The Synthesis and Some of the Pharmacological Properties of [4-L-Isoleucine]-oxytocin and [4-L-Leucine]-oxytocin*

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SUMMARY

Two analogues of oxytocin in which the glutamine residue at position 4 has been replaced by isoleucine and leucine residues, respectively, have been prepared by total synthesis via the stepwise p-nitrophenyl ester method. The [4-isoleucine]-oxytocin and [4-leucine]-oxytocin were tested for a number of biological activities. [4-Isoleucine]oxytocin possesses about 16% of the avian vasodepressor, 7% of the oxytocic, and 45% of the milk-ejecting activities of oxytocin. This analogue possesses negligible pressor and antidiuretic activities. [4-Leucine]-oxytocin possesses approximately 9% of the avian vasodepressor, 2.5% of the oxytocic, and 16% of the milk-ejecting activities of oxytocin. This analogue has no antidiuretic activity. It has a weak and inconsistent depressor effect on the blood pressure. [4-Isoleucine]-oxytocin and [4-leucine]-oxytocin are both considerably less potent than [4-valine]-oxytocin with respect to their avian vasodepressor, oxytocic, and milk-ejecting activities. Of particular interest is the fact that [4-leucine]-oxytocin exhibits in the rat a potent diuretic and natriuretic effect. Furthermore, it inhibits the antidiuretic activity of arginine-vasopressin.

In previous investigations of the significance of various functional groups to the biological activities of oxytocin, it was found that replacement of the carboxamide group of the glutamine residue at position 4 by hydrogen, accomplished by the synthesis of 4-decarboxamido-oxytocin (4-[α-amino butyric acid]-oxytocin), affords an analogue (Fig. 1) that possesses a high degree of avian vasodepressor and oxytocic activities (1). The milk-ejecting activity of this analogue was later found to be considerable (2). These results led to the conclusion that the carboxamide group at position 4 is important but not essential for the manifestation of these biological activities of oxytocin. On the other hand, the

* This work was supported by Grants HE-01675 and HE-11680 from the National Heart Institute, United States Public Health Service. All optically active amino acid residues are of the L configuration. The abbreviations used are: AcOH, acetic acid; Z-, benzoyloxycarbonyl; ONp, p-nitrophenyl ester; Cys(Bzl), S-benzyleysteine; Tyr(Bzl), O-benzyltyrosine.
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† W. Y. Chan and V. du Vigneaud, unpublished data.

antidiuretic and pressor activities of the 4-decarboxamido analogue were considerably lower than those of oxytocin (1). More recently, a comparison of the dose-response curve of the oxytocic activity of 4-decarboxamido-oxytocin with that of oxytocin has indicated that this replacement of the carboxamide group at position 4 of oxytocin by hydrogen decreases the activity by affecting the affinity of the hormone for the receptor (or receptors) but not by altering its intrinsic activity (3).

The biological activities reported by Guttmann and Boissonneault (4) for [4-alanine]-oxytocin are lower than those for [4-α-amino butyric acid]-oxytocin, except for the milk-ejecting activity. Furthermore, Drabarek reported that [4-glycine]-oxytocin has still lower biological activities (5). On the other hand, [4-valine]-oxytocin (6) was found to be more potent than [4-α-amino butyric acid]-oxytocin with respect to the oxytocic, avian vasodepressor, and milk-ejecting activities and to have negligible antidiuretic activity. A study of the effect of a further increase in the size of the side chain at position 4 on these activities therefore seemed warranted. We report here the syntheses of [4-leucine]-oxytocin and [4-isoleucine]-oxytocin and studies of some of their pharmacological properties.

The syntheses of the two analogues, outlined in Chart I, were carried out by use of the p-nitrophenyl ester method of peptide synthesis.
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Z-Asn-Cys(Bzl)-Pro-Leu-Gly-NH₂ (8)

1. HBr, AcOH
2. Z-Ile-ONp (8)

Z-Ile-Asn-Cys(Bzl)-Pro-Leu-Gly-NH₂

1. HBr, AcOH
2. Z-Ile-ONp

Z-Ile-Ile-Asn-Cys(Bzl)-Pro-Leu-Gly-NH₂

1. HBr, AcOH
2. Z-Tyr(Bzl)-ONp (8)

Z-Tyr(Bzl)-Ile-Ile-Asn-Cys(Bzl)-Pro-Leu-Gly-NH₂

1. HBr, AcOH
2. Z-Cys(Bzl)-ONp

Z-Cys(Bzl)-Tyr-Ile-Ile-Asn-Cys(Bzl)-Pro-Leu-Gly-NH₂

1. sodium, NH₃
2. K₃Fe(CN)₆

Cys-Tyr-Ile-Ile-Asn-Cys(Bzl)-Pro-Leu-Gly-NH₂

[4-Isoleucine]-oxytocin VI

1. sodium, NH₃
2. O₂ (air), K₃Fe(CN)₆

Cys-Tyr-Ile-Ile-Asn-Cys(Bzl)-Pro-Leu-Gly-NH₂

[4-Leucine]-oxytocin XI

The protected pentapeptide N-benzyloxycarbonylasparginyl-S-benzylcysteinyllprolylleucylglycinamide (I) served as starting material for both syntheses. In the preparation of [4-isoleucine]-oxytocin, the benzoyloxycarbonyl group was removed from the protected pentapeptide, I, and the free base was coupled with p-nitrophenyl N-benzyloxycarbonylisoleucinate to give the protected hexapeptide, II. The chain was then lengthened with the appropriate protected amino acid residues to give the protected nonapeptide, III. Similarly, treatment of the protected pentapeptide, I, with p-nitrophenyl N-benzyloxycarbonylittcysteinylltyrosylisoleucylasparaginyl-S-benzylcysteinyllprolylleucylglycinamide (V). Similar treatment of the protected nonapeptide, III, with p-nitrophenyl N-benzyloxycarbonylleucinate, followed by lengthening of the chain as before, afforded the desired protected nonapeptide for the preparation of [4-leucine]-oxytocin, namely, N-benzyloxycarbonyl-S-benzylcysteinylltyrosylisoleucylasparaginyl-S-benzylcysteinyllprolylleucylglycinamide (X).

The protecting groups of the protected nonapeptides were removed by treatment with sodium in liquid ammonia as used in the synthesis of oxytocin (9), and the reduced materials were oxidized to the corresponding oxytocin analogues by titration with a solution of potassium ferricyanide (10). [4-Isoleucine]-oxytocin (VI) was purified by partition chromatography on Sephadex G-25 and by gel filtration on Sephadex G-25. [4-Leucine]-oxytocin (XI) was purified by countercurrent distribution and by gel filtration. Both compounds gave the expected values in the amino acid and elementary analyses.

[4-Isoleucine]-oxytocin and [4-leucine]-oxytocin were tested for biological activities characteristic of oxytocin. The four-point assay design (11) was used in all the bioassays. Avian vasodepressor assays were performed on conscious chickens according to the procedure used by Munsick, Sawyer, and van Dyke (12). Oxytocic assays were performed on isolated uteri from rats in natural estrus according to the method of Holton (13), as modified by Munsick (14), with the use of magnesium-free van Dyke-Hastings solution as the bathing fluid. Milk-ejecting activity was measured on anesthetized rabbits by the method of Cross and Harris (15), as modified by van Dyke, Adams, and Engel (16), and by Chan (17). Pressor assays were carried out on anesthetized male rats as described in the United States Pharmacopeia (18). Assays for antidiuretic activity were performed.
on anesthetized male rats according to the method of Jeffers, Livezey, and Austin (18), as modified by Sawyer (20). All of the assays were carried out against the U.S.P. Posterior Pituitary Reference Standard.

The pharmacological activities of [4-isoleucine]-oxytocin and [4-leucine]-oxytocin are presented in Table I, along with the activities of other analogues of oxytocin discussed earlier in which the glutamine residue at position 4 has been replaced with lipophilic amino acid residues of varying size. Of this series of analogues, [4-valine]-oxytocin possesses the highest avian vasodepressor, oxytocic, and milk-ejecting potencies. [4-Isoleucine]-oxytocin possesses about 35% of the avian vasodepressor, 27% of the oxytocic, and 44% of the milk-ejecting potencies of [4-valine]-oxytocin. [4-Leucine]-oxytocin possesses about 19% of the avian vasodepressor, 9% of the oxytocic, and 16% of the milk-ejecting potencies of [4-valine]-oxytocin. Thus a decrease in the degree of these activities is observed when the size of the side chain at position 4 is increased beyond that of the valine residue. When the size of the side chain is decreased from that of the valine residue, there is also a progressive decrease in the degree of these activities. Thus it is clear from the data in Table I that the size of the side chain at position 4 has a marked influence on the oxytocic, avian vasodepressor, and milk-ejecting activities, those activities most characteristic of oxytocin itself.

Oxytocin also possesses a small degree of the rat pressor and antidiuretic activities which are most characteristic of vasopressin. From the data in Table I it can be seen that in the series of analogues listed these activities are present only at an almost vanishingly small level. Furthermore, in the case of [4-leucine]-oxytocin, the opposite effects are noted, that is, a very weak depressor activity and a diuretic effect without any antidiuretic activity.

When [4-leucine]-oxytocin showed this unexpected diuretic activity, further pharmacological studies were undertaken. As reported by Chan, Hruby, Flouret, and du Vigneaud (23), this analogue was found to possess not only a potent diuretic effect but also a potent natriuretic effect. Furthermore, it was shown that [4-leucine]-oxytocin reduced the arginine-vasopressin-induced free water reabsorption and reversed it to free water clearance. Thus it was concluded that the analogue possesses antidiuretic activity. However, it remains to be ascertained whether the inhibition was at a receptor level. The diuretic-natriuretic effect of [4-leucine]-oxytocin is not vasopressin-dependent, since the effect was demonstrable in rats during water diuresis when endogenous vasopressin was low as well as in rats under vasopressin-induced antidiuresis.

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean activity and standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vasodepressor (bowl)</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>507 ± 23</td>
</tr>
<tr>
<td>[4-Leucine]-oxytocin</td>
<td>44 ± 1</td>
</tr>
<tr>
<td>[4-Isoleucine]-oxytocin</td>
<td>81 ± 2</td>
</tr>
<tr>
<td>[4-Valine]-oxytocin</td>
<td>220 ± 14</td>
</tr>
<tr>
<td>(4-α-Aminobutyric acid)-oxytocin</td>
<td>108 ± 5</td>
</tr>
<tr>
<td>[4-Alanine]-oxytocin</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>[4-Glycine]-oxytocin</td>
<td>5.5 ± 0.2</td>
</tr>
</tbody>
</table>

* See Chan and du Vigneaud (21).
* See Chan, O'Connell, and Pomeroy (22).
* See Chan, Hruby, Flouret, and du Vigneaud (23).
* The depressor response to [4-leucine]-oxytocin was very transient and could be demonstrated consistently only when very large doses, 3 μg or more, were administered.
* After the weak antidiuretic effect, a marked diuresis occurred.
* See Du Vigneaud, Flouret, and Walter (6).
* W. Y. Chan and V. du Vigneaud, unpublished data.
* See Du Vigneaud, Denning, Drabarek, and Chan (1).
* W. Y. Chan and V. du Vigneaud, as reported by Branda and du Vigneaud (2).
* See Guttmann and Boissonnas (4).
* See Drabarek (5).

The synthesis of benzyloxycarbonyl peptide intermediates—The protected peptide intermediates listed in Table II were synthesized by procedures patterned closely after those used to prepare comparable intermediates in the synthesis of [4-α-aminobutyric acid]-oxytocin (1) and [4-valine]-oxytocin (6), procedures which, in turn, were based on the stepwise p-nitrophenyl ester synthesis of oxytocin by Bodansky and du Vigneaud (8).

A stirred solution or suspension of a benzyloxycarbonyl peptide in anhydrous AcOH was treated with an equal volume of HC1 at 110 ° . Stirring was continued for 1 to 2 hours with protection from moisture, during which time all undissolved material went into solution. The peptide hydrobromide was precipitated with a large excess of ether, filtered washed with ether, and dried in a vacuum.

### EXPERIMENTAL PROCEDURE

Synthesis of Benzyloxycarbonyl Peptide Intermediates—The protected peptide intermediates listed in Table II were synthesized by procedures patterned closely after those used to prepare comparable intermediates in the synthesis of [4-α-aminobutyric acid]-oxytocin (1) and [4-valine]-oxytocin (6), procedures which, in turn, were based on the stepwise p-nitrophenyl ester synthesis of oxytocin by Bodansky and du Vigneaud (8).
TABLE II

Benzyloxycarbonyl polypeptide intermediates

<table>
<thead>
<tr>
<th>Compound</th>
<th>Melting point (with decomposition)</th>
<th>Rotation</th>
<th>Empirical formula</th>
<th>Elementary analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[α]D</td>
<td></td>
<td>Calculated</td>
</tr>
<tr>
<td>II</td>
<td>257-258°C</td>
<td>-81.9°</td>
<td>C₆₅H₆₄N₂O₁₈S₂</td>
<td>58.7 9.67 13.4</td>
</tr>
<tr>
<td>III</td>
<td>248.5-250°C</td>
<td>-87.0°</td>
<td>C₆₅H₆₄N₂O₁₈S₂</td>
<td>59.3 7.30 13.2</td>
</tr>
<tr>
<td>IV</td>
<td>256.5-258°C</td>
<td>-42.7°</td>
<td>C₆₅H₆₄N₂O₁₈S₂</td>
<td>62.8 7.02 11.6</td>
</tr>
<tr>
<td>VII</td>
<td>234-236°C</td>
<td>-55.3°</td>
<td>C₆₅H₆₄N₂O₁₈S₂</td>
<td>60.5 6.85 11.8</td>
</tr>
<tr>
<td>VIII</td>
<td>242-243°C</td>
<td>-57°</td>
<td>C₆₅H₆₄N₂O₁₈S₂</td>
<td>58.7 6.97 13.4</td>
</tr>
<tr>
<td>IX</td>
<td>230-231°C</td>
<td>-48.8°</td>
<td>C₆₅H₆₄N₂O₁₈S₂</td>
<td>62.8 7.02 11.6</td>
</tr>
<tr>
<td>X</td>
<td>247-249°C</td>
<td>-55.4°</td>
<td>C₆₅H₆₄N₂O₁₈S₂</td>
<td>60.5 6.85 11.8</td>
</tr>
</tbody>
</table>

* The abbreviation used is: DMF, dimethylformamide.

To prepare the free base from the hydrobromide salt (except during the synthesis of Compound V (Chart I)), the salt was dissolved in methanol and treated with an anion exchange resin (Resin X 201 or Amberlite IRA 410), followed by isolation of the product by evaporation. The free base was then dissolved in dimethylformamide, an equivalent or slightly larger amount of benzyloxycarbonyl amino acid p-nitrophenyl ester was added, and the condensation was allowed to proceed for 18 to 24 hours. For preparation of V, the hydrobromide derived from IV was dissolved in dimethylformamide and titrated to pH 5 with triethylamine before the addition of N-benzyloxycarbonyl-S-benzylethyl cysteine p-nitrophenyl ester. In each case the reaction mixture was triturated with an excess of ethyl acetate at the end of the reaction period. The product was filtered off, washed with ethyl acetate and ethanol or ethano and other, and dried in a vacuum before use in a subsequent step. The yields of the protected polypeptide intermediates were 85 to 95%.

For preparation of analytical samples, the compounds were dissolved in hot dimethylformamide and precipitated with water. The melting points, rotations, and analytical data for each of the intermediates are given in Table II.

[4-Isoleucine]-oxytocin (VI)—A stirred, boiling solution of V (262 mg) in 175 ml of anhydrous ammonia (freshly distilled from sodium) was treated with sodium until a blue coloration persisted for 30 sec. The ammonia was removed by evaporation and lyophilization. The resulting colorless powder was dissolved in 500 ml of deaerated water containing 0.5 ml of triethylamine before the addition of N-benzyloxycarbonyl-S-benzylethyl cysteine p-nitrophenyl ester. The reaction mixture was stirred for 15 min, the resin was filtered off, and the solution was lyophilized. The resulting colorless powder was dissolved in 21 ml of the upper phase of the solvent system butanol-benzene-3.5% AcOH in 1.5% aqueous pyridine (6:1:7) and subjected to partition chromatography by the method of Yamashiro (26) and Yamashiro, Gilles, and du Vigneaud (27) on a column, 2.5 × 65 cm, of Sephadex G-25. The 160 mg of purified material obtained (Rf 0.55) was subjected to a second purification by a procedure which was identical except that the components of the solvent system were mixed in the proportion 3:1:4. The 107 mg of product (Rf 0.40) obtained after lyophilization was dissolved in 5 ml of 0.2 N AcOH and subjected to gel filtration (28) on Sephadex G-25. Fractions corresponding to the major peak of Folin-Lowry color values (29) were pooled and lyophilized: yield, 104 mg; [α]D -18.7° (c, 0.5, in 1 N AcOH). For amino acid analysis, a 24-hour hydrolysis time was necessitated by difficulty in hydrolysis of the isoleucyl-isoleucine peptide bond (30, 31). The following molar ratios were obtained: Asp 1.0, Pro 1.0, Gly 1.6, Cys 1.05, Ile 2.0, Leu 1.0, Tyr 1.0, NH₂ 2.1. For elementary analysis, a sample was dried to constant weight at 100°C over P₂O₅ in a vacuum, a loss of weight of 4.5% being observed. Calculated for C₆₄H₆₄N₂O₁₈S₂·H₂O: C 52.3, H 6.89, N 15.2; found: C 52.4, H 6.89, N 15.2.

[4-Leucine]-oxytocin (XI)—Compound XI was prepared from the protected nonapeptide, X (300 mg), with the conditions used for the synthesis of [4-valine]-oxytocin (6). After removal of ferri- and ferrocyanide ions, the resulting solution was concentrated in a flash evaporator to approximately 20 ml, placed in the first two tubes of a 10-ml, 200-tube countercurrent distribution apparatus (32), and subjected to 600 transfers in the solvent system butanol-benzene-0.5% AcOH in 0.1% aqueous pyridine (3:2:5). The distribution pattern, as indicated by the Folin-Lowry color values, showed a main peak with maximum at tube 105 (Rf 0.21). The theoretical curve calculated for the Rf value of 0.21 was in good agreement with our plot of the Folin-Lowry color values. The solutions from tubes 95 to 115 from the central portion of the peak were combined, concentrated to a small volume, and lyophilized to give 58 mg of the analogue, which was further purified by gel filtration on Sephadex G-25 in 0.2 N AcOH; [α]D -11° (c, 0.5, in 1 N AcOH). Amino acid analysis (24-hour hydrolysis) gave the following molar ratios: Asp 1.0, Pro 1.0, Gly 1.0, Cys 0.93, Ile 0.93, Leu 1.0, Tyr 0.86, NH₂ 2.2. For elementary analysis, a sample was dried to constant weight at 100°C over P₂O₅, a loss in weight of 10% being observed. Calculated for C₆₄H₆₄N₂O₁₈S₂·C₂H₄O₂: C 52.5, H 6.99, N 14.6; found: C 52.3, H 6.91, N 14.9.

Paper electrophoresis, with pyridine-AcOH buffer, pH 5.6, for 20 hours, showed [4-leucine]-oxytocin to have a mobility similar to that of oxytocin and to travel as a single spot. When [4-leucine]-oxytocin was subjected to descending paper chromatography in two different solvent systems, butanol-acetic acid-water
4-soleucine-oxytocin and 4-Leucine-oxytocin (4:1:5) and pyridine-acetic acid-water (10:7:3), it traveled as a single spot with $R_p$ 0.71 and $R_F$ 0.82, respectively.

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