# 2-Isoleucine-oxytocin and Deamino-2-isoleucine-oxytocin: Their Synthesis and Some of Their Pharmacologic Activities

LUIS A. BRANDA, VICTOR J. HRUBY, AND VINCENT DU VIGNEAUD[1]

*Department of Biochemistry, Cornell University Medical College, New York, New York, 10021*

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SUMMARY

2-Isoleucine-oxytocin, an analog in which the tyrosine residue at position 2 in the hormone has been replaced by an isoleucine residue, and deamino-2-isoleucine-oxytocin, an analog in which a further change has been made by replacing the half-cystine residue at position 1 by a β-mercaptopropionic acid residue, have been synthesized and assayed for their biological activities. 2-Isoleucine-oxytocin was found to possess 20 ± 0.5 units/mg of oxytocic activity, 48.3 ± 2.5 units/mg of avian vasodepressor activity, about 0.1 unit/mg of rat pressor activity, and approximately 50 units/mg of milk-ejecting activity. Deamino-2-isoleucine-oxytocin was found to possess about 22 units/mg of oxytocic activity, 81.2 ± 7.3 units/mg of avian vasodepressor activity, about 0.1 unit/mg of rat pressor activity and approximately 80 units/mg of milk-ejecting activity. These biological activities are considerably greater than would have been expected on the basis of the activities of 2-leucine-oxytocin, which are very low.

## INTRODUCTION

The neurohypophyseal principle, oxytocin (the structure of which is shown in Fig. 1), was first synthesized in 1953 (1, 2), and since that time many analogs of oxytocin have been prepared in this and other laboratories, including a series of analogs in which one of the eight amino acid residues that make up the octapeptide structure of the hormone has been replaced by a different residue. The extent to which certain pharmacologic properties of these analogs differ from the corresponding properties of oxytocin has been determined and the results of such studies are being applied to the question of how the molecular structure of oxytocin is related to its biological properties. Of these properties the two with which we shall be mainly concerned in this communication are the oxytocic and avian vasodepressor activities of oxytocin. Its oxytocic activity is 546 ± 18 units/mg (3) and its avian vasodepressor activity, the activity on which the official method of the United States Pharmacopeia for the assay of oxytocin is based (4), is 507 ± 23 units/mg (5).

We now wish to report the synthesis of an analog of oxytocin in which the tyrosine residue at position 2 has been replaced by an isoleucine residue, and which possesses the two activities mentioned to a reduced, but still appreciable, degree. The mean potencies of 2-isoleucine-oxytocin expressed in units/mg and the standard error with $n$ indicating the number of sets of four-point assays, were found to be 20 ± 0.5 ($n = 11$) for the oxytocic activity, and 48.3 ± 2.5 ($n = 5$) for the avian vasodepressor activity. The rat pressor activity was found to be about 0.1 unit/mg, and the milk-ejecting activity about 50 units/mg. The possession of the first two of these activities by 2-isoleucine-oxytocin to the degree manifested is of particular interest since thus far it had been found that the replace-

[1] To whom reprint requests should be sent.

ment of the tyrosine residue by an aliphatic amino acid residue results in almost complete elimination of the two biological activities under discussion. For example 2-serine-oxytocin (6), 2-leucine-oxytocin (7), 2-α-aminoadipic acid-oxytocin and 2-α-aminoadipamide-oxytocin (8), and 2-glycine-oxytocin (9) all possess extremely weak oxytocic and avian vasodepressor activities.

Deamino-2-isoleucine-oxytocin was also prepared. This analog was of interest since it previously had been found that the replacement of the free amino group at position 1 in oxytocin with hydrogen (10, 11)

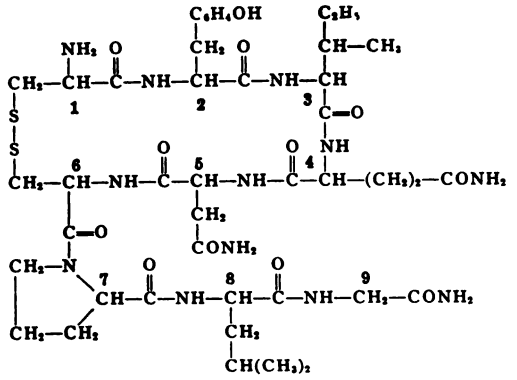

FIG. 1. *Oxytocin, with numbers indicating the positions of the component amino acid residues*

caused no lowering of oxytocic or avian vasodepressor activity. Indeed, deamino-oxytocin is even more potent than oxytocin in its manifestation of these activities (3, 5, 10–13). A comparable result has presently been obtained on replacement of the free amino group in 2-isoleucine-oxytocin by hydrogen. Deamino-2-isoleucine-oxytocin, synthesized by replacing the half-cystine residue at position 1 in 2-isoleucine-oxytocin by a β-mercaptopropionic acid residue, has been found to possess approximately 22 units/mg of oxytocic activity and 81.2 ± 7.3 units/mg ($n = 8$) of avian vasodepressor activity. About 0.1 unit/mg of rat pressor activity and about 80 units/mg of milk-ejecting activity were found. Considerable difficulty was encountered in carrying out the bioassays on this deamino analog. In the avian vasodepressor assay, the responses to the analog were abnormally unstable. It was necessary to adjust and to readjust the dose ratio between sets of four-point assays. This difficulty is reflected in the large standard error. The usual standard error for the avian vasodepressor assay in this laboratory is less than 5%. For the oxytocic activity, only an approximation can be given because of the potentiating effect of the analog. It potentiated the oxytocic responses to subsequent injections of the U.S.P. standard and of the analog itself. The potentiating effect lasted for a long period of time, about 10 minutes, despite thorough and repeated washings of the tissue with the bathing fluid. Infrequently, the analog caused a few contractions instead of a single contraction as normally seen under the conditions of the assay. It also affected the basal tone of the tissue. Normally the baseline is a straight line. Under the effect of deamino-2-isoleucine-oxytocin, the baseline may take the form of a quivering line.

2-Isoleucine-oxytocin and deamino-2-isoleucine-oxytocin were prepared by the methods used for the synthesis of oxytocin (14, 15) and deamino-oxytocin (10, 11), respectively, in which the intermediate peptides were prepared by the stepwise *p*-nitrophenyl ester method. 2-Isoleucine-oxytocin was isolated by partition chromatography on Sephadex (16) and was further purified by gel filtration on Sephadex (17). Deamino-2-isoleucine-oxytocin was purified by subjection to partition chromatography on Sephadex in two different solvent systems described in the experimental section.

## EXPERIMENTAL PROCEDURE[2]

*N-Carbobenzoxy-*L*-isoleucyl-*L*-isoleucyl-*L*-glutaminyl-* L *-asparaginyl-S-benzyl-* L *-cysteinyl-*L*-prolyl-*L*-leucylglycinamide.* The protected heptapeptide *N*-carbobenzoxy-L-isoleucyl- L -glutaminyl- L -asparaginyl- *S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (15), 1.93 g, was dissolved in 20 ml of anhydrous acetic acid, and 20 ml of a

[2] All melting points are capillary melting points and are corrected.

solution of hydrogen bromide in acetic acid (5.3 N) was added. The mixture was stirred for 1 hr at room temperature and then 200 ml of anhydrous ether was added. The precipitated hydrobromide salt was filtered, washed with ether (2 × 100 ml), and dissolved in 50 ml of methanol. The methanol solution was rapidly passed through a short column of Rexyn RG-1 (OH cycle) (Fisher Scientific Company, New York). The resin was then washed with methanol and the combined methanol solutions were evaporated *in vacuo* to dryness. The residue was dissolved in 15 ml of dimethylformamide (DMF), and 0.80 g of *p*-nitrophenyl *N*-carbobenzoxy-L-isoleucinate (15) was added. After the mixture had been allowed to stand for 16 hr at room temperature, 200 ml of ethyl acetate was added, and the precipitate was filtered off and washed with ethyl acetate (2 × 50 ml), ethanol (2 × 100 ml) and ethyl acetate (2 × 75 ml). After the product had been dried *in vacuo* it weighed 2.01 g (93%); m.p. 253–254° (with dec), $[\alpha]_D^{21}$ — 45° (*c*, 1, in DMF).

$C_{52}H_{77}N_{11}O_{12}S$
Calculated: C57.8, H7.18, N14.3
Found: C57.5, H7.28, N14.1

*N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-isoleucyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide.* The preceding protected octapeptide, 1.08 g, was dissolved in 10 ml of warm anhydrous acetic acid. The warm solution was cooled to room temperature and treated with 12 ml of a solution of hydrogen bromide in acetic acid (5.3 N). The mixture was stirred for 80 min and then 100 ml of anhydrous ether was added. The hydrobromide salt was separated by filtration, washed with ether (2 × 75 ml), and dissolved in 100 ml of methanol. The solution was rapidly passed through a column of Rexyn RG-1 (OH cycle) and the column was washed with methanol (2 × 50 ml). The combined eluate and washings were evaporated to dryness *in vacuo*. The colorless powder was dissolved in 15 ml of DMF and 0.47 g of recrystallized *p*-nitrophenyl *N*-carbobenzoxy-*S*-benzyl-L-cysteinate (Cyclo Chemical Corp., Los Angeles; Lot K-5099A) was added. The mixture was allowed to stand at room temperature for about 2 days and then 200 ml of ethyl acetate was added. The product was filtered off, washed with ethyl acetate (75 ml), ethanol (2 × 75 ml), and ethyl acetate (75 ml) and dried *in vacuo*. The product, 1.07 g, was dissolved in 20 ml of DMF and reprecipitated with water. The precipitate was filtered off, washed with ethanol (2 × 25 ml), and dried *in vacuo*. The product weighed 1.00 g (78%); m.p. 262–263° (with dec); $[\alpha]_D^{19}$ — 58.5° (*c*, 0.5, in DMF).

$C_{63}H_{88}N_{12}O_{13}S_2$
Calculated: C58.5, H6.96, N13.2
Found: C58.1, H7.05, N12.9

*2-Isoleucine-oxytocin.* To a solution of the preceding protected nonapeptide (0.256 g) in 175 ml of boiling anhydrous ammonia (freshly distilled from sodium) were added small amounts of sodium until a blue coloration persisted for about 3 min. The ammonia was removed by evaporation to a volume of 30 ml and then by lyophilization. The colorless residue was dissolved in 500 ml of water containing 0.50 ml of trifluoroacetic acid, the pH was adjusted to 8.2 by addition of 1 N ammonium hydroxide, and the resulting solution was treated with excess 0.01 N potassium ferricyanide. After ½ hr 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 AG-3-X4 resin (Bio-Rad Laboratories, Richmond) in the trifluoroacetate cycle. The solution obtained after removal of the resin was lyophilized. The colorless powder was taken up in 10 ml of the upper phase of the solvent system water (containing 3.5% acetic acid and 1.5% pyridine)-1-butanol (1:1), and put on a column of Sephadex G-25 (100–200 mesh) of 3.60 $cm^2$ cross section and 112 cm length that had been equilibrated with the upper phase according to the method of Yamashiro (16). The column was eluted with the organic phase, and one hundred 9.6-ml fractions were collected. Folin-Lowry color values

(18) of aliquots from every third fraction were plotted, and the fractions corresponding to the principal peak ($R_F$ 0.32) were pooled, and 400 ml of water was added. The mixture was concentrated to 65 ml and lyophilized to give 128.4 mg of product. This material was dissolved in 3 ml of 0.2 N acetic acid and placed on a Sephadex G-25 (200–270 mesh) column of 6.24 $cm^2$ cross section and 64 cm length that had been equilibrated (17) with 0.2 N acetic acid. One hundred and fifty 2.95-ml fractions were collected. Folin-Lowry color values for every fifth tube were plotted, and the fractions corresponding to the major peak which had its maximum at Fraction 102 were pooled (a minor peak was observed which had its maximum at Fraction 79). The pooled fractions were concentrated and lyophilized to give 95.2 mg (50%) of colorless powder; $[\alpha]_D^{21} - 29.8°$ (*c*, 0.5, in 1 N acetic acid).

$C_{40}H_{68}N_{12}O_{11}S_2$
Calculated: C50.2, H7.16, N17.6
Found: C50.2, H7.29, N17.2

The product was hydrolyzed for 22 hr in 6 N HCl at 110° and analyzed (19) on a Beckman/Spinco amino acid analyzer. The following molar ratios were obtained, with the value of glutamic acid taken as 1: aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.0; glycine, 0.82; cystine, 0.92; isoleucine, 1.3; leucine, 1.0; and ammonia, 3.0. When the hydrolysis time was increased to 90 hr the following results were obtained with the value of glutamic acid taken as 1: aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.2; glycine, 0.84; cystine, 1.0; isoleucine, 2.0; leucine, 1.0; and ammonia, 3.0. It can be concluded that the low ratio of isoleucine encountered after the shorter hydrolysis time was due to the resistance of the isoleucyl-isoleucine peptide bond to hydrolysis. It has previously been reported that this bond is difficult to hydrolyze (20).

The 2-isoleucine-oxytocin thus obtained was found to possess $20 \pm 0.5$ units/mg ($n = 11$) of oxytocic activity, $48.3 \pm 2.5$ units/mg ($n = 5$) of avian vasodepressor activity, about 0.1 unit/mg of pressor activity and about 50 units/mg of milk-ejecting activity.

*S-Benzyl-β-mercaptopropionyl-L-isoleucyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide.* A warm solution of 0.65 g of the protected octapeptide *N*-carbobenzoxy-L-isoleucyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide in 8 ml of anhydrous acetic acid was cooled to room temperature and treated with 10 ml of hydrogen bromide in acetic acid (5.3 N). The mixture was stirred for 90 min and 100 ml of ether was added. The colorless precipitate was filtered off, washed with ether ($2 \times 50$ ml), dried, and dissolved in 100 ml of methanol. This solution was passed through a short column of Rexyn RG-1 (OH cycle). The column was washed with methanol ($2 \times 50$ ml) and the combined eluate and washings were evaporated to dryness. The residue was dissolved in 20 ml of DMF and 0.23 g of *p*-nitrophenyl *S*-benzyl-β-mercaptopropionate (11) was added. The mixture was stirred for 2 days at room temperature, and then 150 ml of ethyl acetate was added. The precipitate was filtered off and washed with ethyl acetate (50 ml), ethanol ($2 \times 50$ ml), and ethyl acetate (50 ml). After the material had been dried over $P_2O_5$ *in vacuo* it weighed 0.59 g (86.8%); m.p. 263–264° (with dec); $[\alpha]_D^{21} - 57.8°$ (*c*, 0.5, in DMF). A sample was prepared for analysis by precipitation with water from a DMF solution.

$C_{54}H_{81}N_{11}O_{11}S_2$
Calculated: C57.6, H7.26, N13.7
Found: C57.1, H7.26, N13.3

*Deamino-2-isoleucine-oxytocin.* The protected polypeptide just described, 0.225 g, was dissolved in 175 ml of boiling, anhydrous ammonia, and then treated with sodium until a blue coloration persisted for about 1 min. The mixture was concentrated to a volume of about 30 ml, and the remaining solution was lyophilized. To the colorless product was added 500 ml of water containing 0.50 ml of trifluoroacetic acid, and the pH was adjusted to 8.3 with 1 N

ammonium hydroxide. To this stirred mixture was added 42.0 ml of 0.01 N potassium ferricyanide. Thirty minutes later the pH was adjusted to 6.8 with dilute trifluoroacetic acid, and the ferrocyanide and excess ferricyanide ions were removed from the solution by treatment with AG-3-X4 resin (chloride cycle). After removal of the resin by filtration, the colorless solution was transferred to a 3-l flask and lyophilized. The colorless product was dissolved in 12 ml of the upper phase of the solvent system water (containing 3.5% acetic acid and 1.5% pyridine)-1-butanol-benzene (4:3:1) and placed on a column of Sephadex G-25 (100–200 mesh) of 110 cm length and 3.60 $cm^2$ cross section that had been equilibrated with both lower and upper phase. The column was eluted with upper phase, and sixty 10-ml fractions were collected. Folin-Lowry color values were determined, and a major peak at $R_F$ 0.72 was found. The fractions corresponding to this peak were pooled, 150 ml of water was added, the mixture was concentrated to a volume of 50 ml, and the solution that remained was lyophilized. The material thus obtained was dissolved in 20 ml of the upper phase of the solvent system water (containing 3.5% acetic acid and 1.5% pyridine)-1-butanol-benzene (5:2:3) and placed on the column previously used after it had been re-equilibrated with the lower and upper phases. One hundred and fifty 5-ml fractions were collected, and Folin-Lowry color values were determined on aliquots from every fifth tube. The fractions corresponding to the major peak, $R_F$ 0.19, were pooled (a minor peak was located at $R_F$ 0.34), 200 ml of water was added, the mixture was evaporated to a volume of about 40 ml, and the solution was lyophilized. The white powder of deamino-2-isoleucine-oxytocin weighed 72.7 mg (39%); $[\alpha]_D^{19}$ — 104.9° ($c$, 0.5, in 1 N acetic acid).

$C_{40}H_{67}N_{11}O_{11}S_2$
Calculated: C51.0, H7.17, N16.4
Found: C50.9, H7.26, N16.2

The product was hydrolyzed for 90 hr at 110° in 6 N HCl and analyzed on a Beckman/Spinco amino acid analyzer. The following molar ratios of amino acids were found, with the value of glutamic acid taken as 1.0: aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.0; glycine, 1.0; isoleucine, 2.0; leucine, 1.0; cystine, 0.2; the mixed disulfide of β-mercaptopropionic acid and cysteine, 0.61; and ammonia, 2.9.

The deamino-2-isoleucine-oxytocin was found to possess about 22 units/mg of oxytocic activity, $81.2 \pm 7.3$ units/mg ($n = 8$) of avian vasodepressor activity, about 0.1 unit/mg of pressor activity and about 80 units/mg of milk-ejecting activity.

*Methods of biological assay.* Avian vasodepressor activities were measured in conscious chickens by the method of Munsick, Sawyer, and van Dyke (21). Assays of oxytocic activity were performed on isolated uteri from rats in natural estrus by the procedure of Holton (22) as modified by Munsick (23) with the use of magnesium-free van Dyke-Hastings solution as bathing fluid. Rat pressor assays were carried out on male rats as described in the United States Pharmacopeia (24). The milk-ejecting assays were performed according to the method of Cross and Harris (25) as modified by van Dyke, Adamsons, and Engel (26) and by Chan (27).

All assays were performed with the United States Pharmacopeia posterior pituitary reference powder as the standard, and all were of four-point design. At least three animals were used for each assay. From each animal, three or more sets of four-point were obtained. The average value from each animal constitutes one four-point assay. The final potency is the mean value of the four-point assays. Standard error of the assay was calculated according to the statistical method of Bliss (28).

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