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Synthetic Metabolites of Neurohypophyseal Hormones. [Des-9-glycinamide]oxytocin and [Des-9-glycinamide,des-8-leucine]oxytocin[1]

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Syntheses and biological properties are reported for two analogs of oxytocin in which the glycinamide and the leucylglycinamide moiety, respectively, have been deleted from the parent hormone. Both [des-9-glycinamide]oxytocin and [des-9-glycinamide,des-8-leucine]oxytocin are weak agonists in the rat uterotonic and antidiuretic assays but possess no detectable rat pressor activity. In addition, [des-9-glycinamide]oxytocin is an inhibitor of the oxytocin-induced vasodepressor response in fowl but is a potent agonist in the hydroosmotic assay of the toad urinary bladder.

It has been suggested that the milk-ejecting hormone, oxytocin,[2a] can serve as precursor or prohormone for the enzymatic generation in vivo of biologically active fragments.[2b-4] There is an ever-increasing number of peptides both synthetic and isolated from natural sources which comprise N-terminal and C-terminal sequences of oxytocin and vasopressin and which exhibit endocrine and extra-endocrine effects clearly distinct from those of the neurohypophyseal hormones.[5] The isolation from natural sources of peptides is compatible with results obtained from studies of the metabolism of neurohypophyseal hormones by proteolytic enzymes present in tissue extracts, body fluids, and intact animals. These studies have shown that the peptide bonds between residues 1 and 2 (Cys-Tyr) and 7 and 8 (Pro-Leu, Arg, or Lys) as well as 8 and 9 (Leu, Arg, or Lys-Gly) are the initial sites for hydrolysis of the hormones (for a recent summary see ref 6).

In light of these results it became important to obtain authentic samples of analogs of oxytocin in which the glycinamide and the leucylglycinamide moieties, respectively, have been deleted. In this paper we describe the synthesis of H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-OH (dG-OXY) and H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-OH (dLG-OXY) along with some of their potencies for those biological activities which are characteristic of oxytocin. For the synthesis of dG-OXY, Z-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys (Bzl)-Pro-Leu-resin (I) was obtained by the general solid-phase procedure of Merrifield[7] as applied to the synthesis of vasopressin[8] with further modifications noted in the Experimental Section. Removal of the peptide from the resin and deblocking of the N-CBz and O-Bzl groups were achieved by acidolysis with HBr–F_3CCO_2H to give the hydrobromide of the S-benzylated octapeptide. Debenzylation with Na in boiling NH_3[9] followed by aqueous $K_3Fe(CN)_6$[10] oxidation of the intermediary dithiol to the disulfide yielded crude dG-OXY, which was purified by partition chromatography.[11]

Similarly, dLG-OXY was prepared in a stepwise manner through the protected heptapeptide resin, Z-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro-resin (II). Treatment of II with HBr–F_3CCO_2H produced the dibenzyl heptapeptide hydrobromide which upon conversion to the disulfide and partition chromatography gave homogenous dLG-OXY.

Table I. Comparison of Some Biological Activities of [Des-Gly-NH_2[9]]oxytocin and [Des-Gly-NH_2[9],des-Leu[8]]oxytocin with Those of Oxytocin[a]

	Uterotonic (rat)	Vasodepressor (fowl)	Pressor (rat)	Antidiuretic (rat)	Water flux[b] (toad)
Oxytocin[c]	546[d]	507	3.1	2.1	2.3×10^{-9}
dG-OXY	2.4 ± 0.07	Inhibition	Negl	0.08 ± 0.003	4.0×10^{-9}
dLG-OXY	2.7 ± 0.5	0.10 ± 0.03	Negl	0.06 ± 0.006	

[a] Expressed in USP units/mg ± SEM unless otherwise noted. [b] Molar concentration required for obtaining half-maximal response; data were normalized to the value of crystalline deamino-oxytocin, which also served as standard as described by Eggena et al.[29] [c] Values reported by W. Y. Chan and V. du Vigneaud, *Endocrinology*, 71, 977 (1962). [d] Value reported by R. L. Huguenin and R. A. Boissonnas, *Helv. Chim. Acta*, 49, 695 (1966).

The two analogs of oxytocin have been assayed for biological activities characteristic of neurohypophyseal hormones and some of these data are summarized in Table I. Both dG-OXY and dLG-OXY are weak agonists in the in vitro rat uterotonic assay and the rat antidiuretic assay, and they show no activity either as agonists or antagonists in the rat pressor assay at single doses of as high as 1 mg. In contrast, much of the hydroosmotic potency determined in the toad urinary bladder assay is retained upon deletion of the glycinamide moiety from oxytocin. The fact that dG-OXY retains such a high degree of the activity which oxytocin exhibits in this amphibian system is reminiscent of the high natriferic activity in the frog skin[12] retained by [des-Gly[9]]oxytocin (an analog of oxytocin in which the glycine residue has been omitted, but the terminal carboxamide group was kept).[13] The above results are in line

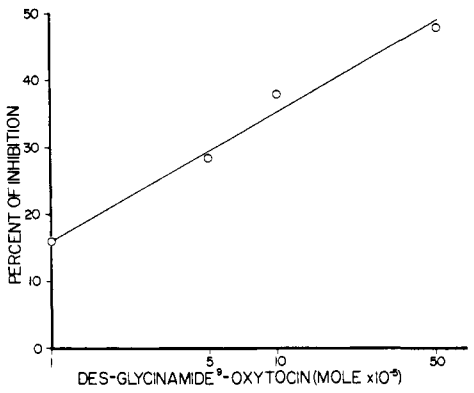

Figure 1. Inhibitory effect of [des-Gly-NH_2^{9}]oxytocin on the vasodepressor response in chicken to oxytocin. The analog and oxytocin were injected simultaneously. The dose of dG-OXY was increased from 10^{-5} to 5×10^{-4} mol, while the concentration of oxytocin was maintained at 5×10^{-11} mol.

with the general contention that the acyclic peptide moiety of neurohypophyseal hormones is critical for biological activity in mammalian vertebrates but not in nonmammalian vertebrates.

The dG-OXY was found to be devoid of any agonistic activity in the avian vasodepressor assay when tested at a dose of as high as 1 mg of analog per injection. However, dG-OXY inhibited in a dose-dependent manner the response to oxytocin (Figure 1). Neither agonistic nor antagonistic activity was detected with dLG-OXY when tested analogously.

Finally, both dG-OXY and dLG-OXY were found ineffective in attenuating puromycin-induced amnesia in mice (see last citation under ref 5).

Experimental Section

All reactions were carried out at room temperature and all products were dried at room temperature over P_2O_5 and KOH in vacuo unless otherwise noted. Optical rotation was determined with a Zeiss photoelectric precision polarimeter set at 0.005°. Peptide hydrolysates were chromatographed on a Beckman/Spinco Model 120 C amino acid analyzer. Each "washing" procedure entailed the use of three 50-ml portions of the solvents in question; the solvents are cited in the order they were used. Yields of the cleaved hydrobromide peptide salts from I, II, and III are based on the titratable Cl^- content of the HCl-Leu-resin and HCl-Pro-resin. TLC was performed on silica gel G (Type Q_1, Quantum Ind.) with *n*-BuOH–pyridine–AcOH–H_2O (15:10:3:12, v/v/v/v) (S_1) as the solvent system and/or the upper phase of the solvent system *n*-BuOH–AcOH–H_2O (4:1:5) (S_2). Material on the plates was detected according to the procedure of Zahn and Rexroth.[30]

Z-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-resin (I). Boc-Leu-resin (polystyrene–2% divinylbenzene resin) (10 g) containing 0.57 mmol of Leu/g of resin was washed with AcOH and then treated with 50 ml of 1.25 *N* HCl in AcOH for 30 min. The resin was washed successively with AcOH, EtOH, and DMF and then treated with 5 ml of Et_3N in 45 ml of DMF for 10 min. The neutralized Leu-resin was washed with DMF and suspended in 50 ml of DMF containing 25 mmol of Boc-Pro-*N*-hydroxysuccinimide ester[14] and 20 mmol of 1,2,4-triazole[15] and the suspension was shaken for 16 hr at room temperature. The Boc-Pro-Leu-resin was washed successively with DMF and EtOH. For the further extension of the peptide resin chain, the cycle of deprotection and neutralization described for Boc-Leu-resin preceded the introduction of each new protected amino acid active ester [i.e., fivefold molar excess of Boc-Cys(Bzl)-ONp,[15] Nps-Asn-ONSu,[16] Nps-Gln-ONSu,[16] Boc-Tyr(Bzl)-ONp,[15] and Z-Cys(Bzl)-ONp[17]. Boc-Ile[18] (25 mmol) was added to the growing peptide chain with DCCI (25 mmol) in 50 ml of CH_2Cl_2 with CH_2Cl_2 washes preceding and following the overnight coupling. After the addition of the Z-Cys(Bzl) residue to the peptide chain, the protected octapeptide resin (I) was washed successively with DMF, EtOH, AcOH, EtOH, and MeOH, and dried: wt 14.15 g.

[Des-Gly-NH_2^{9}]oxytocin (dG-OXY). The protected octapeptide resin I (3 g) was suspended in 40 ml of F_3CCO_2H containing 5 ml of anisole and HBr was slowly bubbled into the mixture for 2.5 hr at room temperature. The resin was removed by filtration and washed with F_3CCO_2H. The filtrate and washings were concentrated in vacuo to a yellow residue which dissolved in 200 ml of EtOH. Then 50 ml of Et_2O was added and the precipitate which formed overnight at 4° was filtered, washed with Et_2O, and dried: wt 930 mg (22%). A sample was hydrolyzed in 6 *N* HCl at 110° for 24 hr and subjected to amino acid analysis.[19] The following molar ratios were found: Asp, 1.0; Glu, 1.0; Pro, 1.0; Ile, 1.0; Leu, 1.0; Tyr, 0.9; Cys(Bzl), 2.0; and NH_3, 2.1. The preceding dibenzyl octapeptide hydrobromide (300 mg) was dissolved in 150 ml of boiling anhydrous NH_3, freshly distilled from Na. A fresh Na stick was introduced intermittently into the solution until a blue color persisted for 45 sec. The NH_3 was removed by lyophilization, and the resultant residue was dissolved in 600 ml of 0.05% F_3CCO_2H. The pH of the solution was adjusted to 8.3 with 3% NH_4OH and 0.01 *N* $K_3Fe(CN)_6$ was added with stirring until a yellow color persisted (39 ml). Stirring was continued for 30 min and then the pH was adjusted to 6 with dilute F_3CCO_2H. AG3-X4 resin (Bio-Rad Laboratories) (Cl form) was added and stirring was continued for 30 min to remove ferrocyanide and excess ferricyanide ions. The resin was removed by filtration, and the solution was concentrated in vacuo to 5 ml. This aqueous solution was mixed with 3 ml of the upper phase of the solvent system *n*-BuOH–1-PrOH–H_2O containing 0.125% pyridine and 0.625% AcOH (6:1:8) and applied to a Sephadex G-25 column (2.8 × 90 cm) that had been equilibrated with the lower phase of the solvent system. The column was eluted with the upper phase, and the chromatogram obtained by plotting the Folin–Lowry[20] color values of the fractions showed a major peak with an R_f of 0.29. The fractions corresponding to this peak were combined, diluted with twice their volume of water, concentrated on a rotary evaporator, and lyophilized: yield 56 mg (24%); $[\alpha]^{22}D$ −31.5° (*c* 0.5, 1 *N* AcOH). Anal. ($C_{41}H_{62}O_{12}N_{10}S_2$) C, H, N. The analog migrated as one component upon TLC in S_1 (R_f 0.7). Quantitative amino acid analysis after acid hydrolysis gave the following molar ratios: Asp, 1.0; Glu, 1.0; Pro, 1.0; $^1/_2$Cys, 1.9; Ile, 0.94; Leu, 1.0; Tyr, 0.9; and NH_3, 2.2.

[Des-Gly-NH_2^{9},des-Leu^8]oxytocin (dLG-OXY). The protected heptapeptide resin [Z-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro-resin] (II) was obtained as described above using Boc-Pro-resin (10 g) containing 0.35 mmol of Boc-Pro/g of resin. For the introduction of each new *tert*-butyloxycarbonylamino acid derivative, the ten-step cycle of deprotection, neutralization, and coupling described was followed. The peptide was cleaved from the resin and partially deblocked with HBr–F_3CCO_2H. From 6 g of protected peptide resin II, 0.86 g of crude dibenzyl heptapeptide hydrobromide was obtained. This material was dissolved in 5 ml of AcOH and 45 ml of H_2O was added. The solution was left at 4° overnight and the precipitate that formed was collected by filtration, washed with cold H_2O, and dried; yield 400 mg (18%). Quanititative amino acid analysis after acid hydrolysis gave the following molar ratios: Asp, 1.1; Glu, 1.0; Pro, 1.0; Ile, 1.0; Tyr, 0.9; Cys(Bzl), 2.1; and NH_3, 2.1. The preceding heptapeptide hydrobromide (150 mg) was reduced, oxidized, deionized, and concentrated as described for dG-OXY. The resulting aqueous solution (5 ml) containing the hormone analog was purified by partition chromatography on Sephadex G-25 with the same solvent system used for purifying dG-OXY. The hormone analog emerged from the column with an R_f of 0.15 and the appropriate fractions after removal of the organic solvent were lyophilized. The resulting powder was further purified by gel filtration through Sephadex G-25 with 0.2 *N* AcOH: yield 40 mg (35%); $[\alpha]^{25}D$ +1.48° (*c* 0.9, 95% EtOH). Anal. ($C_{35}H_{51}O_{11}N_9S_2$) C, H, N. Quantitative amino acid analysis after acid hydrolysis gave the following molar ratios: Asp, 1.0; Glu, 1.0, Pro, 1.1; $^1/_2$Cys, 2.2; Ile, 1.0; Tyr, 0.9; and NH_3, 2.0. TLC of the analogue in S_2 showed only one spot with an R_f of 0.35.

Bioassay Methods. At least five animals were used for the determination of the individual activities of the two analogs in the different assay systems. For bioassays a four-point design or matches were used employing USP Posterior Pituitary Reference Standard unless otherwise stated. Determinations for

oxytocic activity for each analog were performed on six isolated uterine horns from three rats in natural estrus, determined on the morning of the assay by vaginal smear. The method used was that of Holton[21] as modified by Munsick,[22] utilizing Mg^{2+}-free van Dyke–Hastings solution as bathing fluid. Avian vasodepressor assays were performed on a minimum of three conscious chickens[23] according to the procedure of Coon.[24] The pressor properties of the polypeptides were determined on a minimum of five atropinized, urethane-anesthetized male rats following the procedures of the U.S. Pharmacopeia.[25] The antidiuretic acitivty was examined on six Inactin- and ethanol-anesthetized, hydrated male Sprague–Dawley rats, according to the method of Jeffers et al.,[26] as modified by Sawyer;[27] maximal depression of the rate of urine flow was taken as the effective response. Water transport across the toad urinary bladder was measured according to the method of Bentley[28] as modified by Eggena et al.[29]

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References and Notes

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β-Lactam Antibiotics with *N*-Oxide Side Chains. 1. Quinoxaline *N*-Oxides

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A series of penicillin derivatives of quinoxaline di-*N*-oxide carboxylic acids was prepared. These compounds were prepared from the acid chlorides and mixed anhydrides of the quinoxaline di-*N*-oxides. The compounds prepared exhibited minimal antibacterial activity against gram-negative organisms.

An important aspect of current research in β-lactam antibiotics is the attempt to design compounds with enhanced activity against gram-negative bacteria. One approach is side-chain modification. We have observed exceptional activity against *Salmonella* and *Proteus* species with certain quinoxaline di-*N*-oxides[1] and felt that synthesis of amides of 6-aminopenicillanic acid (6-APA) and 7-aminodesacetoxycephalosporanic acid (7-ADCA) from quinoxaline di-*N*-oxide carboxylic acids might give compounds with increased gram-negative activity. Quinacillin (**1**), an example of a quinoxaline penicillin, is without gram-negative activity.[2] Few other examples of