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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-

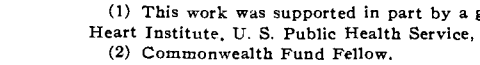
(1) This work was supported in part by a grant from the National Heart Institute, U. S. Public Health Service, Grant No. H-1675.

(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:



(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.

(12) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts and P. G. Katsoyannis, THIS JOURNAL, **76**, 3115 (1954).

pound has a partition coefficient (K) of approximately 0.58 in this system, whereas the value for oxytocin is approximately 0.43.[7]

The O-methyl-oxytocin was tested for avian depressor,[13] rat uterine-contracting[14] and rat pressor[15] activities by comparison with laboratory standards of highly purified oxytocin and arginine vasopressin. The avian depressor activity was found to be approximately 1% that of oxytocin.[16] Although the responses obtained in the isolated uterine strip test showed considerable variation, the potency of O-methyl-oxytocin in this test appeared to be of the same order of magnitude as its potency in the avian depressor test. The derivative showed no pressor activity in the rat with doses as high as 117 μg. but was found to possess anti-vasopressin activity, inhibiting the pressor response of arginine-vasopressin. For example, when 30 μg. of O-methyl-oxytocin was injected simultaneously with 0.016 μg. of arginine-vasopressin, the expected pressor response to that amount of hormone was reduced by approximately 50%. This was confirmed repeatedly. When this amount of O-methyl-oxytocin was injected along with 0.008 μg. of arginine-vasopressin, complete inhibition resulted. Furthermore, a lapse of up to 1 hr. was required before the full pressor response to 0.016 μg. of arginine-vasopressin was obtained.

Experimental[17]

Methyl *p*-Methoxy-L-Phenylalaninate Hydrochloride.—To 20 ml. of dry methanol cooled to −10°, 2.25 ml. of thionyl chloride[18] was added dropwise with stirring, followed, after 5 minutes, by 4.8 g. of *p*-methoxy-L-phenylalanine, prepared according to Izumiya and Nagamatsu.[19] The suspension was allowed to warm to room temperature and stirred for 22 hr. After this time the mixture was heated for 2 minutes on a water-bath. The solution was evaporated to a small volume at room temperature and 40 ml. of dry ether was added. The precipitate was filtered off and washed with 20 ml. of dry ether. The crude product, m.p. 184–188° dec., was recrystallized from methanol by the addition of ether; wt. 5.77 g. (96%), m.p. 186.5–188.5° dec. Paper chromatography on Whatman No. 1 paper with butanol–acetic acid–water (4:1:5) gave one ninhydrin-positive spot at R_f 0.62.

A sample recrystallized again from methanol–ether melted at 188.5–190° with decomposition, $[\alpha]^{20}D$ +74.0° (*c* 1, pyridine); lit. m.p.[20] 180–182°.

Anal. Calcd. for $C_{11}H_{15}O_3N\cdot HCl$: C, 53.8; H, 6.56; N, 5.70. Found: C, 53.8; H, 6.59; N, 5.71.

Methyl S-Benzyl-N-carbobenzoxy-L-cysteinyl-*p*-methoxy-L-phenylalaninate (I).—Methyl *p*-methoxy-L-phenylalaninate hydrochloride (4.8 g., 19.5 mmoles) was dissolved in 20 ml. of water and excess sodium bicarbonate was added. The ester was extracted with three 20-ml. portions of ethyl acetate and the solution was dried over magnesium sulfate, filtered and concentrated to a small volume at room temperature, leaving an oil which was dissolved in 10 ml. of dimethylformamide. To this solution, 6.4 g. (18.5 mmoles) of S-benzyl-N-carbobenzoxy-L-cysteine[21] was added. The solution was cooled to −5° and 4.2 g. (20.5 mmoles) of dicyclohexylcarbodiimide was added in 4 portions at 0.5-hr. intervals. The reaction mixture was stirred at 0° overnight. Acetic acid (0.25 ml.) was then added and the mixture was allowed to warm to room temperature and filtered. The filtrate was poured slowly into 150 ml. of 0.5 *N* HCl which was cooled with ice. The resulting precipitate was separated by filtration, washed with 20 ml. of water, and triturated with 30 ml. of 0.5 *N* potassium bicarbonate solution. It was finally washed with 40 ml. of water and air-dried. Recrystallization from 160 ml. of methanol gave 6.98 g. (69%) of I as fine needles, m.p. 132.5–134.5°. A sample recrystallized again from methanol melted at 133.5–135°, $[\alpha]^{20}D$ −29.5° (*c* 0.5, dimethylformamide).

Anal. Calcd. for $C_{29}H_{32}O_6N_2S$: C, 64.9; H, 6.01; N, 5.22. Found: C, 65.0; H, 6.02; N, 5.10.

S-Benzyl-N-carbobenzoxy-L-cysteinyl-*p*-methoxy-L-phenylalanine (II).—Compound I (16 g., 30 mmoles) was dissolved in a mixture of 10 ml. of water and 300 ml. of acetone and 23 ml. of 2 *N* sodium hydroxide was added. The mixture was stirred for 3 hr., then 50 ml. of *N* hydrochloric acid was added, and crystallization was induced by addition of water. The acetone was evaporated under reduced pressure, and the product, after separation by filtration and drying *in vacuo* over phosphorus pentoxide, melted at 150–155°. This material was recrystallized from acetone–water (2:1), washed with 30 ml. of acetone–water (1:1), and dried *in vacuo* over phosphorus pentoxide; wt. 10 g. (65%), m.p. 158.5–160.5°.

A sample recrystallized again from acetone–water (2:1) melted at 160–161°, $[\alpha]^{20}D$ −14.4° (*c* 2, pyridine).

Anal. Calcd. for $C_{28}H_{30}O_6N_2S$: C, 64.4; H, 5.79; N, 5.36; neut. equiv., 523. Found: C, 64.4; H, 5.87; N, 5.39; neut. equiv., 522.

S-Benzyl-N-carbobenzoxy-L-cysteinyl-*p*-methoxy-L-phenylalanyl-L-isoleucyl-L-glutaminyl-L-asparagine (III).—Compound II (5.6 g., 10.6 mmoles) was dissolved in 110 ml. of freshly distilled tetrahydrofuran and 1.055 g. (10.5 mmoles) of triethylamine was added with cooling. The mixture was cooled to −15° and 1.44 g. (10.5 mmoles) of isobutyl chloroformate was added with stirring. Triethylamine hydrochloride started to separate immediately.

After 25 minutes at −10°, a solution of 4.12 g. (10.5 mmoles) of L-isoleucyl-L-glutaminyl-L-asparagine hydrate[7] and 1.055 g. of triethylamine in 20 ml. of water was added. The mixture was stirred vigorously and allowed to warm to room temperature for 1 hr. and then to stand at room temperature overnight. (Stirring had been discontinued after 2 hr. because the solution had become extremely viscous.)

The resultant gel was dispersed by the addition of 250 ml. of water and 250 ml. of tetrahydrofuran with shaking. The mixture was acidified to *p*H 1 by the addition of 35 ml. of 3 *N* hydrochloric acid and stirred. The tetrahydrofuran was evaporated under reduced pressure and the semi-gelatinous precipitate was separated by filtration and washed with water until the *p*H of the washings was approximately 4.

The product was found to be contaminated with II, which was removed by trituration and boiling with ethyl acetate. The amorphous residue (III) was air-dried; wt. 5.5 g., m.p. 230–234° dec.

(13) J. M. Coon, *Arch. Intern. Pharmacodynam.*, **62**, 79 (1939); "The Pharmacopeia of the United States of America," 15th revision, Mack Printing Co., Easton, Pa., 1955, p. 555.

(14) P. Holton, *Brit. J. Pharmacol.*, **3**, 328 (1948).

(15) "The Pharmacopeia of the United States of America," 15th revision, Mack Printing Co., Easton, Pa., 1955, p. 776.

(16) Paper chromatographic studies showed that carbobenzoxy-*p*-methoxyphenylalanine was slowly converted to tyrosine by the action of hydrogen bromide in acetic acid. It would therefore be expected that similar treatment of the protected nonapeptide intermediate IV for O-methyl-oxytocin would remove the carobenzoxy group and the O-methyl group, giving rise to the S,S'-dibenzyl derivative of reduced oxytocin. Cleavage of the benzyl groups from this product by reduction with sodium in liquid ammonia followed by aeration should then yield oxytocin [S. Gordon and V. du Vigneaud, *Proc. Soc. Exptl. Biol. Med.*, **84**, 723 (1953)]. Thus, this series of reactions on IV ought to yield material with a pharmacological activity much greater than that obtained following conversion of IV to O-methyl-oxytocin. Such was found to be the case. An amount of the protected nonapeptide intermediate IV equivalent to 200 avian depressor units of O-methyl-oxytocin gave rise to 4000 units of avian depressor activity when subjected to treatment with hydrogen bromide in acetic acid followed by reduction and aeration.

(17) Capillary melting points were determined for all compounds and are corrected.

(18) M. Brenner and W. Huber, *Helv. Chim. Acta*, **36**, 1109 (1953); R. A. Boissonnas, St. Guttmann, P.-A. Jaquenoud and J.-P. Waller, *ibid.*, **39**, 1421 (1956).

(19) N. Izumiya and A. Nagamatsu, *Bull. Chem. Soc. Japan*, **25**, 265 (1952).

(20) B. R. Baker, J. P. Joseph and J. H. Williams, THIS JOURNAL, **77**, 1 (1955).

(21) C. R. Harington and T. H. Mead, *Biochem. J.*, **30**, 1598 (1936).

For purification of a sample for analysis, it was dissolved in hot dimethylformamide, precipitated by the addition of hexane and washed with large volumes of ethyl acetate. The residue was dried in air and then over phosphorus pentoxide *in vacuo* at 100°, m.p. (after placement in the bath at 180°) 232–236° dec., $[\alpha]^{20}D$ −22.5° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{43}H_{55}O_{11}N_7S$: C, 58.8; H, 6.31; N, 11.2. Found: C, 58.6; H, 6.33; N, 11.0.

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).—S - Benzyl - L - cysteinyl - L - prolyl- L - leucylglycinamide hydrate[7,10,11] (2.2 g., 4.33 mmoles) was dissolved in 10 ml. of dimethylformamide and III (3.15 g., 3.60 mmoles) was added, followed by 20 ml. of dimethylformamide. To this semi-gelatinous mixture, 2.6 g. (12.6 mmoles) of dicyclohexylcarbodiimide was added and the reaction mixture was allowed to stir at room temperature for 4 days when 2.5 ml. of acetic acid was added, and then 400 ml. of water with cooling. The mixture was allowed to stand for 20 minutes before the precipitate was separated by filtration, washed with 100 ml. of water and dried *in vacuo* over phosphorus pentoxide.

N,N'-Dicyclohexylurea was removed by repeatedly boiling the product with methanol and washing it with boiling methanol. The dried residue weighed 2.4 g., m.p. 230–234°. Further treatment in this manner gave a product weighing 2.08 g. and melting from 236 to 238° with decomposition after sintering at 233°. This material was dissolved with heating in 20 ml. of dimethylformamide, plus several drops of acetic acid, and the solution was filtered. Water (400 ml.) was added to the filtrate with cooling and the resulting precipitate was filtered off and dried; wt. 2.03 g., m.p. 238–240° dec., with sintering from 233°. The methanol treatment was repeated on this material to give IV; wt. 1.42 g., m.p. (when placed in the bath at 190°) 240–241° dec. (s. 235°), $[\alpha]^{20}D$ −48° (*c* 1, dimethylformamide). Before analysis the compound was dried *in vacuo* over phosphorus pentoxide at 100° for 7 hr.

Anal. Calcd. for $C_{68}H_{88}O_{14}N_{12}S_2$: C, 59.3; H, 6.63; N, 12.6. Found: C, 59.4; H, 6.67; N, 12.5.

Preparation of O-Methyl-oxytocin by Reduction of IV and Oxidation of the Resulting Sulfhydryl Peptide.—The technique followed was essentially the same as that previously described in the synthesis of oxytocin.[12] Four 200-mg. batches of IV were reduced separately with sodium in liquid ammonia (100–200 ml.) and the resulting sulfhydryl nonapeptide was dissolved in water (400 ml.) after removal of the ammonia. Acetic acid (2 drops) was added to the solution to adjust the *p*H to 6.5 and the solutions were aerated for 1 hr., filtered and tested for biological activity.

The four solutions possessed 2.2, 1.7, 2.2 and 2.0 avian depressor units for each mg. of starting nonapeptide IV. Acetic acid was added to bring the *p*H of the solutions to 4, and they were combined and concentrated to a small volume (15 ml., containing 650 avian depressor units) by use of a rotary evaporator. This material was subjected to countercurrent distribution for 769 transfers with recycling in the system butanol–ethanol–0.05% acetic acid (4:1:5). The distribution was followed by measurement of the Folin–Lowry color[22] and the avian depressor activity[13] (in the latter case after removal of the butanol and ethanol by evaporation at room temperature). The activity was all contained in one peak with a *K* of 0.58.

The contents of the fifty tubes representing the peak were combined and concentrated on the rotary evaporator. Two-thirds of the concentrate was lyophilized and gave 73 mg. of material. The other third of the concentrate was placed in the first tube of a 20-tube countercurrent distribution apparatus and distributed for 30 transfers in the same solvent system, particular care being taken to avoid transfer of the lower phase. Distribution patterns, obtained by plotting the values for weight, Folin–Lowry color and avian depressor activity, were in agreement with a theoretical curve calculated for a *K* value of 0.58.

The contents of the eight tubes at the center of the peak were combined, concentrated and lyophilized to give 20 mg. of product; total yield: 93 mg. (approximately 500 units of avian depressor activity).

Anal. Calcd. for $C_{44}H_{68}O_{12}N_{12}S_2 \cdot C_2H_4O_2$: C, 51.1; H, 6.80; N, 15.5. Found: C, 51.9; H, 6.87; N, 15.6.

A sample was hydrolyzed in 6 *N* HCl at 105° for 16 hr. under nitrogen and analyzed by the starch column procedure of Moore and Stein.[23] The following ratios between the constituent amino acids were obtained (with the molar ratio for glycine arbitrarily taken as 1): leucine–isoleucine 2.2, tyrosine 0.7, proline 0.9, glutamic acid 1.0, aspartic acid 0.9, glycine 1.0, cystine 0.9 and ammonia 3.2. Results obtained earlier by Turner and Gordon[3] in studies on methylated oxytocin had indicated that demethylation of *p*-methoxyphenylalanine to tyrosine could be avoided by hydrolysis with sulfuric acid. When the O-methyl-oxytocin was hydrolyzed in 1.8 *M* sulfuric acid for 23 hr. at 130°, no tyrosine appeared in the chromatogram and the molar ratio for the leucine–isoleucine peak was increased by 1. Chromatography of an authentic sample of *p*-methoxy-L-phenylalanine showed it to appear with the leucine and isoleucine in this solvent system.

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(22) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(23) S. Moore and W. H. Stein, *ibid.*, **178**, 53 (1949).