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Solid Phase Synthesis of [3,4-Dileucine]-oxytocin and a Study of Some of Its Pharmacological Properties†

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[3,4-Dileucine]-oxytocin has been synthesized by the solid phase method and its pharmacological properties compared with those of [4-leucine]-oxytocin, an analog of the hormone which possesses a marked inhibitory effect on the antidiuretic activity of arginine-vasopressin (anti-ADH). [3,4-Dileucine]-oxytocin was found to possess very weak oxytocic and avian vasodepressor activities and negligible pressor activity. The analog had no antidiuretic activity and no demonstrable anti-ADH activity. However, it had marked natriuretic activity.

[3,4-Dileucine]-oxytocin has been synthesized by the method described in the Experimental Section, and certain pharmacological properties have been studied to explore further the relationship of structure to the marked inhibitory effect exerted by [4-leucine]-oxytocin[1-3] on the antidiuretic activity of arginine-vasopressin, the antidiuretic hormone of the posterior pituitary gland. We have referred to this inhibitory action as anti-ADH. [3,4-Dileucine]-oxytocin ($\overset{1}{\text{Cys}}$-$\overset{2}{\text{Tyr}}$-$\overset{3}{\text{Leu}}$-$\overset{4}{\text{Leu}}$-$\overset{5}{\text{Asn}}$-$\overset{6}{\text{Cys}}$-$\overset{7}{\text{Pro}}$-$\overset{8}{\text{Leu}}$-$\overset{9}{\text{Gly}}$-NH_2) is an analog of the hormone oxytocin ($\overset{1}{\text{Cys}}$-$\overset{2}{\text{Tyr}}$-$\overset{3}{\text{Ile}}$-$\overset{4}{\text{Gln}}$-$\overset{5}{\text{Asn}}$-$\overset{6}{\text{Cys}}$-$\overset{7}{\text{Pro}}$-$\overset{8}{\text{Leu}}$-$\overset{9}{\text{Gly}}$-NH_2) in which leucine residues replace the Ile and Gln residues of oxytocin in the 3 and 4 positions, respectively.

The effects of [3,4-dileucine]-oxytocin on renal excretion of H_2O and electrolytes were studied in rats by the techniques used by Chan and coworkers.[2,3] Like [4-leucine]-oxytocin, the 3,4-dileucine analog had no antidiuretic activity and had a marked natriuretic activity both in water diuresis and during vasopressin-suppressed water diuresis. Unlike [4-leucine]-oxytocin, the 3,4-dileucine analog had only weak diuretic and no demonstrable anti-ADH activity.

Among the natriuretic oxytocin analogs we have so far studied, [3,4-dileucine]-oxytocin exhibited the highest natriuretic activity. When a single dose (0.7 μg/100 g body weight) was injected iv into a rat under water diuresis, an increase in urinary excretion of Na^+ was noted within 2 min. The peak response was noted between 3 and 6 min *post*injection. For [3,4-dileucine]-oxytocin the average increase in Na^+ excretion at the peak was 300%. For [4-leucine]-oxytocin the increase was 215%,[3] for [2,4-diisoleucine]-oxytocin[4] 75%,[3] and for [2,4-dileucine]-oxytocin[5] 60%.§ It is also of interest to note that Rudinger and coworkers have reported that another 4-leucine analog, [4-leucine, 8-isoleucine]-oxytocin, possesses natriuretic and diuretic properties rather than antidiuretic activity.[6]

[3,4-Dileucine]-oxytocin was also assayed for oxytocic,# avian vasodepressor,** and pressor†† activities against the USP Posterior Pituitary Reference Standard. It was found to possess weak oxytocic (~1.0 unit/mg) and avian vasodepressor (~2 units/mg) potencies and negligible pressor potency (<0.005 unit/mg). No inhibition of the oxytocic or pressor activities of oxytocin was detected. In the case of [3,4-dileucine]-oxytocin, it had been found to have a negligible pressor and no oxytocic activity but to possess a weak antioxytocic activity.

It is interesting that additional leucine substitution of [4-leucine]-oxytocin at the 3 or 2 position produces selective changes in the renal activity of the polypeptide. [4-Leucine]-oxytocin is strongly natriuretic and possesses potent anti-ADH activity. These two activities are responsible for the marked diuretic effect of this analog. [3,4-Dileucine]-oxytocin is more potent than [4-leucine]-oxytocin in its natriuretic activity but it has no anti-ADH effect. The 2,4-dileucine analog has only a weak natriuretic activity and shows no anti-ADH activity. These exptl findings suggest a receptor specificity and that it may be possible to synthesize neurohypophysial analogs with highly selective activity.

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§W. Y. Chan, unpublished data.

#Oxytocic assays were performed according to the method of Holton[7] as modified by Munsick[8] on isolated uteri from rats in natural estrus with the use of Mg-free van Dyke–Hastings solution.

**Avian vasodepressor responses were measured by the method of Coon[9] as modified by Munsick, *et al.*,[10] using conscious prepns.

††Pressor assays were carried out on anesthetized male rats as described in "The Pharmacopeia of the United States of America."[11]

Experimental Section‡‡

Cys(Bzl)-Tyr(Bzl)-Leu-Leu-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (I). The protected nonapeptide amide I was prepd by the solid-phase method of Merrifield and coworkers[12] as applied to the synthesis of deamino-oxytocin,[13] except that the Boc-glycyl resin used was in the unnitrated form as had been employed by Manning[14] in the synthesis of oxytocin. Boc-glycyl resin (2.05 g, contg 0.165 mmole of glycine/g of esterified resin) was placed in the Merrifield reaction vessel. For the introduction of each new amino acid residue, the cycle of deprotection, neutralization, and coupling described previously was followed.[13]

For the washing steps, 10-ml portions of the appropriate solvent were used. In the deprotection step 10 ml of 1 *N* HCl in glacial AcOH was used, and neutralization of the hydrochloride was accomplished by treatment with 1.4 ml of Et_3N in 10 ml of DMF. In the coupling steps 0.89 mmole of the appropriate Boc-amino acid in 7 ml of CH_2Cl_2 and 0.89 mmole of *N,N'*-dicyclohexylcarbodiimide in 3 ml of CH_2Cl_2 were used. The coupling reaction involving Boc-asparagine was carried out *via* its *p*-nitrophenyl ester[15] (0.89 mmole in 10 ml of dist DMF) with a reaction time of 24 hr.

Following the incorporation of the last amino acid residue, the Boc protecting group was removed with 1 *N* HCl in glacial AcOH, and the hydrochloride was neutralized by treatment with Et_3N in DMF. The polypeptide-resin was further washed with three 10-ml portions each of EtOH, glacial AcOH, EtOH, and CH_2Cl_2. The product was removed from the flask and dried *in vacuo* over $CaCl_2$ to give 2.26 g.

The ammonolytic cleavage was effected according to Manning.[14] Dry NH_3 was bubbled into a stirred suspension of the protected polypeptide-resin (2.26 g) in anhyd MeOH (100 ml) at 0° for 2.5 hr. The reaction mixt was stirred overnight at 0–4°. The MeOH and NH_3 were removed under aspirator vacuum. DMF (100 ml) was added to the dry residue and the suspension was stirred overnight. The resin was removed by filtration and washed 3 times with 20-ml portions of DMF. H_2O was gradually added to the combined filtrate and washings until the soln appeared turbid. The soln was allowed to stand in an ice bath for 2 hr. The gelatinous white solid was collected by filtration, washed with H_2O and EtOH, and dried *in vacuo* over $CaCl_2$ to yield 296 mg (69% based on Boc-glycyl resin); mp 225–228° uncor, $[\alpha]^{21}D$ −39.9° (*c* 1, DMF). *Anal.* ($C_{65}H_{89}N_{11}O_{11}S_2$) C, H, N.

A sample was hydrolyzed in 6 *N* HCl at 110° for 64 hr and analyzed on a Beckman/Spinco amino acid analyzer according to the method of Spackman, *et al.*[16] The following molar ratios were obtd: Asp 1.0, Pro 1.1, Gly 1.0, Leu 2.8, Tyr 0.8, Cys(Bzl) 2.2, and NH_3 1.9.

[3,4-Dileucine]-oxytocin. The preceding protected nonapeptide amide (232 mg) was dissolved in 100 ml of stirred boiling liq NH_3 (distd from Na in an all-glass app). A fresh Na stick was momentarily introduced intermittently until the blue color persisted for 1 min. A small amt of NH_4Cl was added to disperse the blue color. The NH_3 was evapd at the water aspirator, and the resultant residue was dissolved in 400 ml of 0.03% aq F_3CCO_2H. The pH of the soln was adjusted to 6.8 with dil aq F_3CCO_2H and 0.1 *N* $K_3Fe(CN)_6$ was added dropwise with stirring until a yellow color persisted. During this addn the pH fell to 4.6. Stirring was contd for 30 min, then the pH was adjusted to 6.7 with concd NH_4OH. AG3-X4 resin (Bio-Rad Laboratories) (trifluoroacetate form) was added and stirring was contd for 30 min to remove ferrocyanide and excess ferricyanide ions. The resin was removed by filtration, and the soln was lyophilized. The lyophilisate was dissolved in 6 ml of the upper phase of the solvent system 1-BuOH–PhH–3.5% aq AcOH contg 1.5% pyridine (3:1:4) and applied to a Sephadex G-25 (100–200 mesh) column (2.81 × 65 cm) that had been equilibrated with the lower and upper phases of the solvent system. The column was eluted with the upper phase, and 35 fractions of 9.05 ml each were collected. The chromatogram obtained by plotting the Folin-Lowry color values[17] of the fractions showed a major peak with a max at fraction 26 (R_f 0.34). The fractions corresponding to this peak were pooled, twice the vol of H_2O was added, and the resulting soln was concd under reduced pressure to about 20 ml and lyophilized.

The lyophilized powder (69 mg) was dissolved in 6 ml of 0.2 *N* AcOH and subjected to gel filtration on a Sephadex G-25 (200–270 mesh) column (2.82 × 68 cm) that had been equilibrated with 0.2 *N* AcOH. The column was eluted with 0.2 *N* AcOH and 100 fractions of 6.6 ml each were collected. A plot of the absorption (275 mμ) of the various fractions showed a single peak with max at fraction 50 but with a slight shoulder at fraction 45. Fractions 47–54 were pooled and lyophilized. The lyophilized powder was dissolved in 4 ml of 0.2 *N* AcOH and subjected to gel filtration again, but on a longer Sephadex G-25 (200–270 mesh) column (2.15 × 118 cm). The column was eluted with 0.2 *N* AcOH, and 150 fractions of 3.0 ml each were collected. The plot of the absorption (275 mμ) of the various fractions showed a single peak with max at fraction 102. The fractions corresponding to this peak (94–113) were pooled and lyophilized to give a white powder; 65.9 mg (20% based on Boc-glycyl resin), $[\alpha]^{29}D$ −22.5° (*c* 1, 1 *N* AcOH). *Anal.* ($C_{44}H_{69}N_{11}O_{11}S_2 \cdot CH_3CO_2H$) C, H, N.

Quant amino acid anal. after acid hydrolysis (52 hr) gave the following molar ratios: Asp 1.0, Pro 1.1, Gly 1.0, half-Cys 1.8, Leu 3.0, Tyr 0.9, and NH_3 1.7.

‡‡Capillary melting points were taken on a Thomas-Hoover melting point apparatus. Where anal. are indicated only by symbols of the elements, analytical results obtained for the elements were within ±0.4% of the theoretical values.

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