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# Synthesis and Biological Properties of 9-Sarcosine Oxytocin[1]

WILLIAM D. CASH, LOGAN McCULLOCH MAHAFFEY, ALFRED S. BUCK, DONALD E. NETTLETON, JR., CHRISTOS ROMAS, AND VINCENT DU VIGNEAUD

*Department of Biochemistry, Cornell University Medical College, New York, N. Y*

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The analog of oxytocin containing sarcosinamide in position 9 in the place of glycinamide was synthesized. The protected nonapeptide S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylsarcosinamide was prepared by the dicyclohexylcarbodiimide coupling of the appropriate pentapeptide and tetrapeptide derivatives. The protected nonapeptide was converted to 9-sarcosine oxytocin by removing the protecting groups with sodium in liquid ammonia and aerating an aqueous solution of the resulting sulfhydryl peptide to form the cyclic disulfide. Oxytocin itself was prepared by an analogous series of reactions. Both peptides were purified by countercurrent distribution. The substitution of sarcosinamide for glycinamide in the oxytocin molecule, which is in effect the substitution of a methyl group for the hydrogen atom on the imino nitrogen of the terminal peptide bond in the side chain, markedly reduced but did not completely eliminate the biological effects of the hormone. Furthermore, the various biological properties were affected to different extents.

The analog of oxytocin containing sarcosinamide in position 9 in the place of glycinamide was synthesized as part of a continuing effort to probe the relationship between the chemical structures of the posterior pituitary hormones oxytocin and vasopressin and their biological activities. The substitution of sarcosinamide for glycinamide is in effect the substitution of a methyl group for the hydrogen atom on the imino nitrogen of the peptide bond between leucine and

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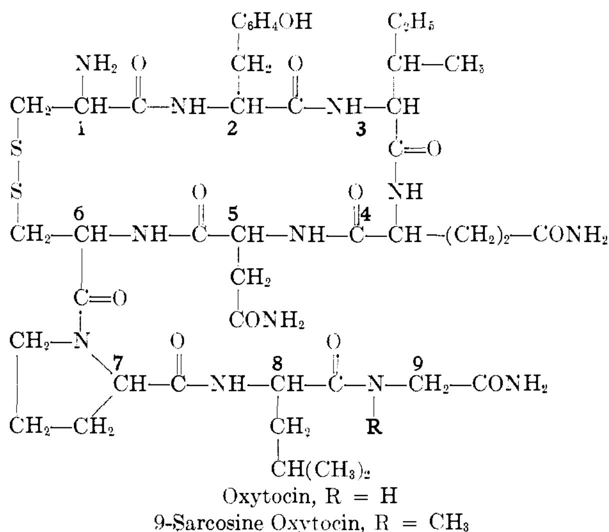
glycinamide in the oxytocin side chain. The synthesis of the 9-



Oxytocin, R = H
9-Sarcosine Oxytocin, R = $CH_3$

sarcosine analog was undertaken with the hope of gaining some indication of the relationship of this particular hydrogen atom to the biological properties of the hormone.[2]

The key intermediate in the synthesis was the protected nonapeptide S - benzyl - N - tosyl - L - cysteinyl - L - tyrosyl - L - isoleucyl - L-glutaminyl - L - asparaginyl - S - benzyl - L - cysteinyl - L - prolyl - L-leucylsarcosinamide. This intermediate was synthesized by condensing with the aid of dicyclohexylcarbodiimide[3] the protected pentapeptide S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparagine with the decarbobenzoxylation product of the protected tetrapeptide S-benzyl-N-carbobenzoxy-L-cysteinyl-L-prolyl-L-leucylsarcosinamide. The protected pentapeptide in turn was prepared by the mixed anhydride procedure[4] from S-benzyl-N-tosyl-L-cysteinyl-L-tyrosine[5,6] and L-isoleucyl-L-glutaminyl-L-asparagine.[7] The synthesis of the protected tetrapeptide involved the preparation of the protected tripeptide carbobenzoxy-L-prolyl-L-leucylsarcosinamide. The latter compound was prepared by the mixed anhydride

(2) When the synthesis of the 9-sarcosine analog of oxytocin was well underway, the synthesis of the corresponding analog of lysine-vasopressin was undertaken. The synthesis and some of the biological properties of 9-sarcosine lysine-vasopressin already have been reported [J. Meienhofer and V. du Vigneaud, *J. Am. Chem. Soc.*, **83**, 142 (1961)].

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

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

(5) J. Honzl and J. Rudinger, *Collection Czechoslov. Chem. Communs.*, **20**, 1190 (1955).

(6) V. du Vigneaud, M. F. Bartlett, and A. Jöhl, *J. Am. Chem. Soc.*, **79**, 5572 (1957).

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

method from carbobenzoxy-L-prolyl-L-leucine[8,9] and sarcosinamide hydrobromide. It was also prepared by using ethyl sarcosinate hydrochloride instead of sarcosinamide hydrobromide and then subjecting the resulting protected tripeptide ester to treatment with ammonia in methanol. The carbobenzoxy group was removed from the protected tripeptide with hydrogen and palladium-charcoal catalyst. The resulting free tripeptide reacted with *p*-nitrophenyl S-benzyl-N-carbobenzoxy-L-cysteinate[7] to form the protected tetrapeptide.

9-Sarcosine oxytocin was prepared by removing the protecting groups from the protected nonapeptide with sodium in liquid ammonia and converting the resulting sulfhydryl peptide into the cyclic disulfide by aeration in dilute aqueous solution.[10] The crude product was purified by countercurrent distribution. The purified material was shown to be homogeneous by paper chromatography and paper electrophoresis. Elementary analysis and analysis for amino acids and ammonia gave the expected results for 9-sarcosine oxytocin.

At the time these experiments were begun a protected nonapeptide intermediate for oxytocin had not been synthesized by coupling a pentapeptide and a tetrapeptide. In order to be sure that oxytocin itself could be prepared without difficulty by this approach, the unmodified hormone was synthesized along with the 9-sarcosine analog. The protected nonapeptide S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide, previously prepared by a different pathway,[11] was synthesized by the dicyclohexylcarbodiimide coupling of the protected pentapeptide and S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide.[12,13] The protected nonapeptide was successfully converted to oxytocin by removal of the protecting groups with sodium in liquid ammonia and subsequent aeration of the sulfhydryl peptide. No difficulty was encountered in purification of the hormone by countercurrent distribution. The avian depressor activity of the purified product was in the range of 500 U.S.P. units per mg., a value in close agreement with the reported avian depressor activity of highly purified oxytocin of both natural and synthetic origin.[7,10,14,15]

(8) G. W. Anderson and F. M. Callahan, *J. Am. Chem. Soc.*, **82**, 3359 (1960).

(9) W. D. Cash, *J. Org. Chem.*, **26**, 2136 (1961).

(10) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, and P. G. Katsoyannis, *J. Am. Chem. Soc.*, **76**, 3115 (1954).

(11) J. Rudinger, J. Honzl, and M. Zaoral, *Collection Czechoslov. Chem. Communs.*, **21**, 202 (1956).

(12) C. Ressler and V. du Vigneaud, *J. Am. Chem. Soc.*, **76**, 3107 (1954).

(13) M. Zaoral and J. Rudinger, *Collection Czechoslov. Chem. Communs.*, **20**, 1183 (1955).

(14) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

(15) J. G. Pierce, S. Gordon, and V. du Vigneaud, *J. Biol. Chem.*, **199**, 929 (1952).

9-Sarcosine oxytocin showed detectable activity when assayed for each of the following biological effects: depression of blood pressure in the chicken,[16] contraction of the isolated rat uterus in the absence of magnesium,[17,18] increase in rabbit mammary gland pressure,[19,20] increase in blood pressure of the rat[21] and inhibition of diuresis in the hydrated rat.[22,23] However, in all of these assays the activity of 9-sarcosine oxytocin was only a small fraction of the activity of oxytocin itself. The 9-sarcosine analog was more active in the rabbit mammary gland pressure assay than in either of the other four assays. It exhibited about one-ninth of the activity of oxytocin when tested for this effect. In contrast to oxytocin which shows as much avian depressor and uterine-contracting activity as milk let-down activity,[20,24] 9-sarcosine oxytocin showed only about one-fifth as much avian depressor and about two-thirds as much uterine-contracting as milk let-down activity. The activity of 9-sarcosine oxytocin in the rat pressor and rat antidiuretic assays was very much lower than in the three assays just described. Oxytocin itself exhibits very low pressor and antidiuretic activity in relation to its milk let-down, avian depressor and uterine-contracting activity.[20,24] However, while pressor and antidiuretic activities of oxytocin are about the same,[24] the pressor activity of 9-sarcosine oxytocin was about one-tenth of its antidiuretic activity.

Thus the substitution of the methyl group for the hydrogen atom attached to the imino nitrogen of the peptide bond between leucine and glycinamide in the oxytocin side chain markedly reduces the biological activity of the hormone but does not eliminate completely any of the five biological actions studied. Furthermore, the various biological effects of oxytocin are lowered to different extents. It appears, then, that the modified structure still possesses the fundamental properties of the oxytocin molecule that enable it to exert its biological effects, but for some reason the new structure is less capable of exerting these effects. It remains to be established whether this lowered capacity to produce the biological actions of oxytocin is attributable to the absence of the hydrogen atom *per se* or instead to the presence of the newly introduced methyl group. This point may

(16) R. A. Munsick, W. H. Sawyer, and H. B. van Dyke, *Endocrinology*, **66**, 860 (1960).
(17) P. Holton, *Brit. J. Pharmacol.*, **3**, 328 (1948).
(18) R. A. Munsick, *Endocrinology*, **66**, 451 (1960).
(19) B. A. Cross and G. W. Harris, *J. Endocrinol.*, **8**, 148 (1952).
(20) H. B. van Dyke, K. Adamsons, Jr., and S. L. Engel, *Recent Progr. in Hormone Research*, **11**, 1 (1955).
(21) "The Pharmacopeia of the United States of America," 16th Revision, Mack Printing Co., Easton, Pa., 1960, p. 793.
(22) W. A. Jeffers, M. M. Livezy, and J. H. Austin, *Proc. Soc. Exp. Biol. Med.*, **50**, 184 (1942).
(23) W. H. Sawyer, *Endocrinology*, **63**, 694 (1958).
(24) R. A. Boissonnas and St. Guttmann, *Helv. Chim. Acta*, **43**, 190 (1960).

be clarified by further studies with 9-sarcosine oxytocin along chemical as well as biological lines and by the synthesis and study of additional analogs.

## Experimental[25]

**S-Benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparagine** (I).—S-Benzyl-N-tosyl-L-cysteinyl-L-tyrosine[5,6] (5.3 g.) was dissolved in tetrahydrofuran (65 ml.). Triethylamine (1.7 ml.) was added and the solution was cooled to $-10°$. Isobutyl chloroformate (1.4 g.) in tetrahydrofuran (55 ml.) was added. The mixture was stirred at $-10°$ for 20 min. Then a solution of L-isoleucyl-L-glutaminyl-L-asparagine[7] (3.7 g.) and triethylamine (1.8 ml.) in water (25 ml.) was added. Stirring was continued without further cooling for 2 hr. Water (50 ml.) was added and the mixture was acidified by the slow addition of concd. hydrochloric acid. Tetrahydrofuran was removed *in vacuo*. The solid product was filtered off and washed with *N* HCl (100 ml.) and water (100 ml.); wt. 7.2 g.

Material at this stage of preparation from two identical experiments (12.7 g.) was dissolved in glacial acetic acid (180 ml.) and ethyl acetate (520 ml.) was added. The precipitated product was filtered off and washed with ethyl acetate (200 ml.); wt. 7.1 g., m.p. 239–241° (dec.), $[\alpha]^{21}_{D} + 5.1°$ (*c* 1.00, dimethylformamide).

*Anal.* Calcd. for $C_{41}H_{53}N_7O_{11}S_2$: C, 55.7; H, 6.04; N, 11.1. Found: C, 55.4; H, 5.99; N, 10.9.

**S-Benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide** (II).—S-Benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide[12,13] (0.60 g.) and dicyclohexylcarbodiimide (0.62 g.) were dissolved in dimethylformamide (6 ml.) and the resulting solution was cooled to 5°. Protected pentapeptide I (0.88 g.) was added. The mixture was stirred at 5° for 1 hr. and at room temperature for 3 days. The reaction mixture was then cooled to 5° and glacial acetic acid (0.61 ml.) was added. Stirring was continued for 20 min. Then water (65 ml.) was added. The precipitated solid was filtered off, washed with water (75 ml.) and dried *in vacuo* over phosphorus pentoxide. It was then finely powdered and washed thoroughly with propanol (100 ml.); wt. 1.10 g. This material was dissolved in dimethylformamide (20 ml.) and precipitated by the addition of water (125 ml.). The precipitated solid was filtered off and washed with water (75 ml.); wt. 1.10 g. It was dissolved in dimethylformamide (16 ml.) and precipitated again by adding propanol (64 ml.) and hexane (16 ml.). The product was filtered off and washed with propanol (20 ml.) and hexane (30 ml.); wt. 0.73 g., m.p. 238–241° (dec.), $[\alpha]^{20}_{D} -33.2°$ (*c* 1.00, dimethylformamide); lit.[11] m.p. 242–244°.

*Anal.* Calcd. for $C_{64}H_{86}N_{12}O_{14}S_3$: C, 57.2; H, 6.45; N, 12.5. Found: C, 57.3; H, 6.51; N, 12.5.

**Oxytocin.**—Protected nonapeptide II (297 mg.) was dissolved in anhydrous liquid ammonia (120 ml.) which was at its boiling point. Very small pieces of sodium were added one at a time until a blue color persisted for 10 min. Ammonium chloride (200 mg.) was added. The ammonia then was removed *in vacuo* from the frozen state. Water (500 ml.) was added to the dry residue and the pH

(25) All melting points were determined in capillary tubes and are corrected. Units of biological activity are based upon the current U.S.P. Standard Powder which was used as the standard in all the biological assays.

of the resulting solution was adjusted to approximately 6.5 by the addition of dilute acetic acid. The solution was extracted 4 times with 60-ml. portions of ether and the ether extracts were discarded. Air which had been passed through concentrated sodium hydroxide solution was bubbled through the solution at pH 6.5 for 1 hr. The pH was adjusted to approximately 3.5 by the addition of glacial acetic acid. Assay of an aliquot of this solution indicated a yield of approximately 250 units of avian depressor activity per mg. of protected nonapeptide II.

In order to remove salts the acidified solution was passed through an Amberlite IRC-50 (XE-64) column (0.9 × 39 cm) in the $H^+$ form.[26] Then the column was washed with 0.1% acetic acid (100 ml.). The peptide was eluted with a 30% pyridine–4% acetic acid solution. Five-ml. portions of eluate were collected and aliquots were analyzed for peptide content by the Folin–Lowry color reaction.[27] The peptide-containing fractions were combined and concentrated *in vacuo* to a viscous liquid residue. Ethanol (6 ml.) was added to the residue and the resulting suspension was filtered. The solid was washed with ethanol (2 ml.). Ethyl acetate (240 ml.) was added to the combined filtrate and washings. The mixture was allowed to stand for 2 hr. The precipitated solid was filtered off, washed with ethyl acetate (60 ml.), quickly placed in a desiccator and dried *in vacuo* over calcium chloride; wt. 126 mg. Assay of a small portion indicated an avian depressor activity of approximately 330 units per mg.

This material was dissolved in 10 ml. of lower phase of the solvent system butanol-ethanol-0.05% acetic acid (4:1:5). The resulting solution was placed in the first 2 tubes of a 200-tube countercurrent distribution apparatus and subjected to 500 transfers in the solvent system just mentioned. Analysis by the Folin–Lowry color reaction indicated a single symmetrical peak with a $K$ of approximately 0.41. The contents of Tubes 130–170 were removed, combined and concentrated in a rotary evaporator to a volume of about 25 ml. The concentrated solution was lyophilized to a white fluffy powder; wt. 83 mg.

The product was assayed repeatedly against the current U. S. P. standard powder for avian depressor activity following the assay directions in the fifteenth revision of the United States Pharmacopeia.[28] These values and their 95% confidence limits expressed as U. S. P. units per mg. of oxytocin were obtained: 575 (525–650), 449 (421–478), 492 (450–525), 475 (411–517) and 472 (427–548).[29]

A portion of the product was applied to strips of Whatman No. 1 paper and subjected to descending chromatography in two solvent systems. The chromatograms were stained with the brom phenol blue–mercuric chloride reagent.[30] The sample of oxytocin traveled as a single spot in butanol–acetic acid–water (4:1:5) ($R_f$ 0.56) and pyridine–acetic acid–water (50:35:15) ($R_f$ 0.82).

Another portion of the product was applied to a strip of Whatman No. 1 paper and subjected to electrophoresis for 18 hr. at 300 v. and 2° in pyridine acetate

(26) H. B. F. Dixon and M. P. Stack-Dunne, *Biochem. J.*, **61**, 483 (1955).

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

(28) "The Pharmacopeia of the United States of America," 15th Revision, Mack Printing Co., Easton, Pa., 1955, p. 555.

(29) These assays were performed during the first 3 months after the oxytocin preparation was completed. After storing the hormone in a desiccator under reduced pressure over phosphorus pentoxide at 5° for 15 months, the activity declined to 384 U.S.P. units per mg. (standard deviation ± 8). Further work on this problem of a loss in biological activity of oxytocin during long periods of storage is under way in this laboratory.

(30) E. L. Durrum, *J. Am. Chem. Soc.*, **72**, 2943 (1950).

buffer at pH 5.6. The paper was stained with the brom phenol blue-mercuric chloride reagent. The oxytocin traveled as a single spot toward the cathode.

A portion of the product was hydrolyzed in 6 $N$ hydrochloric acid at 110° for 17 hr. and the amino acid content of the hydrolysate was determined.[31,32] These expected molar ratios of amino acids and ammonia were obtained (with the value of leucine taken as 1.0): leucine 1.0, isoleucine 0.9, tyrosine 0.9, proline 1.2, glutamic acid 1.0, aspartic acid 1.2, glycine 1.2, cystine 0.9, and ammonia 3.2.

For elementary analysis a sample of the product was heated at 100° *in vacuo* over phosphorus pentoxide until it reached a constant weight. A loss in weight of 7% was observed. The results of the analysis agreed with the calculated values for the free base of oxytocin.

*Anal.* Calcd. for $C_{43}H_{66}N_{12}O_{12}S_2$: C, 51.3; H, 6.60; N, 16.7. Found: C, 50.9; H, 6.65; N, 16.5.

**Carbobenzoxysarcosinamide.**—Carbobenzoxysarcosine[33,34] (20.1 g.) was dissolved in tetrahydrofuran (140 ml.). Triethylamine (15.0 ml.) was added and the resulting solution was cooled to −10°. Isobutyl chloroformate (12.3 g.) in tetrahydrofuran (60 ml.) was added. The mixture was stirred at −10° for 20 min. Then 15 $N$ ammonium hydroxide (17 ml.) and water (20 ml.) were added. Stirring was continued without further cooling for 2 hr. Tetrahydrofuran was removed *in vacuo*. The residue was diluted with water to a total volume of 100 ml. and the solution was allowed to stand at 2° for 3 days. The crystalline product was filtered off and washed with cold water; wt. 9.0 g., m.p. 79–81°.

*Anal.* Calcd. for $C_{11}H_{14}N_2O_3$: C, 59.4; H, 6.35; N, 12.6. Found: C, 59.4; H, 6.40; N, 12.7.

The combined filtrate and washings from the last step were extracted 4 times with 35-ml. portions of ethyl acetate. The ethyl acetate solution was dried over anhydrous magnesium sulfate and filtered. Ethyl acetate was removed *in vacuo* and hexane was added to the residue. The crystalline material was filtered off; wt. 3.7 g. It was recrystallized from ethyl acetate (15 ml.) and hexane (15 ml.); wt. 3.1 g., m.p. 77–79°.

**Sarcosinamide Hydrobromide.**—Carbobenzoxysarcosinamide (8.0 g.) was dissolved in 4 $N$ hydrogen bromide in glacial acetic acid (25 ml.). The solution was allowed to stand at room temperature for 30 min. and then ether (400 ml.) was added. The crystalline product was filtered off and washed with ether; wt. 6.2 g. It was recrystallized from 90% ethanol (105 ml.) and ether (300 ml.); wt. 5.6 g., m.p. 162–163°.

*Anal.* Calcd. for $C_3H_9BrN_2O$: C, 21.3; H, 5.37; N, 16.6. Found: C, 21.6; H, 5.58; N, 16.6.

**Carbobenzoxy-L-prolyl-L-leucylsarcosinamide** (III).—**A.** Carbobenzoxy-L-prolyl-L-leucine[8,9] (14.5 g.) was dissolved in tetrahydrofuran (100 ml.). Triethylamine (6.0 ml.) was added and the solution was cooled to −10°. Isobutyl chloroformate (5.5 g.) in tetrahydrofuran (120 ml.) was added. The mixture was stirred at −10° for 20 min. Then a solution of sarcosinamide hydrobromide (7.4 g.) and triethylamine (6.4 ml.) in water (40 ml.) was added. Stirring was continued without further cooling for 90 min. The reaction mixture was acidified by slow addition of concd. hydrochloric acid. Tetrahydrofuran was removed *in vacuo*. The aqueous residue was extracted 4 times with 40-ml. portions of ethyl

(31) S. Moore, D. H. Spackman, and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958).

(32) D. H. Spackman, W. H. Stein, and S. Moore, *ibid.*, **30**, 1190 (1958).

(33) D. Ben-Ishai and E. Katchalski, *J. Am. Chem. Soc.*, **74**, 3688 (1952).

(34) D. E. Ames, R. E. Bowman, D. D. Evans, and W. A. Jones, *J. Chem. Soc.*, 1984 (1956).

acetate. The combined ethyl acetate extracts were washed successively with three 40-ml. portions each of *N* hydrochloric acid, 5% sodium bicarbonate, and water. The ethyl acetate solution was then dried over anhydrous magnesium sulfate and filtered. Ethyl acetate was removed from the filtrate *in vacuo*. The residue was dissolved in tetrahydrofuran (40 ml.). Hexane (35 ml.) was added to the solution and the crystalline precipitate was filtered off; wt. 7.4 g., m.p. 132–135°. The product was recrystallized from tetrahydrofuran (60 ml.) and hexane (50 ml.); wt. 6.8 g., m.p. 135–137.5°, $[\alpha]^{22}\text{D}$ −74.7° (*c* 2.00, 95% ethanol).

*Anal.* Calcd. for $C_{22}H_{32}N_4O_5$: C, 61.1; H, 7.46; N, 13.0. Found: C, 61.0; H, 7.54; N, 13.0.

**B.** Carbobenzoxy-L-prolyl-L-leucine (12.3 g.) was dissolved in tetrahydrofuran (80 ml.). Triethylamine (4.9 ml.) was added and the solution was cooled to −10°. Isobutyl chloroformate (4.7 g.) in tetrahydrofuran (55 ml.) was added. The mixture was stirred at −10° for 20 min. Then a solution of ethyl sarcosinate hydrochloride[35] (5.8 g.) and triethylamine (5.4 ml.) in water (17 ml.) was added. Stirring was continued without further cooling for 90 min. Tetrahydrofuran was removed *in vacuo*. The aqueous residue was acidified by slow addition of concentrated hydrochloric acid and then was extracted 4 times with 30-ml. portions of ether. The combined ether extracts were washed successively with three 30-ml. portions each of 5% sodium bicarbonate, water, *N* hydrochloric acid, and water. The ether solution then was dried over anhydrous magnesium sulfate and filtered. Ether was removed from the filtrate *in vacuo*. The residue was dissolved in methanol (75 ml.). The methanolic solution was saturated with anhydrous ammonia and allowed to stand at room temperature for 3 days. Methanol and ammonia were removed *in vacuo*. Hexane was added to the residue and the crystalline solid was filtered off and dissolved in tetrahydrofuran (120 ml.). Hexane (95 ml.) was added to the solution and the crystalline precipitate was filtered off; wt. 10.9 g., m.p. 134.5–136°. It was recrystallized from tetrahydrofuran (80 ml.) and hexane (50 ml.); wt. 10.4 g., m.p. 135–136°, $[\alpha]^{20}\text{D}$ −74.6° (*c* 2.00, 95% ethanol).

*Anal.* Found: C, 61.2; H, 7.61; N, 13.0.

**L-Prolyl-L-leucylsarcosinamide** (IV).—Protected tripeptide III (2.0 g.) was dissolved in ethanol (50 ml.). The solution was stirred with 5% palladium-charcoal catalyst (0.5 g.) for 3 hr. with a slow stream of hydrogen flowing over the surface of the mixture. The catalyst was filtered off and washed with ethanol (30 ml.). Ethanol was removed from the combined filtrate and washings *in vacuo*. The residue was dissolved in pyridine (10 ml.) and the solution was diluted with ether (70 ml.). Hexane (80 ml.) was added and the crystalline solid was filtered off; wt. 1.3 g. It was recrystallized from ethyl acetate (25 ml.) and hexane (20 ml.); wt. 1.0 g., m.p. 93–96°, $[\alpha]^{20}\text{D}$ −57.4° (*c* 1.00, *N* hydrochloric acid).

*Anal.* Calcd. for $C_{14}H_{26}N_4O_3$: C, 56.4; H, 8.78; N, 18.8. Found: C, 56.4; H, 8.89; N, 18.7.

**S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-prolyl-L-leucylsarcosinamide** (V).—*p*-Nitrophenyl S-benzyl-N-carbobenzoxy-L-cysteinate[7] (0.93 g.) and free tripeptide IV (0.63 g.) were dissolved in chloroform (2 ml.). The solution was allowed to stand at room temperature for 4 days. Ethyl acetate (50 ml.) was added and the resulting solution was extracted 4 times with 15-ml. portions of *N* hydrochloric acid, once with water (15 ml.), 8 times with 15-ml. portions of *N* ammonium hy-

(35) W. Staudt, *Z. physiol. Chem., Hoppe-Seyler's*, **146**, 286 (1925).

droxide and once with water (15 ml.). The solution was dried over anhydrous magnesium sulfate and filtered. Ethyl acetate and chloroform were removed *in vacuo*. A glassy solid which could not be crystallized readily was obtained: wt. 1.2 g., $[\alpha]^{20}D$ − 61.0° (*c* 2.00, dimethylformamide).

*Anal.* Calcd. for $C_{32}H_{43}N_5O_6S$: C, 61.4; H, 6.93; N, 11.2. Found: C, 61.4; H, 7.11; N, 10.9.

A portion of the product was hydrolyzed in 6 *N* hydrochloric acid at 110° for 17 hr. and the amino acid content of the hydrolysate was determined.[31,32] The following molar ratios of the four amino acids and ammonia were obtained (with the value of leucine taken as 1.0): S-benzylcysteine 1.0, proline 1.0, leucine 1.0, sarcosine 1.0, and ammonia 0.9.

**S-Benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylsarcosinamide** (VI).—Protected tetrapeptide V (3.4 g.) was dissolved in glacial acetic acid (40 ml.) and 4 *N* hydrogen bromide in glacial acetic acid (40 ml.) was added. The solution was allowed to stand at room temperature for 10 min. Ether (900 ml.) was added. The product was filtered off, washed with ether (450 ml.) and then dissolved in water (40 ml.). The resulting solution was extracted 4 times with 15-ml. portions of ether. Potassium bicarbonate (3.5 g.) was added to the aqueous solution and the resulting mixture was extracted with six 15-ml. portions of chloroform. The chloroform extracts were combined and chloroform was removed *in vacuo*. The residue was dissolved in ethyl acetate (50 ml.). The ethyl acetate solution was dried over anhydrous magnesium sulfate and filtered. Ethyl acetate was removed *in vacuo* leaving an amorphous solid; wt. 2.1 g. It was dissolved in dimethylformamide (17 ml.) and the resulting solution was cooled to 5°. Protected pentapeptide I (2.4 g.) and dicyclohexylcarbodiimide (1.6 g.) were added. The mixture was stirred at 5° for 1 hr. and at room temperature for 3 days. The reaction mixture was then cooled to 5° and glacial acetic acid (1.6 ml.) was added. Stirring was continued for 20 min. and water (165 ml.) then was added. The precipitated solid was filtered off, washed with water (165 ml.) and dried *in vacuo* over phosphorus pentoxide. It was then finely powdered and washed thoroughly with propanol (345 ml.); wt. 2.8 g. This material was dissolved in dimethylformamide (60 ml.) and precipitated by the addition of water (300 ml.). The precipitated solid was filtered off and washed with water (200 ml.): wt. 2.7 g. It was dissolved in dimethylformamide (40 ml.) and precipitated again by adding propanol (160 ml.) and hexane (40 ml.). The product was filtered off and washed with propanol (50 ml.) and hexane (50 ml.); wt. 1.4 g., m.p. 236–239° (dec.), $[\alpha]^{20}D$ −37.4° (*c* 1.00, dimethylformamide).

*Anal.* Calcd. for $C_{65}H_{88}N_{12}O_{14}S_3$: C, 57.5; H, 6.53; N, 12.4. Found: C, 57.4; H, 6.54; N, 12.3.

**9-Sarcosine Oxytocin.**—The procedures employed for the conversion of the protected nonapeptide VI to 9-sarcosine oxytocin followed closely those already described for the preparation of oxytocin from the protected nonapeptide II. Compound VI (100 mg.) was dissolved in anhydrous liquid ammonia (70 ml.) and the resulting solution was treated with small pieces of sodium until a blue color persisted for 10 min. Ammonium chloride (90 mg.) was added and after removal of ammonia *in vacuo* from the frozen state the residue was dissolved in water (170 ml.). The pH of the resulting solution was adjusted to approximately 6.5, and the solution was extracted with ether and aerated. Glacial acetic acid (1 ml.) was added to the solution and the peptide was separated from salts with

the use of an Amberlite IRC-50 (XE-64) column. The peptide-containing fractions of the eluate were combined and lyophilized.

The combined dry residues from three identical preparations were triturated with 20 ml. of lower phase of the solvent system butanol-ethanol-0.05% acetic acid (4:1:5). The resulting suspension was filtered and the insoluble residue was washed with an additional 10-ml. portion of lower phase. The filtrate and washings were combined, placed in the first 3 tubes of a 200-tube countercurrent distribution apparatus and subjected to 600 transfers in the solvent system just mentioned. Analysis for peptide content by the Folin–Lowry color reaction indicated one major peak trailed by two smaller peaks one of which slightly overlapped the main peak. In addition a faster-moving, smaller peak was completely removed from the machine by the end of 600 transfers. Only the major peak possessed detectable avian depressor activity. The contents of Tubes 118–199 which represented the biologically-active major component were removed, combined and concentrated in a rotary evaporator to a volume of about 30 ml. The concentrated solution was lyophilized to a white powder; wt. 63 mg.

Material at this stage of purification (137 mg.) was subjected to an additional 500 transfers in the same solvent system. Analysis by the Folin–Lowry color reaction indicated a single symmetrical peak containing material with a $K$ of approximately 0.32. The contents of Tubes 85–155 were removed, combined and concentrated in a rotary evaporator to about 30 ml. The concentrated solution was lyophilized to a white, fluffy powder; wt. 131 mg., $[\alpha]^{20}\text{D}$ $-21.0°$ ($c$ 0.50, $N$ acetic acid).

The product was subjected to paper chromatographic and paper electrophoretic analysis under exactly the same conditions as those already described for oxytocin. 9-Sarcosine oxytocin traveled as a single spot in both chromatographic solvent systems. $R_f$ values of the product were the same as those of oxytocin. 9-Sarcosine oxytocin also traveled as a single spot toward the cathode during electrophoresis and at the same rate as oxytocin.

A portion of the product was hydrolyzed in 6 $N$ hydrochloric acid at 110° for 17 hr. and the amino acid content of the hydrolysate was determined.[31,32] The following expected molar ratios of amino acids and ammonia were obtained (with the value of isoleucine taken as 1.0): isoleucine 1.0, aspartic acid 1.0, glutamic acid 1.0, proline 1.0, cystine 1.0, leucine 1.0, tyrosine 1.0, sarcosine 0.9, and ammonia 2.8.[36,37]

For elementary analysis a sample of the product was heated at 100° over phos-

(36) A small amount of a ninhydrin-positive substance which appeared to be glycine was detected during the amino acid analysis. The molar ratio of the substance when calculated as glycine was 0.02. This observation raised the question of the possible presence of a small amount of oxytocin in the sample of 9-sarcosine oxytocin. The intermediates used in the synthesis were subjected to hydrolysis and amino acid analysis in order to test for the presence of the glycine-like substance. The sarcosine and a hydrolysate of the L-prolyl-L-leucylsarcosinamide were shown to be free of the glycine-like component. The substance could be detected, however, in hydrolysates of the protected tetrapeptide V and the protected nonapeptide VI made from the L-prolyl-L-leucylsarcosinamide. It was concluded that the presence of the glycine-like substance was the result of decomposition during hydrolysis and was not an indication of contamination of the sarcosine peptides by the corresponding glycine peptides.

(37) Dr. David Yphantis of the Rockefeller Institute has determined the molecular weight of the highly purified 9-sarcosine oxytocin, using short column equilibrium centrifugation. At concentrations ranging from 0.25 to 1% in 0.15 $M$ ammonium acetate at pH 5.57 the average molecular weight was 1032 ± 35, assuming a partial specific volume of 0.71. The calculated molecular weight for the free base of 9-sarcosine oxytocin is 1021. The authors wish to thank Dr. Yphantis for this information.

phorus pentoxide *in vacuo* until it reached a constant weight. A loss in weight of 7% was observed. The results of the analysis agreed with the calculated values for the free base of 9-sarcosine oxytocin.

*Anal.* Calcd. for $C_{44}H_{68}N_{12}O_{12}S_2$: C, 51.8; H, 6.71; N, 16.5. Found: C, 52.0; H, 6.87; N, 16.2.

**Biological Activity of 9-Sarcosine Oxytocin.**—9-Sarcosine oxytocin was assayed for 5 biological effects and its activity was compared to that of oxytocin assayed under conditions which were as nearly the same as possible. The approximate activity of 9-sarcosine oxytocin in terms of units per mg. measured by the depression of blood pressure in the chicken[16] is 9, by the increase in mammary gland pressure in the lactating rabbit,[19,20] 55, by the contraction of the isolated rat uterus in the absence of magnesium,[17,18] 36, by the increase in blood pressure of the rat,[21] 0.01, and by the inhibition of diuresis in the hydrated rat,[22,23] 0.1. 9-Sarcosine oxytocin possesses about 2% of the avian depressor activity, 12% of the milk-ejecting activity, 5% of the oxytocic activity, 0.2% of the pressor activity and 2% of the antidiuretic activity of oxytocin.

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# Isomeric 2-Acetoxytropine Methiodides

S. ARCHER, A. M. LANDS, AND T. R. LEWIS

*Sterling-Winthrop Research Institute, Rensselaer, New York*

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The α- and β-isomers of 2-tropanyl acetate methiodide have been prepared and the L-forms examined for muscarine- and nicotine-like action in biological preparations. It has been established that the α-configuration favors muscarinic action and that the β-configuration favors nicotinic action. Comparisons of various results obtained with 3-tropanyl derivatives characterized by blocking action also support the concept that these receptors are structured to favor the *transoid* form for action at the muscarine-sensitive sites and the *cisoid* form for the nicotine-sensitive sites.

## Introduction

The fact that acetylcholine shows both muscarinic and nicotinic effects suggests that there are subtle but fundamental differences in