compounds have not been examined in this regard, but Bellamy has cited cases where they form hydrogen bonds with various donors.[50] With respect to the inert diluents—CCl_4 and DCE—the difference between them is not very great. It is probably due to the effect of the diluent on the extent of organization of polymers of DCA in the two cosolvents. Reeves has shown from nmr studies that DCE will stabilize the monomer form of DCA more than CCl_4. This means that DCA will be more "acidic" in CCl_4 than in DCE.

Some of the results we report here are similar to earlier findings by Lotan, *et al.*[51] These workers examined the helix–coil transitions of some water-soluble polypeptides in formic acid–water mixtures and in formic acid–acetic acid mixtures, as well as in formic acid containing added formate salts. We are limiting our present conclusions to solutions of polypeptides like PBLG in DCA. It is possible, as these authors have assumed, that the polymers they studied are protonated in formic acid solution. This is a solvent which needs investigation on its own. However, it is interesting to note from the curves in their paper that acetic acid is more effective than water as a helix-forming cosolvent for formic acid in the case of at least one of their polymers. Stable heterodimers are not limited to the halogenated acetic acids as one partner; it is known that propionic and acetic acids also form stable heterodimers in the gas phase.[52]

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(50) L. J. Bellamy, H. F. Hallam, and R. L. Williams, *Trans. Faraday Soc.*, **54**, 1120 (1958).
(51) N. Lotan, M. Bixon, and A. Berger, *Biopolymers*, **5**, 69 (1967).
(52) S. D. Christian, *J. Phys. Chem.*, **61**, 1441 (1957).

Oxytocin Analogs with Basic Amino Acid Residues in Positions 4 and 5. Synthesis and Pharmacological Properties of [4-Ornithine]- and [5-Ornithine]-oxytocin[1]

Robert T. Havran, I. L. Schwartz, and Roderich Walter

Contribution from The Mount Sinai Medical and Graduate Schools of The City University of New York, New York, New York 10029, and The Medical Research Center, Brookhaven National Laboratory, Upton, New York 11973.
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Abstract: [4-Ornithine]-oxytocin, an analog of the neurohypophyseal hormone oxytocin in which the glutamine residue at position 4 is replaced by an ornithine residue, and [5-ornithine]-oxytocin, an analog in which the asparagine residue at position 5 is replaced by an ornithine residue, have been synthesized and assayed for their biological activities. The nonapeptides were synthesized stepwise by the activated ester method of peptide synthesis. The chemical properties and biological potencies of [4-ornithine]-oxytocin were identical regardless of whether the δ-amino function of ornithine had been protected during synthesis by a tosyl or a phthalyl group. Upon bioassay the [4-ornithine]-oxytocin was found to possess 163 ± 5 units/mg of avian vasodepressor activity, 58 ± 1.6 units/mg of rat oxytocic activity, 127 ± 7 units/mg of rabbit milk-ejecting activity, less than 0.1 unit/mg of rat pressor activity, and approximately 0.029 unit/mg of rat antidiuretic activity. The hydroosmotic activity of the analog was 1.60 ± 0.10% of that of crystalline deamino-oxytocin upon assay in the toad bladder system. The corresponding activity values for [5-ornithine]-oxytocin were approximately 0.07, 0.24, 0.19, 0.04, and 0.002 unit/mg and 0.046%, respectively. The striking differences in the potencies between [4-ornithine]-oxytocin and [5-ornithine]-oxytocin add further support to the contention that the biological activities of oxytocin are much less affected by structural alterations in position 4 as compared with position 5. However, dose-response analyses on the toad bladder with [5-ornithine]-oxytocin revealed that its intrinsic activity in inducing transepithelial water movement along an osmotic gradient is the same as that of the natural amphibian hormone, [8-arginine]-vasotocin. Thus it can be concluded that the asparagine residue in position 5 of oxytocin is not essential for the manifestation of intrinsic hydroosmotic activity.

In the course of the systematic study in several laboratories of the relation of chemical structure to biological activity of peptide hormones, the role of the side chains of the glutamine and asparagine residues in positions 4 and 5 of the peptide ring of oxytocin (Figure 1) has been explored. From this work[2-4] it can be concluded

(1) This work was supported by U. S. Public Health Service Grant AM-10080 of the National Institute of Arthritis and Metabolic Diseases and by the U. S. Atomic Energy Commission.

(2) (a) R. Walter and I. L. Schwartz, *J. Biol. Chem.*, **241**, 5500 (1966); (b) V. du Vigneaud, G. Flouret, and R. Walter, *ibid.*, **241**, 2093 (1966).
(3) St. Guttmann and R. A. Boissonnas, *Helv. Chim. Acta*, **46**, 1626 (1963).
(4) F. Morel and P. Bastide, in "Symposium on Oxytocin, Vasopressin and their Structural Analogues," J. Rudinger, Ed., Pergamon Press, New York, N. Y., 1964, p 47.

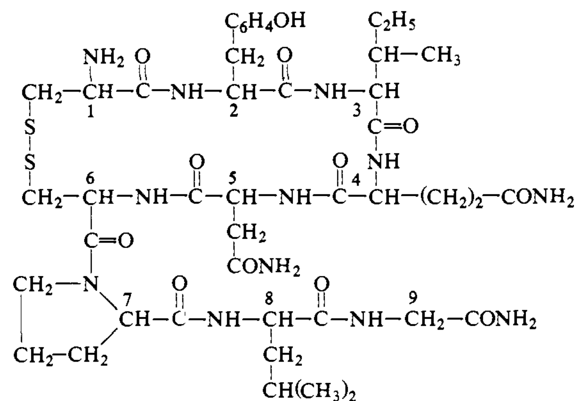

Figure 1. Structure of oxytocin with numbers indicating the position of the individual amino acid residues.

that, in general, position 4 of oxytocin exhibits a considerable tolerance to structural alterations with respect to oxytocin-like activities, *i.e.*, rat uterotonic, milk-ejecting, and avian vasodepressor activities; less tolerance is exhibited with respect to vasopressin-like activities, *i.e.*, rat pressor and rat antidiuretic activities. The single deviation from this trend up to now was noted in studies of the biological properties of [4-glutamic acid]-oxytocin, an analog bearing a carboxyl group in place of the carboxamide group in position 4. This analog exhibited only $^1/_{1000}$ of the avian vasodepressor activity and approximately $^1/_{300}$ of the rat uterotonic activity of oxytocin.[5] In view of the latter findings, we became interested in evaluating the importance of the δ-carbonyl moiety of the glutamine residue for the manifestation of the biological properties of oxytocin. Therefore, we prepared [4-ornithine]-oxytocin, an analog in which this δ-carbonyl moiety of glutamine is formally replaced by a methylene moiety. Moreover, in a continued effort to analyze the importance of the asparagine residue in position 5 of oxytocin, [5-ornithine]-oxytocin was also synthesized and its biological properties were studied.

The [4-ornithine]-oxytocin was obtained by two synthetic approaches. In the first approach, L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide—obtained from the corresponding protected pentapeptide[6]—was allowed to react with *p*-nitrophenyl N^α-carbobenzoxy-N^δ-phthalyl-L-ornithinate[7] to give the required protected hexapeptide. After decarbobenzoxylation this hexapeptide amide was lengthened stepwise with the appropriate protected amino acid *p*-nitrophenyl esters to give the fully protected nonapeptide, N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-N^δ-phthalyl-L-ornithyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. From this intermediate the phthalyl group was removed with hydrazine hydrate. The resulting peptide was decarbobenzoxylated and debenzylated with sodium in liquid ammonia according to the method of Sifferd and du Vigneaud.[8] Following the removal of ammonia the [4-ornithine]-oxytoceine was oxidized to yield [4-ornithine]-oxytocin by treatment with potassium ferricyanide as employed for the synthesis of deamino-oxytocin.[9] The ferricyanide and ferrocyanide ions were removed with a cation-exchange resin. Further purification of [4-ornithine]-oxytocin was effected by partition chromatography on Sephadex G-25 according to the procedure of Yamashiro[10,11] with the solvent system 1-butanol–ethanol–pyridine–0.1 *N* acetic acid (4:1:1:7).

In the second approach to synthesizing [4-ornithine]-oxytocin the free pentapeptide, L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide, was coupled with pentachlorophenyl N^α-carbobenzoxy-N^δ-tosyl-L-ornithinate.[12] We recently investigated the potentialities of derivatives of 1-tosyl-3-amino-2-piperidone for the synthesis of ornithine-containing peptides.[12] In this context the aforementioned free pentapeptide was allowed to react with 1-tosyl-3-carbobenzoxyamino-2-piperidone yielding the protected hexapeptide, less readily, however, as compared with the corresponding pentachlorophenyl ester. The hexapeptide chain was lengthened by the stepwise *p*-nitrophenyl ester method[6] to give the nonapeptide, N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-N^δ-tosyl-L-ornithyl-L-asparaginyl-S-benzyl-L-cysteiny-L-prolyl-L-leucylglycinamide. From this intermediate the tosyl residue was removed simultaneously with the N-carbobenzoxy and S-benzyl residues by treatment with sodium in liquid ammonia; subsequently the [4-ornithine]-oxytoceine was oxidized to [4-ornithine]-oxytocin, which was purified as previously described. Physical properties and biological activities of [4-ornithine]-oxytocin prepared by either method were identical and the yields were comparable.

In the synthesis of [5-ornithine]-oxytocin, S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide[13] was lengthened by successive attachment of the appropriate protected amino acid *p*-nitrophenyl ester to yield the key intermediate, N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-N^δ-tosyl-L-ornithyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. This peptide was converted to [5-ornithine]-oxytocin by the above-described procedure. The hormone analog was isolated after partition chromatography on Sephadex G-25 columns. The highly purified analogs were homogeneous as indicated by paper electrophoresis and thin layer and paper chromatography. Elementary analyses and analysis for ninhydrin-active material following peptide hydrolysis gave the expected values.

The current studies, involving comparable replacements in positions 4 and 5 of oxytocin, focus on the relative importance of these two *loci* for the manifestation of neurohypophyseal hormonal activities. In general, the striking differences in the degree of potencies of [4-ornithine]-oxytocin as compared with [5-ornithine]-oxytocin

(5) I. Photaki and V. du Vigneaud, *J. Am. Chem. Soc.*, **87**, 908 (1965).
(6) M. Bodanszky and V. du Vigneaud, *ibid.*, **81**, 5688 (1959).
(7) M. Bodanszky, M. A. Ondetti, C. A. Birkhimer, and P. L. Thomas, *ibid.*, **86**, 4452 (1964).
(8) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).
(9) D. B. Hope, V. V. S. Murti, and V. du Vigneaud, *ibid.*, **237**, 1563 (1962); V. du Vigneaud, G. Winestock, V. V. S. Murti, D. B. Hope, and R. D. Kimbrough, Jr., *ibid.*, **235**, PC64 (1960).
(10) D. Yamashiro, *Nature*, **201**, 76 (1964).
(11) D. Yamashiro, D. Gillessen, and V. du Vigneaud, *J. Am. Chem. Soc.*, **88**, 1310 (1966).
(12) V. Gut, J. Rudinger, R. Walter, P. A. Herling, and I. L. Schwartz, *Tetrahedron*, **24**, 6351 (1968).
(13) N. I. A. Overweg, I. L. Schwartz, B. M. Dubois, and R. Walter, *J. Pharmacol. Exptl. Therap.*, **161**, 342 (1968); H. C. Beyerman, J. S. Bontekoe, and A. C. Koch, *Rec. Trav. Chim.*, **78**, 935 (1959); M. Zaoral and J. Rudinger, *Coll. Czech. Chem. Commun.*, **20**, 1183 (1955); C. Ressler and V. du Vigneaud, *J. Am. Chem. Soc.*, **76**, 3107 (1954).

Table I. Biological Activities of Oxytocin, [4-Ornithine]-oxytocin, and [5-Ornithine]-oxytocin[a]

Compound	Vasodepressor (fowl)	Oxytocic (rat)	Pressor (rat)	Antidiuretic (rat)	Water flux[b] (toad bladder)	Milk-ejecting (rabbit)
Oxytocin[c]	507 ± 23	546 ± 18[d]	3.1 ± 0.1	2.7 ± 0.2	182 ± 9[e]	410 ± 16
[4-Ornithine]-oxytocin	163 ± 5	58 ± 1.6	<0.1	~0.029 ± 0.006	1.60 ± 0.10	125 ± 7
[5-Ornithine]-oxytocin	~0.07	~0.24	<0.04	~0.002	0.046 ± 0.0018	0.19

[a] Expressed in USP units/mg. [b] As percentage of crystalline deamino-oxytocin on a molar basis; see ref 15. [c] Values reported by W. Y. Chan and V. du Vigneaud, *Endocrinology*, **71**, 977 (1962). [d] Value reported by W. Y. Chan, M. O'Connell, and S. R. Pomeroy, *ibid.*, **72**, 279 (1963). [e] See ref 15.

(Table I) add further support to the contention (for a more detailed discussion see ref 14) that position 4 of oxytocin is less sensitive to structural alterations than position 5. Specifically, the substitution of the δ-carbonyl group in the glutamine residue by a methylene group reduced the rat pressor, rat antidiuretic, and toad bladder hydroosmotic potencies significantly more than the avian depressor, rat uterotonic, and rabbit milk-ejecting potencies. The ratio of avian depressor to rat uterotonic activity in [4-ornithine]-oxytocin is approximately 3:1, while the ratio in oxytocin for these activities is about 1:1. [5-Ornithine]-oxytocin has an extremely low order of biological activity in all assays studied. However, it is noteworthy that upon characterizing the amphibian hydroosmotic activity of [5-ornithine]-oxytocin by dose-response analyses in a newly designed toad bladder bioassay,[15] this analog—in spite of the reduction in potency by a factor of one million when compared to [8-arginine]-vasotocin, the natural water balance principle of the toad—still retained the same intrinsic activity as the hormone. Similar results were obtained previously with [5-valine]-oxytocin.[15] These findings indicate that the presence of the asparagine residue is necessary for the exhibition of an *appreciable degree* of biological activity; but, as demonstrated by the experiments on the toad bladder, the asparagine residue in position 5 of oxytocin is not *essential* for the manifestation of hydroosmotic and probably other hormonal activities.

Experimental Section[16]

N$^{\alpha}$-Carbobenzoxy-N$^{\delta}$-phthalyl-L-ornithyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. A solution of 4.1 g of N-carbobenzoxy-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide[6] in 16.5 ml of glacial acetic acid was treated with 20.5 ml of hydrogen bromide in glacial acetic acid (25%, w/w). After 45 min at room temperature, the solution was poured in 300 ml of cold, dry ether. The precipitated hydrobromide of the free base was washed by decantation with three 300-ml portions of cold ether. After being dried under vacuum over KOH and P_2O_5 overnight, the hydrobromide was dissolved in 120 ml of dry methanol and the resulting solution was passed through a column of strong base anion-exchange resin (Rexyn RGl, Fisher Scientific Co.) in hydroxyl cycle. Subsequently the column was washed with 50 ml of dry methanol. The filtrate was slightly basic and, after acidification with nitric acid, gave a negative test for halides with silver nitrate. The solid obtained after evaporation of the solvent from the eluate and the washings was dried for several hours under vacuum and then dissolved in 8.5 ml of dimethylformamide. To the cold solution, 2.92 g of *p*-nitrophenyl N$^{\alpha}$-carbobenzoxy-N$^{\delta}$-phthalyl-L-ornithinate[7] was added. The product was precipitated with 80 ml of ethyl acetate. The solid was collected by filtration and washed with five 80-ml portions of ethyl acetate and finally with a 100-ml portion of ether. The material was then dried to constant weight over P_2O_5 under vacuum at room temperature, giving 3.91 g, mp 240–241.5° dec, $[\alpha]^{26}D$ −67.1° (*c* 2, 97% formic acid).

Anal. Calcd for $C_{48}H_{59}N_9O_{11}S$: C, 59.4; H, 6.13; N, 13.1 Found: C, 59.3; H, 6.21; N, 13.0.

N-Carbobenzoxy-L-isoleucyl-N$^{\delta}$-phthalyl-L-ornithyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. A suspension of 3.78 g of the protected hexapeptide in 30 ml of glacial acetic acid was treated with 25 ml of 25% HBr in glacial acetic acid according to the procedure already described. After 1 hr, the hydrobromide of the free hexapeptide was isolated, dried, and dissolved in 300 ml of dry methanol. The solution was passed through a column of Rexyn RG1 (in hydroxyl cycle). After removal of the solvent from the eluate and washings, the residue was dried for several hours under vacuum over KOH. The material was taken up in 9 ml of dimethylformamide, and 1.53 g of *p*-nitrophenyl N-carbobenzoxy-L-isoleucinate was added. After 16 hr at room temperature, 100 ml of ethyl acetate was added. The product was collected by filtration and washed three times with 50-ml portions of absolute ethanol. After being dried under vacuum over P_2O_5 at room temperature, the product weighed 3.22 g, mp 236–238°, $[\alpha]^{26}D$ −68.8° (*c* 2, 97% formic acid).

Anal. Calcd for $C_{54}H_{70}N_{10}O_{12}S$: C, 59.9; H, 6.51; N, 12.9. Found: C, 59.6; H, 6.38; N, 12.8.

N-Carbobenzoxy-O-benzyl-L-tyrosyl-L-isoleucyl-N$^{\delta}$-phthalyl-L-ornithyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. The protected heptapeptide, 3.0 g, was suspended in 22.5 ml of glacial acetic acid and treated with 22 ml of 25% HBr in glacial acetic acid. After 45 min at room temperature, the hydrobromide of the free heptapeptide was isolated and dried as described before and then dissolved in 200 ml of dry methanol. The solution was then passed through a column of Rexyn RG1 (in hydroxyl cycle) and washed with an additional 200 ml of dry methanol. After removal of the solvent, the residue was taken up in 10 ml of dimethylformamide and allowed to react with 1.6 g of *p*-nitrophenyl N-carbobenzoxy-O-benzyl-L-tyrosinate. After 24 hr, 200 ml of ethyl acetate was added; the precipitate was collected and triturated five times with 100-ml portions of an ethanol–ether mixture (9:1). After being dried under vacuum at 25° for 12 hr, the compound weighed 2.96 g, mp 248–250°.

Anal. Calcd for $C_{70}H_{85}N_{11}O_{14}S$: C, 62.9; H, 6.41; N, 11.5. Found: C, 62.7; H, 6.44; N, 11.7.

N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-ornithyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. A suspension of 2.77 g of the preceding protected octapeptide in 25 ml of glacial acetic acid was treated with 25 ml of 25% HBr in glacial acetic acid. After 45 min the hydrobromide was precipitated with ether, repeatedly washed with the same solvent, and then dried in a desiccator. The hydrobromide salt was then dissolved in 7 ml of dimethylformamide and brought to pH 7.4 with ethylpiperidine. Then 1.1 g of *p*-nitrophenyl N-carbobenzoxy-S-benzyl-L-cysteinate was added to the mixture. After 2 days at room temperature the product was precipitated by addition of 300 ml of ethyl acetate. The precipitate was collected and washed five times with 50-ml portions of ethanol. After being dried under vacuum at room temperature for 20 hr, the material weighed 2.2 g, mp 244–245° dec, $[\alpha]^{23}D$ −59.6° (*c* 1, 97% formic acid).

(14) R. Walter, J. Rudinger, and I. L. Schwartz, *Am. J. Med.*, **42**, 663f (1967).

(15) P. Eggena, I. L. Schwartz, and R. Walter, *J. Gen. Physiol.*, **52**, 463 (1968).

(16) All melting points (mp) were determined with a Thomas–Hoover capillary melting point apparatus and are corrected. Optical rotations were determined with a Carl Zeiss photoelectric precision polarimeter, 0.005°. The samples for elementary analysis were dried for 12 hr at 100° over P_2O_5 under vacuum. Peptide hydrolysates were chromatographed on a Beckman-Spinco Model 120C amino acid analyzer, using Beckman custom research resin PA-28. The protected peptides were detected according to the procedure by Zahn and Rexroth [*Z. Anal. Chem.*, **148**, 181 (1955)] and the hormone analogs with cadmium-containing ninhydrin reagent.[17]

(17) A. J. Morris, *Biochem J.*, **81**, 606 (1961).

Anal. Calcd for $C_{73}H_{90}N_{12}O_{15}S_2$: C, 60.9; H, 6.30; N, 11.7. Found: C, 60.7; H, 6.20; N, 11.3.

To remove the phthalyl protecting group from the δ-amino function the fully protected nonapeptide (600 mg) was dissolved with heating in dimethylformamide (4 ml). A 1 *M* solution of hydrazine hydrate (1.2 ml) in dimethylformamide was added. After 3 hr, the mixture was acidified with acetic acid (0.6 ml), and the solvent was removed under vacuum. Water (4 ml) was added to the residue, and the next day the precipitate was collected and washed with water, sodium carbonate, and again water. The product was dried at 35° over P_2O_5 to a constant weight of 482 mg, mp 197–198°, $[\alpha]^{22}D$ −51.7° (*c* 1, 97% formic acid).

Anal. Calcd for $C_{65}H_{88}N_{12}O_{13}S_2$: N, 12.8. Found: N, 12.9.

[4-Ornithine]-oxytocin. To a solution of the preceding partially deprotected nonapeptide (0.4 g) in 250 ml of boiling anhydrous ammonia (freshly distilled from sodium) small amounts of sodium were added until a blue coloration persisted for 30 sec. The ammonia was subsequently removed by evaporation to a volume of 30 ml and the rest was removed by lyophilization. The slightly yellow residue was dissolved in 750 ml of water containing 0.20 ml of trifluoroacetic acid, the pH was adjusted to 8.2 by addition of 2 *N* ammonium hydroxide, and the resulting solution was treated with excess 0.01 *N* potassium ferricyanide. After 30 min the pH was adjusted to about 6.5 with dilute trifluoroacetic acid, and the ferrocyanide and excess ferricyanide ions were removed by treatment of the solution with AG3x4 resin (Bio-Rad Laboratories) in the chloride form. The solution obtained after removal of the resin was lyophilized. The resulting powder was taken up in 5 ml of the upper phase of the solvent system 1-butanol–ethanol–pyridine–0.1 *N* acetic acid (4:1:1:7) and applied on a column of Sephadex G-25 (100–200 mesh) of 6.37-cm² cross section and 87-cm length that had been equilibrated with both solvent phases according to the procedure of Yamashiro.[10,11] The column was eluted with the organic phase, and 60 10.5-ml fractions were collected. Folin–Lowry color values[18] of aliquots from every second fraction were plotted; the fractions corresponding to the principal peak (R_f 0.33) were pooled, and 300 ml of water was added. To remove the organic solvent the mixture was concentrated to 50 ml and lyophilized to give 125 mg of product. This material was again dissolved in 5 ml of the upper phase of the above-described solvent system and placed on a Sephadex G-25 (100–200 mesh) column of 6.37-cm² cross section and 60-cm length that had been equilibrated with upper and lower phase of the solvent system. After elution with upper phase the Folin–Lowry color values showed a single symmetrical peak having an R_f of 0.33. The amount of [4-ornithine]-oxytocin isolated from the fractions corresponding to this peak was 114 mg; $[\alpha]^{23}D$ −34.7° (*c* 0.5, 1 *N* acetic acid). When the analog was subjected to paper electrophoresis in 6% acetic acid it traveled as a single spot (8.7 cm/0.67 hr, 2600 V). It also migrated as a single component upon chromatography on silica gel G (R_f 0.30) and Whatman No. 1 paper (R_f 0.32) in the upper phase of the system 1-butanol–acetic acid–water (4:1:5). For elementary analysis a sample was lyophilized from 1 *N* acetic acid.

Anal. Calcd for $C_{43}H_{68}N_{12}O_{11}S_2\cdot(C_2H_4O_2)_2$: C, 50.7; H, 6.88; N, 15.1; O, 21.6. Found: C, 50.6; H, 6.69; N, 15.3; O, 21.9.

A sample was hydrolyzed in 6 *N* HCl under vacuum at 110° for 22 hr and analyzed for ninhydrin-active material according to Spackman, Stein, and Moore.[19] The following molar ratios of amino acids and ammonia were obtained with glycine being taken as 1.00: aspartic acid, 1.02; proline, 1.03; glycine, 1.00; cystine, 1.01; isoleucine, 1.04; leucine, 1.03; tyrosine, 0.94; ornithine, 1.02; and ammonia, 2.16.

N$^\alpha$-Carbobenzoxy-N$^\delta$-tosyl-L-ornithyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. Method A. A solution of 1.8 g of L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide[6] in 5 ml of dimethylformamide was allowed to react with 1.93 g of pentachlorophenyl N$^\alpha$-carbobenzoxy-N$^\delta$-tosyl-L-ornithinate.[12] After 16 hr at room temperature, 100 ml of ethyl acetate was added. The resulting precipitate was collected and washed repeatedly with a ethyl acetate–ethanol mixture. The material dried under vacuum at 25° weighed 2.15 g, mp 199–202°, $[\alpha]^{24}D$ −67.0° (*c* 2, 97% formic acid).

Anal. Calcd for $C_{47}H_{63}N_9O_{11}S_2$: C, 56.8; H, 6.39; N, 12.7. Found: C, 56.6; H, 6.47; N, 12.5.

Method B. A portion of the above-described free pentapeptide (0.443 g) dissolved in 0.9 ml of dimethylformamide was allowed to react with 0.367 g of 1-tosyl-3-carbobenzoxyamino-2-piperidone[12] at 90–100° under anhydrous conditions. The progress of the reaction was followed by thin layer chromatography on silica gel G in the solvent system chloroform–methanol (8:2, v/v); the hexapeptide prepared by method A served as a reference. After 5 days, 100 ml of water was added to the residue which was then collected and washed with ethyl acetate. The hexapeptide was applied onto an acidic alumina column and eluted with a chloroform–methanol mixture (4:1, v/v). After evaporation of the solvent the resulting white powder was dried under vacuum over P_2O_5 at 25°. The hexapeptide (0.1 g) exhibited the following physical properties: mp 199–202°, $[\alpha]^{23}D$ −64.7° (*c* 0.9, 97% formic acid). The material traveled as a single spot upon thin layer chromatography on silica gel G in the solvent system chloroform–methanol (8:2, v/v) and possessed the same R_f as the hexapeptide prepared according to method A.

N-Carbobenzoxy-L-isoleucyl-N$^\delta$-tosyl-L-ornithyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. Free hexapeptide, prepared from 2.05 g of the protected hexapeptide as already described, was taken up in 4.5 ml of dimethylformamide and was allowed to react with 0.88 g of *p*-nitrophenyl N-carbobenzoxy-L-isoleucinate. After 16 hr, 200 ml of ethyl acetate was added; the precipitate was collected and washed with ethyl acetate and then absolute ethanol. After being dried under vacuum over P_2O_5, the product weighed 1.2 g, mp 232–234°, $[\alpha]^{21}D$ −68.1° (*c* 2, 97% formic acid).

Anal. Calcd for $C_{53}H_{74}N_{10}O_{12}S_2$: C, 57.5; H, 6.74; N, 12.7. Found: C, 57.3; H, 6.90; N, 12.9.

N-Carbobenzoxy-O-benzyl-L-tyrosyl-L-isoleucyl-N$^\delta$-tosyl-L-ornithyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. The protected heptapeptide (1.2 g) was decarbobenzoxylated as described and allowed to react with 0.7 g of *p*-nitrophenyl N-carbobenzoxy-O-benzyl-L-tyrosinate. After 16 hr, 200 ml of ethyl acetate was added; the precipitate was collected and triturated five times with 100-ml portions of an ethanol–ethyl acetate mixture (9:1, v/v). After being dried under vacuum at room temperature, the compound weighed 1.1 g, mp 234–236° dec, $[\alpha]^{25}D$ −50.8° (*c* 2, 97% formic acid).

Anal. Calcd for $C_{69}H_{89}N_{11}O_{14}S_2$: C, 60.9; H, 6.59; N, 11.3. Found: C, 60.7; H, 6.48; N, 11.3.

N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-N$^\delta$-tosyl-L-ornithyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. A suspension of 1.0 g of the preceding octapeptide in 7 ml of glacial acetic acid was treated with 6 ml of 25% HBr in glacial acetic acid. After 2 hr, the hydrobromide was precipitated with dry ether and repeatedly washed with the same solvent. The white, dry powder was dissolved in 5 ml of dimethylformamide and the mixture was then cooled to 0°. The pH was adjusted to 7.5 with N-methylmorpholine and then the peptide was allowed to react with 0.425 g of *p*-nitrophenyl N-carbobenzoxy-S-benzyl-L-cysteinate. After 2 days at room temperature, 125 ml of ethyl acetate was added. The mixture was triturated, filtered, and washed with ethyl acetate–ether (7:3, v/v) and ethanol–ether (1:1, v/v). After drying at 100° over P_2O_5, the material weighed 0.826 g, mp 226–227°, $[\alpha]^{23}D$ −56.5° (*c* 1, 97% formic acid).

Anal. Calcd for $C_{72}H_{94}N_{12}O_{15}S_3$: C, 59.1; H, 6.47; N, 11.5. Found: C, 59.1; H, 6.34; N, 11.5.

[4-Ornithine]-oxytocin. The procedure for the reduction of the preceding protected nonapeptide (0.29 g) with sodium in boiling anhydrous ammonia, the oxidation of the disulfhydryl peptide, the deionization of the solution with AG3x4 resin, and the purification by partition chromatography were the same as in the preparation of [4-ornithine]-oxytocin described above. The amount of analog isolated (R_f 0.33) was 88 mg; $[\alpha]^{23}D$ −35.2° (*c* 0.5, 1 *N* acetic acid); the compound behaved as a single component upon chromatography on silica gel G and on paper with identical R_f values as the [4-ornithine]-oxytocin described above. The material was also homogeneous upon paper electrophoresis in 6% acetic acid.

N-Carbobenzoxy-N$^\delta$-phthalyl-L-ornithyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. A solution of 3.0 g of the free tetrapeptide S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide[13] was dissolved in 20 ml of dimethylformamide and then allowed to react with 2.54 g of *p*-nitrophenyl N$^\alpha$-carbobenzoxy-N$^\delta$-phthalyl-L-ornithinate. After 24 hr, 100 ml of ethyl acetate was added, and the precipitate was collected and washed with ethyl acetate and finally with ether. The material was then dried to a constant

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

(19) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

weight over P_2O_5 under vacuum at 25°, giving 3.45 g, mp 229–231°, $[\alpha]^{24}D$ −64.4° (*c* 2, 97% formic acid).

Anal. Calcd for $C_{44}H_{53}N_7O_9S$: C, 61.7; H, 6.24; N, 11.5. Found: C, 61.6; H, 6.23; N, 11.6.

N-Carbobenzoxy-L-glutaminyl-N$^\delta$-phthalyl-L-ornithinyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. A solution of 3.2 g of the preceding protected pentapeptide in 32 ml of glacial acetic acid was treated with 12 ml of 30% HBr in glacial acetic acid. The free pentapeptide amide, obtained as described previously, was dissolved in 4 ml of dimethylformamide. It was then treated with 1.5 g of *p*-nitrophenyl N-carbobenzoxy-L-glutaminate. After 16 hr, 100 ml of ethyl acetate was added, and the precipitate was washed with ethyl acetate, ethanol, and ether. After being dried under vacuum over P_2O_5 at room temperature, the product weighed 3.09 g, mp 236–239°, $[\alpha]^{24}D$ −64.7° (*c* 2, 95% formic acid).

Anal. Calcd for $C_{49}H_{61}N_9O_{11}S$: C, 59.8; H, 6.25; N, 12.8. Found: C, 59.6; H, 6.29; N, 13.0.

N-Carbobenzoxy-L-isoleucyl-L-glutaminyl-N$^\delta$-phthalyl-L-ornithinyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. The free hexapeptide, prepared according to the procedure described above from 3.19 g of the preceding protected hexapeptide, was dissolved in 8 ml of dimethylformamide and then allowed to react with 1.4 g of *p*-nitrophenyl N-carbobenzoxy-L-isoleucinate. After 24 hr, 100 ml of ethyl acetate was added, and the resulting precipitate was isolated and washed with ethyl acetate and finally with absolute ethanol. After being dried under vacuum over P_2O_5 at 28°, the product weighed 2.76 g, mp 216–218°, $[\alpha]^{24}D$ −69.05° (*c* 2, 97% formic acid).

Anal. Calcd for $C_{55}H_{72}N_{10}O_{12}S$: C, 60.6; H, 6.70; N, 12.8. Found: C, 60.3; H, 6.66; N, 12.8.

N-Carbobenzoxy-O-benzyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-N$^\delta$-phthalyl-L-ornithinyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. The free heptapeptide, obtained from 2.49 g of protected heptapeptide according to the method described above, was dissolved in 11 ml of dimethylformamide and 1.3 g of *p*-nitrophenyl N-carbobenzoxy-O-benzyl-L-tyrosinate was added to the solution. After 16 hr, the product was precipitated by the addition of 200 ml of ethyl acetate; the precipitate was collected and triturated five times with 100-ml portions of an ethanol–ethyl acetate mixture (9:1, v/v). After being dried under vacuum over P_2O_5 the product weighed 2.25 g, mp 252–253°, $[\alpha]^{23}D$ −50.2° (*c* 1, 97% formic acid).

Anal. Calcd for $C_{71}H_{87}N_{11}O_{14}S$: C, 63.1; H, 6.56; N, 11.4. Found: C, 63.0; H, 6.40; N, 11.1.

N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-ornithinyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. The preceding protected octapeptide (2.1 g) was decarbobenzoxylated and freed of HBr in the usual manner. The residue obtained from the methanol solution was dissolved in 5 ml of dimethylformamide and allowed to react with 0.8 g of *p*-nitrophenyl N-carbobenzoxy-S-benzyl-L-cysteinate. After 2 days at room temperature the product was precipitated by addition of 200 ml of ethyl acetate, collected, and washed with ethanol and then ether. After being dried under vacuum at room temperature the material weighed 0.72 g, mp 232–235°.

Anal. Calcd for $C_{74}H_{92}N_{12}O_{15}S_2$: C, 61.1; H, 6.44; N, 11.6. Found: C, 60.9; H, 6.24; N, 11.5.

The above fully protected nonapeptide (0.226 g) was dissolved with heating in dimethylformamide (1.6 ml). A molar solution of hydrazine hydrate (0.48 ml) in dimethylformamide was added. After 3 hr, the mixture was acidified with acetic acid (0.3 ml), and the solvent was removed under reduced pressure. Water (1.6 ml) was added to the residue, and the next day the precipitate was collected and washed with water, sodium carbonate, and water. The product was dried at 35° over P_2O_5 to a constant weight of 0.2 g, mp 185–189°, $[\alpha]^{23}D$ −59.9° (*c* 1, 97% formic acid).

Anal. Calcd for $C_{66}H_{90}N_{12}O_{13}S_2$: C, 59.9; H, 6.85; N, 12.7. Found: C, 59.6; H, 6.97; N, 12.7.

[5-Ornithine]-oxytocin. The preceding nonapeptide (0.2 g) was reduced, oxidized, deionized, and lyophilized, similarly to that described for [4-ornithine]-oxytocin. The resulting powder was taken up in 5 ml of the upper phase of the solvent system 1-butanol–ethanol–pyridine–0.1 *N* acetic acid (4:1:1:7) and put on a column of Sephadex G-25 (100–200 mesh) of 6.37-cm² cross section and 86-cm length that had been equilibrated with both phases. Folin–Lowry color values indicated a principal peak with R_f 0.41 and fractions corresponding to this peak were pooled, stripped of solvents under reduced pressure, and lyophilized to give 50 mg of product. This material was then dissolved in 5 ml of the upper phase of the above-described solvent system and placed on a Sephadex G-25 (100–200 mesh) column of 6.37-cm² cross section and 68-cm length that had been equilibrated with both phases of the solvent system. The Folin–Lowry color values showed a single symmetrical peak (R_f 0.43). The amount of [5-ornithine]-oxytocin isolated from the fractions corresponding to this peak was 42 mg; $[\alpha]^{23}D$ −34.5° (*c* 0.5, 1 *N* acetic acid). When the analog was subjected to paper electrophoresis in 6% acetic acid it traveled as a single spot (8.5 cm/0.67 hr, 2600 V) and also migrated as a single component upon chromatography on silica gel G (R_f 0.20) and Whatman No. 1 paper (R_f 0.32) in the upper phase of the system 1-butanol–acetic acid–water (4:1:5). For elementary analysis a sample was lyophilized from water.

Anal. Calcd for $C_{44}H_{72}N_{12}O_{11}S_2 \cdot C_2H_4O_2$: C, 51.7; H, 7.16; N, 15.7; O, 19.5. Found: C, 51.5; H, 6.98; N, 15.6; O, 19.0.

A sample was hydrolyzed in 6 *N* HCl under vacuum at 110° for 22 hr and analyzed for ninhydrin-active components. The following molar ratios of amino acids and ammonia were obtained with glycine taken as 1.00: glutamic acid, 1.05; proline, 1.13; glycine, 1.00; cystine, 1.01; isoleucine, 1.03; leucine, 1.05; tyrosine, 0.92; ornithine, 1.13; and ammonia, 2.08.

Bioassay Methods. Avian vasodepressor assays were performed on conscious chickens according to the procedure employed by Munsick, Sawyer, and van Dyke.[20] Oxytocic assays were performed on isolated uteri from rats in natural estrus according to the method of Holton,[21] modified by Munsick[22] with the use of magnesium-free van Dyke–Hastings solution as the bathing fluid. Rat pressor assays were carried out on atropinized, urethan-anesthetized male rats as described in the United States Pharmacopeia.[23] Assays for antidiuretic activity were performed on anesthetized, hydrated male rats according to the method of Jeffers, Livezey, and Austin[24] as modified by Sawyer.[25] Milk-ejecting activity in the anesthetized, lactating rabbit was determined according to the method of Van Dyke, Adamsons, and Engel[26] as modified by Chan.[27] Assays for hydroosmotic activity were performed on toad bladders (*Bufo marinus*, L.) according to the method by Bentley[28] as modified by Eggena, Schwartz, and Walter.[15] The biological activities were measured against the USP Posterior Pituitary Reference Standard.

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(20) R. A. Munsick, W. H. Sawyer, and H. B. van Dyke, *Endocrinology*, **66**, 860 (1960).
(21) P. Holton, *Brit. J. Pharmacol.*, **3**, 328 (1948).
(22) R. A. Munsick, *Endocrinology*, **66**, 451 (1960).
(23) "The Pharmacopeia of the United States," 17th Revision, Mack Publishing Company, Easton, Pa., 1965, p 749.
(24) W. A. Jeffers, M. M. Livezey, and J. H. Austin, *Proc. Soc. Exptl. Biol. Med.*, **50**, 184 (1942).
(25) W. H. Sawyer, *Endocrinology*, **63**, 694 (1958).
(26) H. B. Van Dyke, K. Adamsons, Jr., and S. L. Engel, *Recent Progr. Hormone Res.*, **11**, 1 (1955).
(27) W. Y. Chan, *J. Pharmacol. Exptl. Therap.*, **147**, 48 (1965).
(28) P. J. Bentley, *J. Endocrinol.*, **17**, 201 (1958).