[CONTRIBUTION FROM THE HORMONE RESEARCH LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY 4, CALIF.]

The Synthesis of L-Histidyl-D-phenylalanyl-L-arginyl-L-tryptophylglycine and its Melanocyte-stimulating Activity

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L-Histidyl-D-phenylalanyl-L-arginyl-L-tryptophylglycine has been synthesized and its melanocyte-stimulating activity has been assayed by three different methods. It was observed that the pentapeptide possesses a considerably higher specific activity than the L-phenylalanine analog.

In previous publications[1,2] on the synthesis of the melanotropically active pentapeptides, L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine, L-histidyl - L - phenylalanyl - L - ornithyl - L - tryptophylglycine and L-histidyl-D-phenylalanyl-L-ornithyl-L-tryptophylglycine, it was noted that substitution of L-arginine by L-ornithine or L-phenylalanine by D-phenylalanine in the ornithine peptide does not alter its melanocyte-stimulating activity. In order to investigate the influence of the D-phenylalanine residue on the biological behavior of these melanotropically active peptides, it was deemed desirable to synthesize L-histidyl-D-phenylalanyl-L-arginyl-L-tryptophylglycine.

Figure 1 presents the steps involved in the synthesis. The tosylation of carbobenzoxy-L-

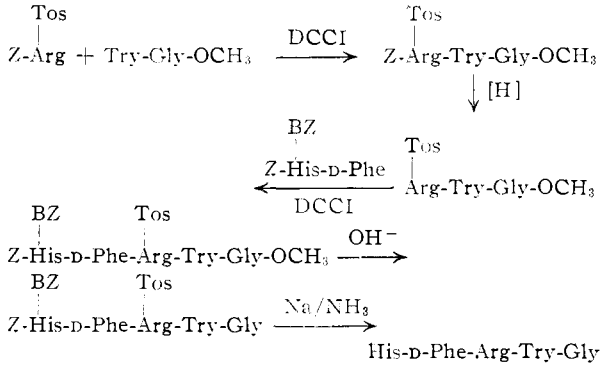

Fig. 1.—Route for the synthesis of L-histidyl-D-phenylalanyl-L-arginyl-L-tryptophylglycine; Z, carbobenzoxy-; Tos, tosyl-; BZ, benzyl.

arginine (Z-Arg), which was carried out as previously described,[1] yielded a clear sirup which solidified after the complete removal of the ethyl acetate either by drying *in vacuo* or by precipitation with anhydrous ether in the cold. The product (Z-Tos

Arg) behaved as a homogeneous component in paper chromatography; the chlorine method[3] again proved to be a helpful tool for establishing the location of the completely blocked amino acids and peptides on the chromatograms. All attempts to crystallize the tosylated Z-Arg failed. A small sample of the material was hydrogenated and the tosylated arginine was isolated as its copper complex. After several crystallizations, the copper was removed by the addition of a cyanide solution[4] and the resulting mixture was treated directly with carbobenzoxy chloride. The tosylated Z-Arg was again obtained in non-crystalline form.

Another attempt to tosylate the carbobenzoxy-L-arginine at *p*H 8.2 led mainly to the formation of an anhydro compound which crystallized in the form of large plates; it was characterized by elementary analysis and by determination of the molecular weight. Moreover, the high R_f-values in both the basic and acidic paper chromatographic systems indicated the absence of any acidic or basic groups. Unfortunately this anhydro compound could not be used in peptide synthesis. The analogous product, dicarbobenzoxy-anhydro-L-arginine, was recently described by Zervas and coworkers[5] as a by-product of the coupling of dicarbobenzoxy-L-arginine with other amino acids esters. The tosyl chloride at this low *p*H must have reacted first with the carboxyl group of the carbobenzoxy-G-tosyl-L-arginine to form a mixed anhydride and this activated carboxyl group must have then interacted with the second reactive center of the guanido group.[6,7] Fortunately, we never, obtained any anhydro compounds from the reaction of carbobenzoxy-tosyl-L-arginine with amino acid esters by the dicyclohexylcarbodiimide[8] (DCCI) method.

The carbobenzoxy-G-tosyl-L-arginyl-L-tryptophylglycine methyl ester was obtained in 80% yield by coupling carbobenzoxyl-G-tosyl-L-arginine with L-tryptophylglycine methyl ester[1] in acetonitrile in the presence of DCCI. The sirupy tripeptide derivative did not crystallize but was obtained as a white amorphous powder after being allowed to stand under ether or after precipitation from its ethyl acetate solution with absolute ether. The material was then submitted to catalytic hydrogenation which proceeded very slowly; this procedure yielded the G-tosyl-L-arginyl-L-tryptophylglycine-methyl ester. The latter derivative was coupled with carbobenzoxy-im-benzyl-L-histidyl-D-phenylalanine[2] to give the protected pentapeptide. The protected pentapeptide could not be obtained in crystalline form; the carbobenzoxy-im-benzyl-L-histidyl-D-phenylalanyl-G-tosyl-L-arginyl - L - tryptophyl - glycine precipitated after saponification. The material was reprecipitated several times from methanol–water. Sodium reduction in liquid ammonia[9] produced the free

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

(2) C. H. Li, E. Schnabel and D. Chung, THIS JOURNAL, **82**, 2062 (1960).

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

(4) H. Zahn, H. Zuber, W. Ditscher, D. Wegerle and J. Meienhofer, *Chem. Ber.*, **89**, 407 (1956).

(5) L. Zervas, T. T. Atani, M. Winitz and J. P. Greenstein, THIS JOURNAL, **81**, 2878 (1959).

(6) K. Felix and K. Dirr, *Z. physiol. Chem.*, **176**, 29 (1928).

(7) D. T. Gish and F. H. Carpenter, THIS JOURNAL, **75**, 5872 (1953).

(8) J. C. Sheehan and G. P. Hess, *ibid.*, **77**, 1067 (1955).

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

pentapeptide which was desalted on a XE-64 ion exchange column. The material behaved as a homogeneous component in paper chromatography and electrophoresis on starch at *p*H 4.9. The action of chymotrypsin liberated only glycine and L - histidyl - D - phenylalanyl - L - arginyl - L - tryptophan, indicating that the phenylalanine had the desired configuration. Hydrolysis of the peptide with trypsin under the same conditions as those used for chymotryptic digestion gave rise to two fragments (Try-Gly and His-Phe-Arg), as would be expected from the specificity of the enzyme. Amino acid analysis gave a molar ratio of His/Phe/Arg/Try/Gly = 1/1.2/0.9/1/0.9.

The peptide was assayed for its melanocyte-stimulating activity by the *in vitro* frog skin method[10] and the *in vitro* lizard skin method,[11] and *in vivo* in hypophysectomized *Rana pipiens*.[12] These three procedures showed L-histidyl-D-phenylalanyl - L - arginyl - L - tryptophylglycine to have a potency 5–10 times greater than that exhibited by the L-phenylalanine analog (Table I).

TABLE I

MELANOCYTE-STIMULATING ACTIVITY OF SYNTHETIC PEPTIDES

Peptide	Frog skin[a]	Lizard skin,[b]	Hypophysectomized *Rana pipiens*,[c]
L-His-D-Phe-L-Arg-L-Try-Gly	3.3×10^5	0.02	2
L-His-L-Phe-L-Arg-L-Try-Gly[d]	3.1×10^4	0.2	10
L-His-D-Phe-L-Orn-L-Try-Gly[e]	2.6×10^4	1.2	20
L-His-L-Phe-L-Orn-L-Try-Gly[e]	2.9×10^4	8.5	50

[a] Performed on the basis of an average of 3 assays; each assay was performed on 4–6 pieces of frog skin (see ref. 10). [b] The minimum effective dose caused color changes in the skin of *Anolis carolinensis in vitro*. (see ref. 11). [c] The single dose caused a rise in melanophore index from 1+ to 3+ within one hour in hypophysectomized *Rana pipiens* (see ref. 12). [d] For the synthesis, see ref. 1. [e] For the synthesis, see ref. 2.

It is of interest to note that when L-phenylalanine is substituted by the D-isomer in the pentapeptide containing L-ornithine, no increase in specific activity is shown by bioassay on the frog skin, but an enhancement of effect is noted when the assay is performed by the lizard skin method. It was demonstrated earlier that alkali–heat treatment abolished the melanocyte-stimulating activity of L - histidyl - D - phenylalanyl - L - ornithyl - L-tryptophylglycine.[2] When L - histidyl - D - phenylalanyl-L-arginyl-L-tryptophylglycine was subjected to the same treatment, there was no alteration of the melanocyte-stimulating potency. Detailed investigations on the chemical changes which take place after alkali–heat treatment of the two pentapeptides (L - histidyl - D - phenylalanyl-L - arginyl - L - tryptophylglycine and L - histidyl-D - phenylalanyl - L - ornithyl - L - tryptophylglycine) should lead to some better understanding of the mysterious phenomenon of prolongation of the darkening effect on the skin of the hypophysectomized frog exerted by treated adrenocorticotropins, melanotropins and synthetic peptides.[13]

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

(11) The method was developed by Dr. A. C. J. Burgers in this Laboratory with the lizard *Anolis carolinensis* used as test animal; a detailed account will be published elsewhere. The authors wish to thank Dr. Burgers for the assay results obtained by this method which are recorded in Table I.

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

Experimental[14]

Nα-Carbobenzoxy-G-Tosyl-L-Arginine. (**A**).—Carbobenzoxy-L-arginine[15] (12.5 g.) was dissolved in a mixture of 40 ml. of 4 *M* NaOH and 320 ml. of acetone. After the mixture was cooled to 0°, a solution of 19 g. of *p*-toluenesulfonyl chloride (tosyl chloride) in 60 ml. of acetone was added dropwise with vigorous stirring. The stirring was continued for 2 more hours at 0° and the mixture was then acidified to *p*H 5. The acetone was removed by evaporation *in vacuo* and the residue was extracted with two 30-ml. portions of ether to remove excess tosyl chloride. The product was extracted into 150 ml. of ethyl acetate and, after being washed with water, the product was extracted into 5% bicarbonate. After being acidified to *p*H 3, the material was extracted with 150 ml. of ethyl acetate and this solution was washed well with 2 *M* HCl followed by several portions of water. After the solution was dried over anhydrous sodium sulfate, the solvent was removed *in vacuo*, leaving a nearly colorless sirup which solidified when allowed to stand over P_2O_5. Precipitation from ethyl acetate with petroleum ether in a Dry Ice–acetone-bath also gave an amorphous product; yield 13.5 g. (73%), m.p. 75–85°. The material behaved as a homogeneous component in paper chromatography ($R_{f,SBA} - 0.40$; $R_{f,BAW} = 0.90$)[16] and gave negative reactions with ninhydrin and the Sakaguchi reagent[17]; $[\alpha]^{25}D$ −1.3° (*c* 2, methanol). A sample was dried at 68° over P_2O_5 for 12 hours for elementary analysis.

Anal. Calcd. for $C_{21}H_{26}N_4O_6S$ (462.52): C, 54.54; H, 5.62; N, 12.11; S, 6.92. Found: C, 54.24; H, 5.60; N, 11.87; S, 7.11.

(**B**).—α-Carbobenzoxy-G-tosyl-L-arginine (1 g.) was dissolved in 50 ml. of methanol and 2 ml. of glacial acetic acid; the solution was submitted to catalytic hydrogenation. After the methanol and acetic acid were removed by evaporation, $CuCO_3$ and $NaHCO_3$ were added to the water solution to give a somewhat gummy precipitate. The water was decanted and the material was recrystallized from methanol–water and acetone–water. The resulting blue needles had a m.p. of 182–185° and decomposed at 315°, yield 0.45 g. (56%). In paper chromatography, $R_{f,BAW}$ = 0.26–0.30.

Anal. Calcd. for $(C_{13}H_{19}N_4O_4S)_2 \cdot Cu\ H_2O$ (736.22): C, 42.41; H, 5.48; N, 15.22. Found: C, 42.01; H, 5.60; N, 15.39.

The above compound was dissolved in a 5% solution of potassium cyanide to give a clear and colorless solution which was acidified with acetic acid and evaporated to dryness. The material was redissolved and carbobenzoxylated at *p*H 9 in the usual manner, and the excess of carbobenzoxychloride was removed with ether. After acidification, the oily product was extracted with ethyl acetate and this solution was washed with 1 *M* HCl and H_2O. The acid was extracted from the organic phase with 5% $NaHCO_3$ and, after acidification, was re-extracted into ethyl acetate. After the solution was dried over anhydrous sodium sulfate and the ethyl acetate removed by evaporation, a colorless sirup remained which solidified over P_2O_5 *in vacuo*.

α-Carbobenzoxy-G-tosyl-anhydro-L-arginine.—Carbobenzoxy-L-arginine (10.4 g.) was dissolved as described above and the solution was cooled to −10° by the addition of Dry Ice. The *p*H of the solution dropped from 12 to 8.2;

(13) C. H. Li, *Lab. Invest.*, **8**, 574 (1959).

(14) Melting points are uncorrected; they were determined on a Fisher–Johns melting point apparatus. Elementary analyses were performed by the Microchemical Laboratory of the Chemistry Department of the University of California.

(15) M. Bergmann and L. Zervas, *Ber. Deut. Chem. Ges.*, **65**, 1192 (1932).

(16) Paper chromatography was carried out with Whatman filter paper no. 1 in the solvent system: 2-butanol–10% ammonia (designated SBA) = 85:15; 1-butanol–acetic acid–water (designated BAW) = 4:1:1.

(17) R. J. Bloch, E. L. Durrum and G. Zweig, "A Manual of Paper Chromatography and Paper Electrophoresis," 2nd Edition, Academic Press, Inc., New York, N. Y., 1958, pp. 128–139.

the tosyl chloride was added in the usual way and the reaction was allowed to proceed for 3 hours at room temperature with vigorous stirring. The mixture was then brought to *p*H 5 with 6 *N* HCl and the acetone–water removed *in vacuo*. A product was obtained in the form of white crystals which were then filtered off and washed with ethyl acetate. The filtrate was carefully extracted with ethyl acetate and pooled with the ethyl acetate used above for the washing. This solution was then washed well with 2 *N* HCl and much water; any crystals obtained after removal of the ethyl acetate by evaporation were pooled with the main crop and the material was recrystallized several times from acetone and ethyl acetate to give 5.2 g. of white plates, m.p. 156–157°, $[\alpha]^{24}D$ −10.5° (*C* 2, acetone). In paper chromatography, $R_{f,SBA}$ = 0.9 and $R_{f,BAW}$ = 0.9.

Anal. Calcd. for $C_{21}H_{26}N_4O_6S$, (444.43): C, 56.75; H, 5.44; N, 12.61; S, 7.20. Found: C, 56.68; H, 5.42; N, 12.53; S, 7.24.

To make certain that this product was the cyclic anhydro compound, the molecular weight was determined cryoscopically with camphor[18]; the values obtained for two determinations were 462 and 433, which are in good agreement with the calculated value above.

From the mother liquors, 2.5 g. of the α-carbobenzoxy-G-tosyl-L-arginine was also obtained.

α-Carbobenzoxy-G-tosyl-L-arginyl-L-tryptophyl-glycine Methyl Ester.—α-Carbobenzoxy-G-tosyl-L-arginine (4.62 g.) and 3.1 g. of L-tryptophyl-glycine methyl ester[1] were dissolved in 100 ml. of acetonitrile and cooled to 0°; DCCI (2.2 g.) was added and the mixture kept in the refrigerator overnight. The urea that formed was removed by filtration, and the acetonitrile was evaporated *in vacuo;* the resulting sirup was dissolved in ethyl acetate and this solution was washed in the usual manner. On evaporation of the ethyl acetate a sirup was obtained which solidified under ether. The ether was decanted and the residue washed carefully with some more ether. The material was dried over P_2O_5 *in vacuo* to give an amorphous product, m.p. 106–112°, yield, 5.75 g. (80%), $[\alpha]^{25}D$ −21.8° (*c* 2 methanol); paper chromatography: $R_{f,BAW}$ = 0.85. A sample was dried *in vacuo* at 68° over P_2O_5 for elementary analysis.

Anal. Calcd. for $C_{35}H_{41}N_7O_8S$ (719.74): C, 58.43; H, 5.74; N, 13.62; S, 4.44. Found: C, 58.58; H, 6.04; N, 13.46; S, 4.55.

G-Tosyl-L-arginyl-L-tryptophyl-glycine Methyl Ester.—α-Carbobenzoxy-G-tosyl-L-arginyl-L-tryptophyl-glycine methyl ester (1.9 g.) was dissolved in 40 ml. of methanol, and after the addition of 0.5 ml. of glacial acetic acid, the solution was submitted to catalytic hydrogenation. Since the hydrogenation was very slow, its progress was followed by paper chromatography. The hydrogenation was carried on for 5 days with the addition of new palladium catalyst every 24 hours until all the material gave a positive reaction with ninhydrin. After the catalyst was filtered off and the methanol and acetic acid were removed by evaporation *in vacuo* at room temperature, a sirupy product was obtained. This product was dried in a desiccator over KOH, and the acetate salt was stirred with ethyl acetate for 2 hours to remove any traces of unreacted material. The ethyl acetate was then decanted, any remaining ethyl acetate removed by evaporation *in vacuo*, and the residue was then suspended in 20 ml. of water. After the suspension was cooled to 0°, triethylamine (0.5 ml.) was added and the mixture was stirred for 10 minutes. The water was then removed by decanting and filtration, and the residue was then washed thoroughly with water to remove the triethylamine hydrochloride and any impurities with a free carboxy group. The remaining water removed by evaporation *in vacuo;* yield 1.07 g. (70%). In paper chromatography, $R_{f,BAW}$ 0.58. The material was used in this form for the next condensation.

Carbobenzoxy-im-benzyl-L-histidyl-D-phenylalanyl-G-tosyl-L-arginyl-L-tryptophyl-glycine Methyl Ester.—Carbobenzoxy-im-benzyl-L-histidyl-D-phenylalanine (870 mg.) and 1070 mg. of the above ester were dissolved in 200 ml. of acetonitrile and after the solution was cooled to 0°, DCCI (370 mg.) was added. The reaction mixture was kept in the refrigerator for 48 hours and the urea that formed (340 mg.) was removed by filtration. The acetonitrile was removed by evaporation *in vacuo* and the sirupy residue was dissolved in 30 ml. of ethyl acetate and washed in the usual manner, except that dilute acetic acid was used instead of HCl because the mineral acid partially precipitated the peptide derivative from the organic phase. The ethyl acetate solution was then evaporated to a small volume; when it was cooled in a Dry Ice–acetone-bath and a large volume of absolute ether added, a precipitate appeared. The amorphous material was filtered off and washed with much ether. It had a m.p. of 115–119° with sintering at 110°, yield, 1.1 g. (61%), $[\alpha]^{26}D$ −26.5° (*c* 2, methanol). A sample was dried *in vacuo* over P_2O_5 at 68° for elementary analysis.

Anal. Calcd. for $C_{57}H_{63}N_{11}O_{10}S$ (1094.23): C, 62.56; H, 5.81; N, 14.08. Found: C, 62.30; H, 5.90; N, 14.00.

Carbobenzoxy-im-benzyl-L-histidyl-D-phenylalanyl-G-tosyl-L-arginyl-L-tryptophylglycine.—The above protected peptide ester derivative (660 mg.) was dissolved in 20 ml. of methanol and kept at room temperature for 2.5 hours with 1.5 ml. of 1 *M* NaOH. One equivalent of 1 *M* HCl was then added to the solution and the methanol and water removed *in vacuo*. During this procedure a precipitate appeared which was freed from salt by being washed thoroughly with much water. The material was reprecipitated from methanol–water; m.p. 148–150° with sintering at 145°, yield 620 mg. (92%), $[\alpha]^{26}D$ −23.5 (*c* 2, methanol). In paper chromatography, $R_{f,SBA}$ = 0.45, $R_{f,BAW}$ = 0.84.

Anal. Calcd. for $C_{56}H_{61}N_{11}O_{10}S \cdot 1/2\ H_2O$ (1080.2): C, 61.84; H, 5.74; N, 14.14. Found: C, 61.70; H, 5.74; N, 13.54.

L-Histidyl-D-phenylalanyl-L-arginyl-L-tryptophylglycine.—The above blocked pentapeptide, (570 mg.) was dissolved in 100 ml. of liquid ammonia and small pieces of metallic sodium were added with vigorous magnetic stirring until the blue color persisted. The excess sodium was then destroyed with ammonium acetate and the ammonia removed by evaporation. The material was dried in the desiccator over H_2SO_4 to remove all traces of ammonia.

The dry residue was dissolved in 0.5 *N* acetic acid and the insoluble material was removed by filtration. The peptide was absorbed onto an Amberlite XE-64 resin column (1.5 × 30 cm.) which had been equilibrated with 0.1 *M* acetic acid. The salts were removed by passing 1000 ml. of distilled water through the column, and the peptide was then eluted with 0.2 *M* ammonium hydroxide. The ultraviolet-absorbing fraction was pooled and lyophilized; yield 280 mg. (69%) $[\alpha]^{27}D$ −20° (*c* 1, 0.1 *M* NH_4OH). In paper chromatography, $R_{f,BAW}$ = 0.05; a positive reaction was obtained with the Ehrlich, Sakaguchi and Pauly reagents and with ninhydrin.[17,19] Elementary analysis was performed.

Anal. Calcd. for $C_{34}H_{45}N_{11}O_6 \cdot 4\ H_2O$: C, 52.77; H, 6.64; N, 19.9. Found: C, 52.88; H, 6.49; N, 19.2.

Analytical Procedures.—Acid hydrolysis of the peptide was carried out at 110° in a sealed evacuated tube with 1 ml. of constant boiling HCl for 24 hours. The amino acid content in the acid hydrolysates of the peptide was estimated by the method of Levy[20]; tryptophan in the intact peptide was determined spectrophotometrically.[21]

The homogeneity of the synthetic peptide was investigated by means of zone electrophoresis on starch by the procedure previously described.[22] The electrophoresis was performed in a cold room at 4° with a potential gradient of approximately 5 volts per cm. in a 0.1 *M* pyridine–acetate buffer of *p*H 4.9 for 48 hours.

Chymotryptic and tryptic digestion of the peptide was performed in a solution of *p*H 9.0 with an enzyme–substrate ratio of 1/100 (w./w.) for 24 hours at 25°. Separation and identification of the fragments derived from the digests were achieved exactly as previously described.[2,23]

Acknowledgment.—The authors wish to acknowledge the able technical assistance of D. Chung and B. Solomon during the course of this work,

(18) K. Rast, *Ber. Deut. Chem. Ges.*, **55**, 1051, 3727 (1922).

(19) A sample was dried thoroughly over P_2O_5 at 60° and immediately submitted to analysis for nitrogen; the value of 22.4% for the content of N is in agreement with the calculated value (21.96%) for N in the anhydrous peptide ($C_{34}H_{45}N_{11}O_6$).

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

(21) T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40**, 628 (1946).

(22) P. Fønss-Bech and C. H. Li, *J. Biol. Chem.*, **207**, 175 (1954).

(23) J. Léonis, C. H. Li and D. Chung, THIS JOURNAL, **81**, 419 (1959).

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE]

Synthesis of 2-*p*-Methoxyphenylalanine Oxytocin (O-Methyl-oxytocin) and Some Observations on its Pharmacological Behavior[1]

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The synthesis of O-methyl-oxytocin, starting from *p*-methoxy-L-phenylalanine methyl ester, is described. The pattern of the synthesis paralleled one used previously for the synthesis of oxytocin and involved the coupling of S-benzyl-N-carbobenzoxy-L-cysteinyl-*p*-methoxy-L-phenylalanyl-L-isoleucyl-L-glutaminyl-L-asparagine with S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. Conversion of the resulting protected nonapeptide amide to O-methyl-oxytocin was effected by reduction with sodium in liquid ammonia followed by aeration. The methyl derivative of oxytocin was purified by countercurrent distribution and then compared with oxytocin as to some of its pharmacological effects. This hormone derivative containing *p*-methoxyphenylalanine in place of tyrosine possessed approximately 1% of the avian depressor and rat-uterine-contracting potency of oxytocin and showed no pressor activity in the rat. Furthermore, the O-methyl-oxytocin inhibited the pressor response of arginine-vasopressin when injected simultaneously with the pressor hormone into the rat.

Early in the course of studies in this Laboratory on the functional groups present in oxytocin, evidence was obtained by Turner and Gordon[3] that treatment of oxytocin with diazomethane resulted in a loss of avian depressor activity. A study of the ultraviolet absorption spectrum of the reaction mixture indicated that the phenolic group of the tyrosine residue had been methylated. Moreover, after hydrolysis of the diazomethane-treated hormone with sulfuric acid, methoxyphenylalanine appeared among the constituent amino acids and no tyrosine was present. Because of the small amount of material available at that time, it was not feasible to attempt to isolate and study the methylated derivative.

In more recent work in this Laboratory an analog of oxytocin was synthesized in which the phenolic hydroxyl group was not present. The phenylalanine residue was substituted for the tyrosine residue in a series of reactions leading to 2-phenylalanine oxytocin.[4,5] This analog was also prepared by Jaquenoud and Boissonnas[6] and was found by both groups to possess about one-eighth of the avian depressor activity and one-sixteenth of the rat uterine-contracting activity of oxytocin itself. The compound also showed a fairly high degree of milk-ejecting activity. It was obvious that while the phenolic hydroxyl group does play a role in the biological activity of oxytocin, its presence is not essential for biological activity.

The present investigation represents a further contribution to the study of the significance of the phenolic group in oxytocin, in that the analog in which the phenolic hydroxyl group is methylated, namely 2-*p*-methoxy-phenylalanine oxytocin[5] (henceforth referred to as O-methyl-oxytocin), has been synthesized, starting from *p*-methoxyphenylalanine methyl ester, and its pharmacological effects have been investigated.

The O-methyl-oxytocin was prepared by a method similar to one previously used for the synthesis of oxytocin.[7] S-Benzyl-N-carbobenzoxy-L-cysteine was coupled with methyl *p*-methoxy-L-phenylalaninate with the use of dicyclohexylcarbodiimide[8] to give methyl S-benzyl-N-carbobenzoxy-L-cysteinyl-*p*-methoxy-L-phenylalaninate (I), which was saponified with sodium hydroxide in an acetone–water solution to S-benzyl-N-carbobenzoxy-L-cysteinyl-*p*-methoxy-L-phenylalanine (II). The latter compound was coupled with L-isoleucyl-L-glutaminyl-L-asparagine[7] by the isobutyl chloroformate method.[9] The resulting S-benzyl-N-carbobenzoxy-L-cysteinyl-*p*-methoxy-L-phenylalanyl-L-isoleucyl-L-glutaminyl-L-asparagine (III) was coupled with the tetrapeptide amide, S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide[7,10,11] by the dicyclohexylcarbodiimide method to yield the protected nonapeptide amide, S-benzyl-N-carbobenzoxy-L-cysteinyl-*p*-methoxy-L-phenylalanyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (IV). Compound IV was then reduced with sodium in liquid ammonia and oxidized by aeration according to the procedure previously employed in the synthesis of oxytocin.[12] The O-methyl-oxytocin was isolated by countercurrent distribution in the system butanol–ethanol–0.05% acetic acid (4:1:5), followed by concentration and lyophilization. The com-

(1) This work was supported in part by a grant from the National Heart Institute, U. S. Public Health Service, Grant No. H-1675.

(2) Commonwealth Fund Fellow.

(3) R. A. Turner and S. Gordon, unpublished data.

(4) M. Bodanszky and V. du Vigneaud, THIS JOURNAL, **81**, 1258, 6072 (1959).

(5) The number indicates the position of the amino acid residue which is replaced in the oxytocin molecule:

H-CyS-Tyr-*i*Leu-Glu(NH$_2$)-Asp(NH$_2$)-CyS-Pro-Leu-Gly-NH$_2$
1 2 3 4 5 6 7 8 9

(6) P.-A. Jaquenoud and R. A. Boissonnas, *Helv. Chim. Acta*, **42**, 788 (1959); H. Konzett and B. Berde, *Brit. J. Pharmacol.*, **14**, 333 (1959).

(7) M. Bodanszky and V. du Vigneaud, THIS JOURNAL, **81**, 2504 (1959).

(8) J. C. Sheehan and G. P. Hess, *ibid.*, **77**, 1067 (1955).

(9) J. R. Vaughan, Jr., and J. A. Eichler, *ibid.*, **75**, 5556 (1953).

(10) C. Ressler and V. du Vigneaud, *ibid.*, **76**, 3107 (1954).

(11) The sample of this compound was kindly provided by Dr. Miklos Bodanszky, who was at that time in this Laboratory.

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