which was subsequently filtered off and air-dried. The precipitate was suspended in a chloroform solution of δ-carbobenzoxy-L-ornithine methyl ester[11] which was prepared by adding an equivalent amount of triethylamine to 3.7 g. of the hydrochloride. The reaction started immediately with evolution of hydrazoic acid. The reaction mixture was kept in the refrigerator for 3 days, and then the amorphous product was filtered off and washed carefully with water and bicarbonate solution. The residue was dissolved in 30 ml. of methanol,[23] and the methanol-soluble material was further purified by dissolving it in hot ethyl acetate and then extracting with 3 portions of boiling water. When the ethyl acetate was evaporated in the oven, the ester crystallized as a white material, m.p. 191–193°, which behaved as a pure component in paper chromatography ($R_{f\ BAW}$ = 0.8); yield 2.1 g. (27%), $[\alpha]^{23}D$ −25.2° (*c* 2, methanol).

Anal. Calcd. for $C_{37}H_{42}N_6O_8$ (698.76): C, 63.59; H, 6.06; N, 12.03. Found: C, 63.88; H, 6.10; N, 12.09.

Carbobenzoxy-L-histidyl-L-phenylalanyl-δ-carbobenzoxy-L-ornithine (III).—Carbobenzoxy-L-histidyl-L-phenylalanyl-δ-carbobenzoxy-L-ornithine methyl ester, 2 g., was dissolved in 75 ml. of methanol; 50 ml. of 0.5 *N* $Ba(OH)_2$ was added and the mixture was allowed to stand at room temperature for 3 hours. After acidification with 25 ml. of 1 *N* HCl, the mixture was kept at 0° overnight and the amorphous precipitate that formed was filtered off. The mother liquor was evaporated *in vacuo* and the salt was removed by repeated washing with water. The residue and the filtered product were pooled and dissolved in methanol, leaving some crystalline material.[24] From the methanolic solution an amorphous product was obtained which crystallized when water was added (m.p. 125–129°, sintering from 122°). After several recrystallizations from acetone–water and methanol, the yield of III was 960 mg. (49%), m.p. 163–166°. An amino acid analysis showed the presence of histidine, phenylalanine and ornithine in a ratio of 1:1:1; $R_{f\ SBA}$, 0.33, $[\alpha]^{25}D$ −15.6° (*c* 2, methanol). For elementary analysis III was dried over P_2O_5 at 80° for 5 hours.

Anal. Calcd. for $C_{36}H_{40}N_6O_8$ (684.73): C, 63.29; H, 5.89; N, 12.37. Found: C, 62.32; H, 6.06; N, 12.10. Calcd. for $C_{36}H_{40}N_6O_8 \cdot {}^1/_2H_2O$: C, 62.32; H, 5.96; N, 12.11.

Carbobenzoxy-L-tryptophyl-glycine Benzyl Ester.—Carbobenzoxy-L-tryptophan cyanomethyl ester,[14] 10.5 g., was dissolved in 50 ml. of tetrahydrofuran and the solution was mixed with a solution of glycine benzyl ester which was prepared by adding 5.8 ml. of triethylamine to a suspension of 13.5 g. of glycine benzyl ester benzenesulfonate[25] in 50 ml. of tetrahydrofuran. After the reaction had been allowed to proceed for 24 hours at room temperature, the solvent was removed in a vacuum and the oily residue was dissolved in ethyl acetate. The ethyl acetate solution was washed thoroughly with 1 *N* HCl, H_2O, 5% $NaHCO_3$ and H_2O and dried over sodium sulfate. The ethyl acetate was evaporated under reduced pressure and the remaining yellow oil triturated with the aid of ether; 9.2 g. of white needles was obtained (70%), which were recrystallized from ethyl acetate–petroleum ether and ethyl acetate–ether; yield 5.5 g. (42%), m.p. 124–125°, $[\alpha]^{24}D$ −20° (*c* 2, methanol).

Anal. Calcd. for $C_{28}H_{27}N_3O_5$ (485.52): C, 69.26; H, 5.61; N, 8.65. Found: C, 69.55; H, 5.51; N, 8.72.

L-Tryptophyl-glycine Benzyl Ester (IV).—L-Tryptophyl-glycine benzyl ester was prepared by decarbobenzoxyl-

(23) Some insoluble material proved to be carbobenzoxy-L-histidyl-L-phenylalanine (m.p. 226–228°).

(24) R_f SBA 0.03; amino acid analysis showed that this material did not contain phenylalanine, indicating that Curtius degradation had occurred during the azide synthesis.

(25) H. K. Miller and H. Waelsch, THIS JOURNAL, **74**, 1092 (1952).

ation of 2.2 g. of carbobenzoxy-L-tryptophyl-glycine benzyl ester with 4 *N* HBr in glacial acetic acid for 15 minutes[13]; the hydrobromide salt was precipitated by the addition of ethyl ether and the precipitate was repeatedly washed by decantation. The free base was obtained by stirring the hydrobromide salt with 6 ml. of a 50% solution of potassium carbonate with ethyl acetate at 0°. After the solution was dried and the ethyl acetate evaporated, there remained 0.7 g. of IV (39%), $R_{f\ sBA}$ 0.64, $R_{f\ BAW}$ 0.55.

Carbobenzoxy-L-histidyl-L-phenylalanyl-δ-carbobenzoxy-L-ornithyl-L-tryptophyl-glycine Benzyl Ester (V).—Carbobenzoxy-L-histidyl-L- phenylalanyl-δ-carbobenzoxy-L-ornithine (III), 690 mg., was dissolved in 20 ml. of dimethylformamide and the solution was added to a solution of 500 mg. of L-tryptophyl-glycine benzyl ester (IV) in 10 ml. of dimethylformamide; 250 mg. of dicyclohexylcarbodiimide was added and the reaction mixture was kept in the refrigerator for 36 hours. The dicyclohexylurea formed was removed by filtration and the solvent was evaporated *in vacuo*. The oily residue was dissolved in ethyl acetate and the solution washed in the usual manner. Evaporation of the solvent yielded an amorphous material which was recrystallized several times from ethyl acetate and finally from methanol. The yield was 600 mg. (59%), m.p. 198–201°. A small sample was crystallized once more from methanol for analysis; m.p. = 203–205°, $[\alpha]^{23}D$ −7.5° (*c* 2, glacial acetic acid), $R_{f\ sBA}$ = 0.90; a positive reaction was obtained with the Pauly and Ehrlich reagents.

Anal. Calcd. for $C_{56}H_{59}N_3O_{10}$ (1018.1): N, 12.38. Found: N, 12.41.

L-Histidyl-L-phenylalanyl-L-ornithyl-L-tryptophyl-glycine (I).—The pentapeptide derivative V obtained from the preceding step, 560 mg., was dissolved in 75 ml. of hot methanol, and after the addition of 1.5 ml. of glacial acetic acid, the solution was hydrogenated with palladium as the catalyst at 60° for 24 hours. The catalyst was removed by filtration and the solvent was evaporated under reduced pressure to give a sirup which solidified when trituration with the aid of ethyl acetate was performed; yield 240 mg. of the peptide diacetate (56%). The material contained some minor impurities, with $R_{f\ BAW}$ = 0.02 and 0.5. The mixture was purified by countercurrent distribution in the acetic acid–pyridine system of Craig and Konigsberg.[26] After 40 transfers, three components[27] were present. The contents of the peak tubes (K = 0.96) were combined and the solvent was removed by a rotary evaporator; the residue was redissolved in diluted acetic acid and lyophilized to yield the pentapeptide diacetate in the form of a white powder, yield 110 mg. (56%), $R_{f\ BAW}$ 0.07. According to the results of paper electrophoresis in an acetate–collidine buffer of *p*H 7.0, the material was homogeneous; furthermore, it gave positive reactions with ninhydrin and the Ehrlich and Pauly reagents; $[\alpha]^{25}°D$ −7.8° (*c* 1, H_2O).

Anal. Calcd. for $C_{37}H_{43}N_9O_{10}\cdot H_2O$ (797.85): C, 55.70; H, 6.44; N, 15.80. Found: C, 56.03; H, 6.66; N, 15.80.

The diacetate was converted to the free peptide by resin (XE-64) column chromatography.

Carbobenzoxy-im-benzyl-L-histidyl-L-phenylalanine Methyl Ester.—Carbobenzoxy-im-benzyl-L-histidine,[15] 2.0 g., was dissolved in 70 ml. of dimethylformamide with the aid of heat. After the solution was cooled to 0°, the L-phenylalanine methyl ester prepared from 2.0 g. of the hydrochloride as described by Hofmann, *et al.*,[10] was added, together with 1.24 g. of DCCI. The reaction mixture was kept in the refrigerator overnight, the urea was filtered off, and the solvent was removed in a vacuum. The sirupy residue was dissolved in ethyl acetate, and acidic and basic impurities were removed by washing in the usual manner. When the ethyl acetate was evaporated, carbobenzoxy-im-benzyl-L-histidine-L-phenylalanine methyl ester[28] crystallized in the form of white needles, m.p. 136–140°, and was recrystallized from ethyl acetate, m.p. 144–145°, $[\alpha]^{25}D$ +5.2° (*c* 2, methanol), yield 2.2 g. (77%).

Anal. Calcd. for $C_{31}H_{32}N_4O_5$ (540.6): C, 68.87; H, 5.97; N, 10.36. Found: C, 69.02; H, 5.96; N, 10.47.

Carbobenzoxy-im-benzyl-L-histidyl-L-phenylalanine.—The ester derived from the preceding step, 2.3 g., was dissolved in 50 ml. of methanol and 85 ml. of 0.5 *N* $Ba(OH)_2$ was added. More methanol was added to clear the cloudy solution. When the clear solution was allowed to stand at room temperature, crystallization occurred in the form of needles, which were filtered off after 3 hours and washed well with water and ether. The barium salt was recrystallized from a small volume of methanol; m.p. 218–220°, yield 1.8 g. (71%).

Anal. Calcd. for (594.24) $C_{30}H_{29}N_4O_5Ba_{1/2}$: N, 9.43. Found: N, 9.24.

The crystalline barium salt (1.75 g.) was acidified to *p*H 5.3 with 1 *N* HCl and water was added to precipitate the acid. After the suspension was allowed to stand in the cold overnight, 1.5 g. (88%) of carbobenzoxy-im-benzyl-L-histidyl-L-phenylalanine[29] was recovered. It was crystallized from a large volume of ethyl acetate; m.p. 114–118°. After further drying over P_2O_5 at 80°, the material took the form of needles, m.p. 127–130° (sintering at 123°), $[\alpha]^{25}D$ +15.6° (*c* 2, methanol).

Anal. Calcd. for $C_{30}H_{30}N_4O_5$ (526.57): C, 68.42; H, 5.74; N, 10.64; neut. equiv., 526.6. Found: C, 68.18; H, 5.83; N, 10.78; neut. equiv., 552.5.

Carbobenzoxy-im-benzyl-L-histidyl-L-phenylalanyl-δ-carbobenzoxy-L-ornithine Methyl Ester.—Carbobenzoxy-im-benzyl-L-histidyl-L-phenylalanine, 730 mg., was dissolved in 100 ml. of tetrahydrofuran and mixed with a solution of δ-carbobenzoxy-L-ornithine methyl ester[11] in ethyl acetate, which was prepared from 900 mg. of the ester-hydrochloride with a K_2CO_3 solution; DCCI, 340 mg., was added, the reaction mixture was kept in the refrigerator for 24 hours and then filtered free of the urea that formed. The filtrate was evaporated in a vacuum, the residue was redissolved in ethyl acetate and was then purified in the usual manner to yield 700 mg. (60%) of the desired compound, m.p. 143–145°. The material was recrystallized several times from methanol and finally from dioxane; yield 500 mg. (43%), m.p. 157–158°, $[\alpha]^{25}D$ −17° (*c* 1, dioxane).

Anal. Calcd. for $C_{44}H_{48}N_6O_8$ (788.87); C, 66.99; H, 6.12; N, 10.65. Found: C, 66.79; H, 6.25; N, 10.43.

Carbobenzoxy-im-benzyl-L-histidyl-L-phenylalanyl-δ-carbobenzoxy-L-ornithine (IIIa).—The ester obtained from the previous step, 2.2 g., was dissolved in 35 ml. of dioxane and treated with 6 ml. of *N* NaOH for 1.5 hours at room temperature. The mixture was then acidified to *p*H 5.2 and evaporated under reduced pressure to give a somewhat gelatinous product which crystallized from methanol; m.p. 135–140°, yield 1.6 g. (75%); IIIa was then recrystallized from dioxane and acetonitrile; yield 1.5 g. (69%), m.p. 149–150°, $[\alpha]^{25}D$ −17.5° (*c* 2, dimethylformamide), $R_{f\ sBA}$ 0.59.

Anal. Calcd. for $C_{43}H_{46}N_6O_8$ (774.85): C, 66.65; H, 5.98; N, 10.85. Found: C, 66.36; H, 5.79; N, 10.77.

Carbobenzoxy-im-benzyl-L-histidyl-L-phenylalanyl-δ-carbobenzoxy-L-ornithyl-L-tryptophyl-glycine Benzyl Ester

(26) L. C. Craig and W. Konigsberg, *J. Org. Chem.*, **22**, 1345 (1957).

(27) An amino acid analysis of the slow moving component (K = 0.15) showed the absence of phenylalanine, another indication that Curtius degradation had occurred during the azide synthesis in a heterogeneous medium. The fast-moving impurity (K = 3.2) seemed to be L-histidinyl-L-phenylalanyl-L-ornithine-dicyclohexylurea, since only traces of glycine were found.

(28) This compound may also be obtained by the reaction of carbobenzoxy-im-benzyl-L-histidine and DL-phenylalanine methyl ester: Carbobenzoxy-im-benzyl-L-histidine, **2 g.**, was coupled with DL-phenylalanine methyl ester by means of reaction with DCCI as described above; after extraction in the usual manner, crystals with m.p. of 120–125° were obtained. After repeated recrystallization from ethyl acetate, a product with a m.p. of 144–145° was obtained which did not manifest a lowering of the melting point when mixed with the L,L-compound, yield 820 mg.

(29) Alternatively, the free acid may be obtained from the chloride salt which was prepared as follows: 910 mg. of the barium salt was acidified to *p*H 3.5 by the addition of 1 *N* HCl. The solvent was then removed *in vacuo* and the residue extracted several times with ethyl acetate and water to remove unreacted material and the $BaCl_2$. The chloride salt was then crystallized from dioxane; m.p. 186–194°, yield 520 mg. (55%). The product was further purified by dissolving the crystals in dimethylformamide, adding ethyl acetate to produce a precipitate, and finally recrystallizing the material from pyridine–ethyl acetate; m.p. 208° dec. with darkening from 203°, $[\alpha]^{25}D$ −1.5° (*c* 2, dimethylformamide), $R_{f\ sBA}$ 0.4. *Anal.* Calcd. for $C_{36}H_{31}ClN_4O_8$ (563.04): C, 63.99; H, 5.55; N, 9.95; Cl, 6.30. Found: C, 63.90; H, 5.80; N, 10.16; Cl, 6.33.

(**Va**).—Compound IIIa, 1.5 g., was dissolved in 50 ml. of dioxane and after the solution was cooled to 0°, 0.91 g. of IV and 0.46 g. of DCCI were added. The reaction mixture was kept in the refrigerator for 3 days. After the urea that formed was removed by filtration, the filtrate was evaporated *in vacuo*, redissolved in ethyl acetate, and washed in the usual manner. The residue which was left after evaporation of the ethyl acetate weighed 1.25 g. (56%). Va was recrystallized several times from ethyl acetate and methanol; m.p. 160–162° with sintering at 152°, $[\alpha]^{25}D$ −10.5° (*c* 1, dioxane); yield 950 mg. (43%).

Anal. Calcd. for $C_{63}H_{85}N_9O_{10}$ (1108.22): C, 68.28; H, 5.91; N, 11.38. Found: C, 68.14; H, 5.83; N, 11.11.

L-Histidyl-L-phenylalanyl-L-ornithyl-L-tryptophyl-glycine (I).—Compound Va (875 mg.) was subjected to catalytic hydrogenation in a mixture of 100 ml. of methanol and 3 ml. of glacial acetic acid at 60°. The reaction was allowed to continue until the material gave a positive Pauly reaction after paper chromatography (3 days), since the benzyl group attached to the imidazo group was split off only very slowly. After the catalyst was removed by filtration, the solvent was evaporated in a vacuum and the resulting sirup was triturated with the aid of ethyl acetate. The product was dissolved in water and adsorbed onto an XE-64 resin column. The column was washed, first with water and then with NH_4OH. The NH_4OH eluate was lyophilized to give 340 mg. of I (65%). This peptide was identical with the product obtained in the first synthesis.

Carbobenzoxy-im-benzyl-L-histidyl-D-phenylalanine Methyl Ester.—Carbobenzoxy-im-benzyl-L-histidine, 2 g., was coupled by the DCCI method with D-phenylalanine methyl ester, which was prepared from 2 g. of the hydrochloride as described above, and purified in the usual manner. The product was crystallized several times from ethyl acetate; yield 1.9 g. (67%), m.p. 122–123°, $[\alpha]^{23}D$ +11° (*c* 2, methanol).

Anal. Calcd. for $C_{31}H_{32}N_4O_5$ (540.6): C, 68.87; H, 5.97; N, 10.36. Found: C, 69.13; H, 5.80; N, 10.36.

Carbobenzoxy-im-benzyl-L-histidyl-D-phenylalanine (VI).—The ester obtained from the preceding step, 2 g., was dissolved in 50 ml. of methanol, and 2 ml. of 1 *N* NaOH was added, and the mixture was stirred at room temperature for 1.5 hours. The *p*H was then adjusted to 5.3 and the free acid precipitated by the addition of water; yield 1.6 g. (82%), m.p. 156–159°; VI was crystallized several times from methanol; yield 1.4 g. (73%), m.p. 167–168°, $[\alpha]^{25}D$ +5.4° (*c* 2, methanol). For analysis the material was dried *in vacuo* over P_2O_5 at 80°.

Anal. Calcd. for $C_{30}H_{30}N_4O_5$ (526.57): C, 68.42; H, 5.74; N, 10.64. Found: C, 68.28; H, 5.79; N, 10.44.

Carbobenzoxy-δ-tosyl-L-ornithine.—δ-Tosyl-L-ornithine,[30] 2.66 g., was suspended in 25 ml. of water at 0°, and 1 *N* NaOH was added until the *p*H was 11. Then 1.74 g. of carbobenzoxy chloride was added dropwise with vigorous stirring; 1 *N* NaOH was added when necessary, to maintain the *p*H at 11. When the *p*H no longer dropped (*ca.* 1 hr.) an additional 0.5 g. of carbobenzoxy chloride was added and the mixture was stirred an hour longer at 0°. The sodium salt of the product precipitated out during the acylation. The mixture was then diluted with an equal volume of water and extracted with ethyl ether to remove the excess carbobenzoxy chloride. The aqueous phase was then acidified to *p*H 3 with 6 *N* HCl, producing a white, cloudy suspension. The suspension was then extracted with ethyl ether, and the ether layer was dried over anhydrous sodium sulfate and then evaporated to dryness *in vacuo*. An amorphous material remained, which could not be crystallized. Chromatographically this material (2.78 g., 70%) was homogeneous (R_f BAW 0.79, R_f SBA 0.45), when developed by the chlorine method.[17] There was no ninhydrin-reactive material present. This compound was used directly in the following condensation.

Carbobenzoxy-δ-tosyl-L-ornithine-L-tryptophyl-glycine Methyl Ester.—Carbobenzoxy-L-tryptophyl-glycine methyl ester,[7] 2.3 g., was dissolved in 100 ml. of methanol, and 1 ml. of 5.7 *N* HCl (1 equivalent) was added; hydrogenation was carried out in the presence of 0.2 g. of palladium–charcoal mixture. After the hydrogenation was complete (*ca.* 1 hr.), the methanol was evaporated in a vacuum at 30–40°, and the residue was dissolved in 50 ml. of water. The aqueous solution was extracted with ethyl acetate to remove any unreacted material and cooled to 0°; 50 ml. of saturated K_2CO_3 was then added. The suspension was vigorously stirred for 0.5 hr. at 0° with 100 ml. of ethyl acetate. The suspension was re-extracted with another 50 ml. of ethyl acetate. The ethyl acetate extracts, containing the L-tryptophyl-glycine methyl ester, were pooled, dried over anhydrous sodium sulfate, and then evaporated to dryness in a vacuum at 30°; yield 1.53 g.

The L-tryptophyl-glycine methyl ester was then dissolved in 100 ml. of ethyl acetate. This solution was mixed with a solution of 2.3 g. of carbobenzoxy-δ-tosyl-L-ornithine in 100 ml. of ethyl acetate at 0° and 1.23 g. of DCCI was added. The reaction mixture was stirred until all the DCCI was dissolved, and was then stored overnight at 4°. After the dicyclohexylurea was removed by filtration, the ethyl acetate solution was washed in the usual manner, dried over anhydrous sodium sulfate and evaporated in a vacuum. The carbobenzoxy-δ-tosyl-L-ornithyl-L-tryptophyl-glycine methyl ester was crystallized from ethyl acetate–petroleum ether; yield 2.48 g. (67%), m.p. 115–120°, $[\alpha]^{23}D$ −3.4° (*c* 2, glacial acetic acid).

Anal. Calcd. for $C_{34}H_{39}O_8N_5S_1$: C, 60.30; H, 5.75; N, 10.32. Found: C, 60.36; H, 5.82; N, 10.29.

Carbobenzoxy-im-benzyl-L-histidyl-D-phenylalanyl-δ-tosyl-L-ornithyl-L-tryptophyl-glycine Methyl Ester (VIII).—Carbobenzoxy-δ-tosyl-L-ornithyl-L-tryptophyl-glycine methyl ester, 1.35 g., was subjected to catalytic hydrogenation, as above, to obtain 1.05 g. of the HCl salt of the decarbobenzoxylated tripeptide ester. Treatment of the HCl salt in cold saturated K_2CO_3 and subsequent extraction with ethyl acetate produced 0.885 g. of VII. This tripeptide ester was then dissolved in 50 ml. of tetrahydrofuran and added to a solution of 0.790 g. of VI in 50 ml. of warm tetrahydrofuran. The combined solutions were cooled to 0°, and 0.350 g. of DCCI was added with stirring until the DCCI was dissolved. After the reaction mixture had stood in a refrigerator for 3 days, the dicyclohexylurea that formed was filtered off and the tetrahydrofuran was evaporated *in vacuo*. The residue was redissolved in ethyl acetate and then purified by being washed in the usual manner. After the ethyl acetate was removed by evaporation, the residue was crystallized from ethyl acetate–petroleum ether to yield a product (VIII) with m.p. 135–140°, yield 0.818 g. (51%).

Anal. Calcd. for $C_{56}H_{61}O_{10}N_9S_1$: C, 64.0; H, 5.80; N, 12.00. Found: C, 64.11; H, 5.95; N, 12.05.

L-Histidyl-D-phenylalanyl-L-ornithyl-L-tryptophyl-glycine (II).—The blocked pentapeptide methyl ester VIII was saponified by dissolving 0.710 g. in 40 ml. of methanol, adding 6.8 ml. of 1.0 *N* NaOH, and allowing the solution to stand at room temperature for 1 hour. Chromatography on paper of the acidified solution in SBA showed complete saponification when developed by the chlorine method.[17] The solution was then transferred to a two-necked round-bottomed flask and then evaporated to dryness in a vacuum. The residue was then further dried over P_2O_5 in a vacuum to ensure removal of all water. It was then dissolved in anhydrous liquid ammonia, and reduction with small pieces of metallic sodium was carried out in the usual manner.[18] After the ammonia was removed by evaporation, the peptide was extracted into 0.1 *M* acetic acid. Chromatography on an Amberlite XE-64 resin column was effective in removing all salts and side products. A column, measuring 2 × 10 cm. and containing 50 ml. of resin that had been washed with 0.1 *M* acetic acid and water, was used. The material was applied to the column in a buffer of 0.1 *M* acetic acid, and elution with water was performed until the optical density at 270 mμ was zero and all the salts had been removed. The free peptide was then eluted from the column with 0.1 *M* NH_4OH. Lyophylization of the main component in the NH_4OH eluate yielded a substance that was chromatographically and electrophoretically homogeneous on paper and gave a positive reaction with ninhydrin and the Ehrlich and Pauly reagents. The yield was 0.25 g. (60%), $[\alpha]^{23}D$ +6.1° (*c* 0.5, water). Elementary analysis indicated that this material was the dihydrate of peptide II.

Anal. Calcd. for $C_{33}H_{45}O_8N_9$: C, 57.0; H, 6.48; N, 18.13. Found: C, 56.73; H, 6.93; N, 18.40.

Amino Acid Analysis.—Peptides (1 to 3 mg.) were hydrolyzed at 110° in a sealed evacuated tube with 1 ml. of

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constant boiling (5.7 *N*) HCl for 24 hours. The amino acid content in the hydrolysates was determined by the method of Levy.[19] Since tryptophan is destroyed by acid hydrolysis, this amino acid was estimated from the ultraviolet absorption spectrum of the intact peptide according to the procedure of Goodwin and Morton.[31]

Paper Chromatography and Paper Electrophoresis.—Paper chromatography was carried out on Whatman No. 1 filter paper. The solvents used were *n*-butyl alcohol–acetic acid–water, 4:1:1 (BAW), and *sec*-butyl alcohol–10% NH_3, 85:15 (SBA). Zone electrophoresis on paper (Whatman 3 MM) was performed in a Spinco apparatus[32] for 8 hours at 200 volts with a collidine–acetic acid buffer of *p*H 7. Both paper electrophoresis and paper chromatography were conducted at room temperature. Color reactions were used to confirm the presence of histidine[33] and tryptophan.[34]

Enzymic Studies.—Crystalline α-chymotrypsin and trypsin were obtained from the Armour Laboratories; digestion of I and II with these enzymes was carried out at 25° for 24 hours in a solution of *p*H 9.0, with an enzyme–substrate ratio of $^1/_{100}$ (w./w.). A preparation of leucine aminopeptidase was kindly furnished by Drs. R. L. Hill and E. L. Smith; digestion was carried out at 25° for 8 hr. in a bicarbonate buffer of *p*H 8.8 containing 0.002 *M* $MgCl_2$, and with an enzyme–substrate ratio of $^1/_{100}$ (w./w.).

Assay of Melanocyte-stimulating Activity.[35]—The melanocyte-stimulating activity of the synthetic peptides was determined by the method described by Shizume, *et al.*,[21] with isolated skins of *Rana pipiens;* the unit of activity used is the same as that defined by these investigators. Bioassay was also carried out with hypophysectomized *Rana pipiens* (not more than 4 days after operation) as described by Hogben and Slome.[22]

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Resolution of N-Carbobenzoxy Amino Acids. Alanine, Phenylalanine and Tryptophan

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Racemic N-carbobenzoxy derivatives of alanine, phenylalanine and tryptophan were resolved, respectively, with ephedrine, α-phenylethylamine and quinine. The resolution of N-carbobenzoxy-DL-alanine with inexpensive (−)-ephedrine provided what is regarded as the most convenient source of N-carbobenzoxy-D-alanine and of D-alanine. The reciprocal resolution of DL-ephedrine with N-carbobenzoxy-L-alanine easily provided (+)-ephedrine. These resolutions afford routes to all active forms of the acid and base. The resolutions of carbobenzoxyphenylalanine and tryptophan were less convenient and gave only moderate yields of pure isomers.

The carbobenzoxy derivatives of D- and L-amino acids are widely used for peptide synthesis because of their relatively simple preparation and the eventual easy removal of the carbobenzoxy group by reductive procedures or by the use of anhydrous acids. However, the requisite D-amino acids and many of the L-forms must now first be obtained through resolution of some other racemic derivative and subsequent hydrolysis to the free amino acid prior to conversion to the desired optically active carbobenzoxy derivative. A saving in time and material would be possible by preparation of the desired optically active derivatives by direct chemical resolution of carbobenzoxy-DL-amino acids. Because of the facile removal of the carbobenzoxy group, the method might also be of advantage as a source of free amino acids in instances where other resolutions are difficult or removal of acyl radicals from other types of derivatives is not satisfactory.

Several carbobenzoxy amino acids have been resolved through the asymmetric biosynthesis of the anilides.[2-6] Hunt and du Vigneaud[7] resolved the carbobenzoxy derivatives of β-amino-*n*-butyric acid and β-aminoisobutyric acid using (+)- and (−)-α-phenylethylamine.

In the present studies we sought to provide a broader test of the method with representative examples by preparing the pure D- and L-forms of carbobenzoxyalanine, carbobenzoxyphenylalanine and carbobenzoxytryptophan. Natural (−)-ephedrine forms a less soluble diastereoisomeric salt with N-carbobenzoxy-D-alanine, and this was obtained pure in high yields, providing an excellent source of D-alanine. Furthermore, DL-ephedrine was found to be easily resolved with active carbobenzoxyalanine. Therefore, starting with either N-carbobenzoxy-L-alanine or (−)-ephedrine and the corresponding racemic compounds, both active forms of each were easily produced by reciprocal resolutions. The process provides (+)-ephedrine, a rare and little studied compound.

N-Carbobenzoxy-DL-phenylalanine was resolved with α-phenylethylamine. Both active forms of

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