[2,4-Dileucine]oxytocin. A Polypeptide with Natriuretic and Diuretic Activities, and an Inhibitor of the Oxytocic Response of Oxytocin[1a,b]

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Received March 31, 1971

[2,4-Dileucine]oxytocin, an analog of oxytocin in which the tyrosine in position 2 and the glutamine in position 4 have both been formally replaced by leucine, has been synthesized from the corresponding protected nonapeptide intermediate. The analog was found to possess negligible pressor activity and no antidiuretic or oxytocic activities. However, [2,4-dileucine]oxytocin has a natriuretic activity and a weak diuretic effect. Furthermore, it was shown to be an inhibitor to oxytocin in the oxytocic *in vitro* assay system.

In two recent communications[2,3] it was reported that the replacement of the Gln residue at position 4 of oxytocin (Figure 1) by a Leu residue afforded an analog ([4-leucine]oxytocin) that possessed no antidiuretic activity or pressor activity. It had a weak oxytocic activity. However, [4-leucine]oxytocin was found to possess considerable diuretic and natriuretic effects during both water diuresis and vasopressin-suppressed water diuresis, as well as anti-ADH activity (inhibitory to the antidiuretic effect of vasopressin).[2-4]

More recently it was reported[5] that [2,4-diisoleucine]-oxytocin, an analog of oxytocin in which the Tyr residue at position 2 and the Gln at position 4 are both formally replaced by an Ile residue, has a natriuretic–diuretic effect, and negligible oxytocic activity. It has also been reported that [4-aspartic acid]oxytocin, [4-serine]-oxytocin, and [8-isoleucine]oxytocin can block the antidiuretic effect of ADH.[6,7]

To assess further the structural features which led to the above mentioned results, we have prepared [2,4-dileucine]oxytocin. In this highly lipophilic compound, the Tyr residue at position 2 and the Gln residue at position 4 of oxytocin are formally replaced by Leu residues.

For the synthesis of [2,4-dileucine]oxytocin the protected heptapeptide *N*-Z-Ile-Leu-Asn-*S*-Bzl-Cys-Pro-Leu-Gly(NH_2) was prepared and served as starting material. It was synthesized in a similar manner to that reported in the synthesis of [4-leucine]oxytocin,[2] except that the protected tripeptide intermediate, *N*-Z-Pro-Leu-Gly(NH_2) was prepared by the method of Cash.[8] The *N*-benzyoxycarbonyl protecting group of the heptapeptide was removed by HBr in AcOH, the heptapeptide salt was neutralized, and the next residue, Leu, was added as *N*-Z-Leu(ONp).[9,10] This general procedure was repeated except that the free octapeptide was treated with *N*-Z-*S*-Bzl-Cys(ONp)[9,10] to give the desired protected nonapeptide *N*-Z-*S*-Bzl-Cys-Leu-Ile-Leu-Asn-*S*-Bzl-Cys-Pro-Leu-Gly(NH_2). The nonapeptide was treated with Na in liq NH_3[11] to cleave the *S*-benzyl and *N*-benzyloxycarbonyl protecting groups. The disulfhydryl compound was than oxidized in aq soln with $K_3Fe(CN)_6$,[12] and the desired product, [2,4-dileucine]oxytocin was purified by partition chromatog on Sephadex G-25[13,14] in the solvent system 3.5% aq AcOH (containing 1.5% pyridine)–1-BuOH–C_6H_6 (3:2:1), and by gel filtration[15] on Sephadex G-25 in 0.2 *N* AcOH (see Experimental Section).

The [2,4-dileucine]oxytocin was assayed for oxytocic activity according to the method of Holton[16] as modified by Munsick[17] on isolated uteri from rats in natural estrus with the use of Mg-free van Dyke–Hastings soln. Pressor assays were performed on anesthetized male rats as described in the USP,[18] and antidiuretic and diuretic assays were performed on anesthetized male rats according to the method of Jeffers, *et al.*,[19] as modified by Sawyer.[20] The USP Posterior Pituitary Reference Standard served as a reference for all activities assayed. Urinary Na^+ concns were determined by a Baird-Atomic flame photometer, with Li_2SO_4 as an internal standard.

[2,4-Dileucine]oxytocin was found to possess a very low pressor activity of less than 0.1 unit/mg, and no inhibitory activity was detected in this assay. The analog was found to possess no antidiuretic activity. However it was found to have a natriuretic effect in rats both during water diuresis and during AVP-sup-

(1) (a) This work was supported by Grant GB-11781 (V. J. H.) from the National Science Foundation and Grant HE-09795 (W. Y. C.) from the National Heart and Lung Institute, U. S. Public Health Service. (b) All optically active amino acids are of the L variety. (c) Recipient of Career Development Award, 1-K4-HE-38,849, U. S. Public Health Service.

(2) V. J. Hruby, G. Flouret, and V. du Vigneaud, *J. Biol. Chem.*, **244**, 3890 (1969).

(3) W. Y. Chan, V. J. Hruby, G. Flouret, and V. du Vigneaud, *Science*, **161**, 280 (1968).

(4) W. Y. Chan and V. du Vigneaud, *J. Pharmacol. Exp. Ther.*, **174**, 541 (1970).

(5) V. J. Hruby, V. du Vigneaud, and W. Y. Chan, *J. Med. Chem.*, **13**, 185 (1970).

(6) H. Datta and R. R. Chaudhury, *J. Endocrinol.*, **46**, 117 (1970).

(7) H. Datta and R. R. Chaudhury, *ibid.*, **42**, 345 (1968).

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

(9) M. Bodanszky and V. du Vigneaud, *Nature (London)*, **183**, 1324 (1959).

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

(11) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).

(12) D. B. Hope, V. V. S. Murti, and V. du Vigneaud, *ibid.*, **237**, 1563 (1962).

(13) D. Yamashiro, *Nature (London)*, **201**, 76 (1964).

(14) D. Yamashiro, D. Gillessen, and V. du Vigneaud, *J. Amer. Chem. Soc.*, **88**, 1310 (1966).

(15) J. Porath and P. Flodin, *Nature (London)*, **183**, 1657 (1959).

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

(17) R. A. Munsick, *Endocrinology*, **66**, 451 (1960).

(18) "The Pharmacopeia of the United States of America," 17th rev, Mack Publishing Co., Easton, Pa., 1965, p 749.

(19) W. A. Jeffers, M. M. Livezey, and J. H. Austin, *Proc. Soc. Exp. Biol. Med.*, **50**, 184 (1942).

(20) W. H. Sawyer, *Endocrinology*, **63**, 694 (1958).

pressed water diuresis. No anti-ADH activity was detected for the compound. Urine output, however, was slightly increased due to the natriuretic activity.

[2,4-Dileucine]oxytocin has no oxytocic activity in the *in vitro* assay system. Rather it was found to possess inhibitory activity to the hormone oxytocin. Preliminary experiments show that approximately a 250:1 ratio of inhibitor to oxytocin (w/w) produced a 50% inhibition of the response to a 10 mU dose of oxytocin (bath volume 10 ml). The effect was found to be Ca^{2+} dependent. Thus when the Ca^{2+} concn of the bathing fluid was raised from 0.5 m*M*/l. to 1.0 m*M*/l., the inhibitory activity of [2,4-dileucine]oxytocin was diminished 50%.

It is interesting to note that [4-leucine]oxytocin has an anti-ADH activity but has no antioxytocic activity, whereas [2,4-dileucine]oxytocin has no anti-ADH activity but is antioxytocic. [2-Leucine]oxytocin has neither anti-ADH nor antioxytocic activities, but rather possesses small oxytocic and negligible antidiuretic activities.[21,22]

Experimental Section[23]

***N*-Z-Leu-Ile-Leu-Asn-*S*-Bzl-Cys-Pro-Leu-Gly(NH_2).**—A slurry of 1.14 g (1.2 mmoles) of *N*-Z-Ile-Leu-Asn-*S*-Bzl-Cys-Pro-Leu-Gly(NH_2)[2] in 7 ml of anhyd AcOH was stirred with 7 ml of 5.4 *N* HBr in glacial AcOH for 1 hr at room temp. Anhyd Et_2O (200 ml) was added, and the white ppt was filtered off, washed with Et_2O (three 40-ml portions), and dried *in vacuo*. The salt was dissolved in 75 ml of MeOH and passed through a short column of Rexyn 201 (OH cycle) (11 g). The column was washed with three 40-ml portions of MeOH and the combined MeOH solns were evapd to dryness *in vacuo*. The free heptapeptide (0.95 g) so obtd was dissolved in 7 ml of DMF. Then 0.54 g (1.5 mmoles) of *N*-Z-Leu(ONp)[9,10] was added, and the mixt was stirred for 22 hr at room temp. The product was triturated with 75 ml of EtOAc, cooled, filtered off, and washed with 20 ml of EtOAc, two 20-ml portions of EtOH, and 20 ml of Et_2O. The product was dried *in vacuo* to give 1.21 g of a white powder: mp 264.5–266° dec, $[\alpha]^{25}D$ −85.7° (*c* 0.5, AcOH). *Anal.* ($C_{53}H_{80}N_{10}O_{11}S$) C, H, N.

***N*-Z-*S*-Bzl-Cys-Leu-Ile-Leu-Asn-*S*-Bzl-Cys-Pro-Leu-Gly-(NH_2).**—A slurry of 0.54 g (0.5 mmole) of the preceding protected octapeptide in 7 ml of anhyd AcOH was stirred with 7 ml of 5.4 *N* HBr in AcOH for 1 hr at room temp. Then 200 ml of anhyd Et_2O was added, and the ppt was filtered off and washed with three 40-ml portions of Et_2O. The powder was dried *in vacuo*, and dissolved in 9 ml of DMF. The pH was adjusted to 7.0 with *N*-methylmorpholine, and 0.33 g (0.7 mmole) of *N*-Z-*S*-Bzl-Cys(ONp)[9,10] was added with stirring. The mixt was stirred for 36 hr at room temp, and the slurry was triturated with 75 ml of EtOAc. The ppt was filtered off and washed with 15 ml of EtOH, 15 ml of 50% aq EtOH, 15 ml of EtOH, and 15 ml of Et_2O. The product was dried *in vacuo* to give 0.58 g of white powder: mp 257–259°, $[\alpha]^{26}D$ −67.4° (*c* 0.5, HOAc). *Anal.* ($C_{63}H_{91}N_{11}O_{12}S_2$) C, H, N.

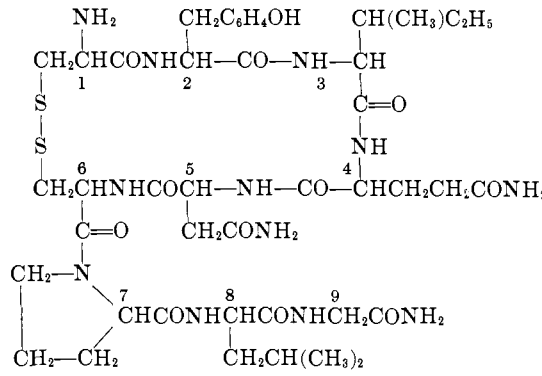

Figure 1.—Structure of oxytocin, with numbers indicating the position of individual amino acid residues.

[2,4-Dileucine]oxytocin.—A soln of 252 mg (0.2 mmole) of the preceding protected nonapeptide in 175 ml of boiling, freshly distd anhyd NH_3 was treated with Na until a blue coloration persisted for 45 sec. The NH_3 was removed by evapn and lyophilization and the residue was dissolved in 400 ml of deaerated, deionized H_2O contg 0.4 ml of AcOH. The pH of the soln was adjusted to 8.0 with 2 *N* NH_4OH, and 43 ml of 0.01 *N* $K_3Fe(CN)_6$ was added. The mixt was stirred for 30 min, the pH was adjusted to 5.0 with AcOH, and the ferrocyanide and excess ferricyanide ions were removed by treatment with ion-exchange resin Rexyn 203 (Cl^- cycle). The mixt was stirred for 15 min, the resin was filtered off, and the soln was lyophilized. The powder was dissolved in 8 ml of the upper phase and 3 ml of the lower phase of the solvent system 3.5% aq AcOH (contg 1.5% pyridine)–1-BuOH–C_6H_6 (3:2:1), and placed on a 2.8 × 63 cm column of Sephadex G-25 (100–200 mesh) that had been equilibrated with both the lower and upper phases according to the method of Yamashiro.[13,14] Eighty 7.7-ml fractions were collected and fractions corresponding to the major peak (R_f 0.56) as detd by plotting of the Folin–Lowry color values[24] were pooled. Then 225 ml of deionized H_2O were added, and the mixt was concd to 40 ml *in vacuo* and lyophilized. The resulting powder (86.2 mg) was dissolved in 4 ml of 0.2 *N* AcOH and placed on a 2.8 × 67 cm column of Sephadex G-25 (200–270 mesh) in 0.2 *N* AcOH for gel filtration.[15] One hundred 4.7-ml fractions were collected, and the fractions corresponding to the major peak (tubes 59–68) as detd by plotting Folin–Lowry color values were pooled and lyophilized to give 78.9 mg of [2,4-dileucine]oxytocin as a white powder, $[\alpha]^{25}D$ −43.1° (*c* 0.48, 1 *N* AcOH). *Anal.* ($C_{41}H_{71}N_{11}O_{10}S_2 \cdot 2H_2O$) C, H, N.

The sample was hydrolyzed for 90 hr in 6 *N* HCl at 110° and analyzed on a Technicon Amino Acid Analyzer. The molar ratios obtained with glycine taken as 1.0 were: Asp, 1.0; Pro, 1.1; Gly, 1.0; Cys Cys, 1.1; Ile, 1.0; Leu, 2.8; and NH_3, 1.9. Prolonged hydrolysis was necessitated by the difficulty in the hydrolysis of an Ile–Leu peptide bond.[25]

Acknowledgments.—The authors wish to thank Mrs. Nydia Rivera for technical assistance.

(21) K. Jošt, J. Rudinger, and F. Sŏrm, *Collect. Czech. Chem. Commun.*, **26**, 2496 (1961).

(22) V. J. Hruby and V. du Vigneaud, *J. Med. Chem.*, **12**, 731 (1969).

(23) Capillary melting points were determined and are corrected. Where analyses are indicated only by symbols of the elements, anal. results obtained for the elements were within ±0.4% of the theor values.

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

(25) H. Takashima, V. J. Hruby, and V. du Vigneaud, *J. Amer. Chem. Soc.*, **92**, 677 (1970).