Four Cyclic Disulfide Pentapeptides Possessing the Ring of Vasopressin[1a,b]

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Abstract: Pressinoic acid, the cyclic disulfide of cysteinyltyrosylphenylalanylglutaminylasparaginylcysteine which contains the pressin ring structure of the posterior pituitary hormones arginine- and lysine-vasopressin, has been synthesized by the stepwise *p*-nitrophenyl ester method. The closely related compounds deaminopressinoic acid, pressinamide, and deaminopressinamide have been synthesized by similar methods, and all have been tested for some of the biological activities shown by the parent hormones. None of the compounds showed any pressor or avian vasodepressor activities. All except pressinoic acid exhibited a slight degree of oxytocic activity, in the range of 0.05–0.5 unit/mg. No inhibitory properties were noted in the systems studied.

Establishment of the structure of the posterior pituitary hormone oxytocin (Figure 1) through synthesis[2] stimulated interest in knowing whether or not the ring portion of the molecule alone would possess any of the biological properties of oxytocin. Accordingly, Ressler synthesized a cyclic pentapeptide amide consisting of the 20-membered disulfide ring moiety of oxytocin with a primary amide group replacing the prolylleucylglycinamide side chain.[3] The compound was found to have low but significant oxytocic and milk-ejecting potencies (3.3 units/mg and 1.1 units/mg, respectively) but no avian vasodepressor (AVD) activity.

The same pentapeptide ring compound was later synthesized by a different method by Kaurov, Martynov, and Popernatskii,[4] and also by Hruby, Ferger, and du Vigneaud, who proposed the name "tocinamide" for it.[5] The latter authors also synthesized deaminotocinamide, in which the free amino group of tocinamide was formally replaced by hydrogen, and found it to have 34.2 ± 3.0 units/mg of oxytocic activity, ten times the activity of tocinamide. It possessed no AVD activity.

Recently Papsuevich and Cipens[6] synthesized the cyclic pentapeptide amide containing the 20-membered disulfide ring of the vasopressins (Figure 1). They reported it to have insignificant oxytocic activity and extremely low pressor activity. The present study reports a different synthesis of the same compound, here called pressinamide (Figure 2), and parallel syntheses of deaminopressinamide, pressinoic acid, and deaminopressinoic acid. The names follow the terminology previously proposed for these compounds.[5] These four compounds were synthesized from either *Z*-Cys(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-OBzl (I) or β-mercaptopropionyl(Bzl) - Tyr(Bzl) - Phe - Gln - Asn - Cys(Bzl)-OBzl (II). These protected peptides were prepared from suitably protected amino acid *p*-nitrophenyl esters,[7] using the stepwise method of peptide synthesis as developed in a synthesis of oxytocin.[8]

For preparation of pressinamide, the benzyl ester I was converted to the corresponding amide by refluxing in anhydrous NH_3. Subsequently this solution was treated with Na for the removal of the remaining protecting groups,[2,9] and the resulting disulfhydryl intermediate was converted by aeration to the desired cyclic disulfide compound.[2] Deaminopressinamide was made from ester II through ammonolysis and deprotection as just described and ring closure by treatment of the disulfhydryl intermediate with the Weygand reagent ICH_2CH_2I.[10] The two ring amides were purified on Sephadex G-25 by partition chromatography[11] and gel filtration.[12] Deaminopressinamide crystallized from H_2O as rosettes of needles.

For preparation of pressinoic acid, a solution of ester I in anhydrous NH_3 was treated with Na without prior refluxing in order to attain simultaneous cleavage of all protecting groups. The resulting disulfhydryl intermediate was oxidized to the cyclic disulfide by aeration. Partition chromatography of the product revealed a major peak of the desired product and a smaller peak that was identified as pressinamide. Further purification of the pressinoic acid fractions by gel filtration and recovery by lyophilization gave a white powder which crystallized readily from H_2O in the form of clusters of fine needles. Deaminopressinoic acid was prepared from ester II by cleavage of protecting groups with Na in liquid NH_3, followed by conversion of the disulfhydryl intermediate to the cyclic disulfide by treatment with ICH_2CH_2I. The product was separated from a small amount of deaminopressinamide by partition chromatography and further purified by gel filtration.

The four compounds prepared for the present study were tested for oxytocic,[13] AVD,[14] and rat pressor[15]

(1) (a) This work was supported in part by Grant No. HE-11680 from the National Heart Institute, U. S. Public Health Service. (b) All optically active amino acid residues are of the L variety. The symbols for amino acid residues follow the tentative rules of the IUPAC–IUB Commission on Biochemical Nomenclature.

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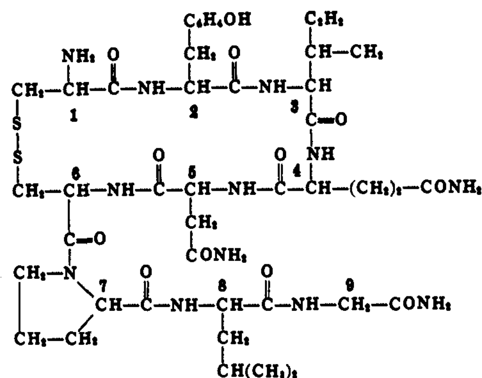

Figure 1. Structure of *oxytocin*, with numbers indicating the position of the individual amino acid residues. *Arginine-vasopressin* and *lysine-vasopressin* both have a phenylalanine residue instead of that of isoleucine at position 3 and an arginine or lysine residue, respectively, instead of a leucine residue at position 8.

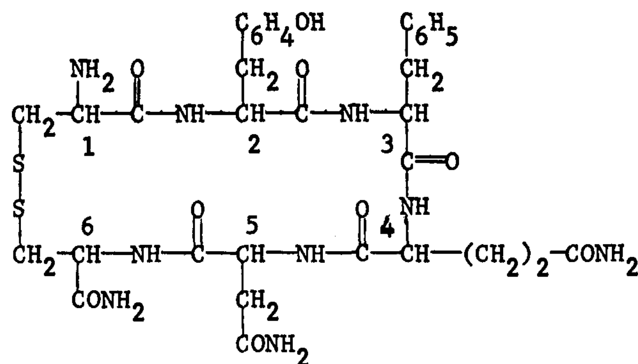

Figure 2. Structure of *pressinamide* with numbers indicating the position of individual amino acid residues. In *deaminopressinamide* the NH_2 at position 1 is replaced with H. In *pressinoic acid* the $CONH_2$ at position 6 is replaced with COOH. In *deaminopressinoic acid* the NH_2 at position 1 is replaced with H and the $CONH_2$ at position 6 is replaced with COOH.

activity and also for inhibitory properties in these systems. The four-point assay design[16] was used whenever measurable activity was encountered, and the U.S.P. posterior pituitary reference standard was used throughout. None of the compounds showed any detectable AVD or pressor activity. However, all except pressinoic acid exhibited a very slight degree of oxytocic activity, in the range of 0.05–0.5 unit/mg. No inhibition of the effects of the U.S.P. standard was noted in any of the assay systems.

The nearly total absence of biological activity of these compounds in the systems studied is in marked contrast to the high potencies of the naturally occurring hormones which contain the pressin ring plus a tripeptide amide side chain: lysine-vasopressin possesses approximately 50 units/mg of AVD activity,[17] 266 units/mg of pressor activity,[18] and 7 units/mg of oxytocic activity,[18] while arginine-vasopressin possesses approximately 65 units/mg, 435 units/mg, and 17 units/mg of the same activities, respectively.[19] Thus, the studies on the pentapeptide pressin ring compounds demonstrate, as in the case of oxytocin, the important contribution of their respective tripeptide amide side chains to the biological activity of these posterior pituitary hormones.

Experimental Section[20]

Boc-Asn-Cys(Bzl)-OBzl (III). HTos·Cys(Bzl)-OBzl[21] (8.33 g, 17.6 mmol) was suspended in peroxide-free tetrahydrofuran (100 ml) and dimethylformamide (DMF) (20 ml), cooled to 0°, treated with *N*-methylmorpholine (17.6 mmol) and then with Boc-Asn-ONp (5.65 g, 16 mmol), and stirred at room temperature for 48 hr. The product was precipitated with H_2O (600 ml), collected, and dissolved in EtOAc (700 ml). The resulting solution was washed with 0.2 *N* H_2SO_4, H_2O, and brine, then dried ($MgSO_4$), and evaporated to 250 ml. Crystallization occurred on addition of Et_2O (400 ml). The product was recrystallized from boiling acetone and washed with Et_2O: wt 5.48 g (66%); mp 145–147°; $[\alpha]^{23}D$ −37.7° (*c* 1, DMF); homogeneous by tlc (A). *Anal.* Calcd for $C_{26}H_{33}N_3O_6S$: C, 60.56; H, 6.46; N, 8.15. Found: C, 60.44; H, 6.65; N, 8.11.

Boc-Gln-Asn-Cys(Bzl)-OBzl. A solution of III (6.45 g, 12.5 mmol) in anhydrous trifluoroacetic acid (TFA) (19 ml) was stirred for 1 hr, then evaporated to dryness *in vacuo*. The oily residue was triturated with Et_2O, evaporated to dryness, then triturated again under Et_2O until it solidified, collected, washed well with Et_2O, and dried over KOH *in vacuo*. This product was dissolved in DMF (32 ml) and treated with *N*-methylmorpholine (12.5 mmol) at 0° and with Boc-Gln-ONp (4.61 g, 12.6 mmol). After stirring for 20 hr the mixture was filtered. The precipitate was washed with DMF (two 5-ml portions) and with Et_2O (two 50-ml portions) and dried *in vacuo*: wt 4.70 g (58%); mp 199–201°; $[\alpha]^{26}D$ −25.0° (*c* 0.5, DMF); homogeneous by tlc (A, B). A second crop (1.02 g, 13%) of equal purity was obtained by addition of H_2O (2 vol) to the DMF filtrate and washing of the precipitated product with EtOH, EtOAc, and Et_2O. A sample was crystallized from hot DMF by addition of two volumes of acetone. *Anal.* Calcd for $C_{31}H_{41}N_5O_8S$: C, 57.84; H, 6.42; N, 10.88. Found: C, 57.87; H, 6.45; N, 10.88.

Synthesis of the Subsequent Protected Tetra-, Penta-, and Hexapeptides. Each remaining protected peptide needed in this study was prepared in a similar manner by deprotection of the next lower Boc-peptide with TFA, addition of 1 equiv of *N*-methylmorpholine to the resulting salt in DMF solution, and condensation with a 10% excess of the appropriate nitrophenyl ester. As described in the next paragraph, disappearance of the free amino group was followed with the ninhydrin reagent of Troll and Cannan[22] as modified by Kaiser, *et al.*[23] In some cases it was found that the condensation reaction proceeded rapidly at first and then slowed markedly and apparently stopped. Addition of an amount of *N*-methylmorpholine equivalent to the unreacted free amine resulted in satisfactory completion of the condensation reaction. The work-up and characterization of each peptide are given in subsequent paragraphs.

Ninhydrin Color Test on Condensation Mixtures. The TFA salt of a peptide in DMF solution (0.002 m*M*) was used for preparation of a standard curve. Aliquots (5–35 μl) of this solution were incu-

(13) Oxytocic assays were performed on rats in natural estrus according to the method of P. Holton, *Brit. J. Pharmacol. Chemother.*, 3, 328 (1948), as modified by R. A. Munsick (*Endocrinology*, **66**, 451 (1960)) with the use of Mg-free van Dyke–Hastings solution as the bathing fluid.

(14) Avian vasodepressor assays were performed on conscious chickens by the method of J. M. Coon (*Arch. Pharm.*, **62**, 79 (1939)), as modified by R. A. Munsick, W. H. Sawyer, and H. B. van Dyke, *Endocrinology*, **66**, 860 (1960).

(15) Pressor assays were carried out on anesthetized male rats as described in "The Pharmacopeia of the United States of America," 18th rev, Mack Publishing Co., Easton, Pa., 1970, p 771.

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(20) All melting points were determined in capillary tubes and are uncorrected. Thin-layer chromatography was performed on silica gel G in the following solvent systems: (A) $CHCl_3$–CH_3OH, 9:1; (B) $CHCl_3$–CH_3OH–AcOH, 95:5:3; (C) $CHCl_3$–CH_3OH, 1:1; (D) $CHCl_3$–CH_3OH, 8:2; (E) *n*-BuOH–AcOH–H_2O, 3:1:1; (F) *n*-BuOH–pyridine–H_2O, 20:10:11; (G) $CHCl_3$–CH_3OH–AcOH, 8:1:1; (H) *sec*-BuOH–3% aqueous NH_4OH, 100:44.

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bated for 5 min at 100° with three drops each of three test reagents,[23] and then diluted with 95% EtOH (1.00 ml) and read after 10 min at 590 mμ against a reagent blank. During the course of the condensation reactions, appropriate aliquots of the reaction mixture were removed, tested in similar fashion, and compared to the standard curve for estimation of the amount of the free amine peptide remaining in solution. *N*-Methylmorpholine, the various *p*-nitrophenyl esters, DMF, and TFA did not interfere in the color test in the amounts present in the condensation mixtures.

Boc-Phe-Gln-Asn-Cys(Bzl)-OBzl. The semisolid condensation mixture obtained during synthesis of this compound on a scale of 9 mmol was stirred with EtOAc (160 ml). The product was separated by centrifugation, washed by centrifugation from EtOAc (60 ml) and then from Et_2O (60 ml), and finally crystallized from hot DMF by the addition of acetone (1:1): wt 4.80 g (68%); mp 213–216°; $[\alpha]^{24}D$ −22.6° (*c* 0.5, DMF); tlc (C) showed a trace of one contaminant. *Anal.* Calcd for $C_{40}H_{50}N_6O_9S$: C, 60.74; H, 6.37; N, 10.62; S, 4.05. Found: C, 60.62; H, 6.31; N, 10.62; S, 3.77.

Boc-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-OBzl. The condensation mixture obtained during synthesis of this compound on a scale of 5.1 mmol was stirred with 95% EtOH (four volumes) and centrifuged. The precipitate was washed in turn with 120 ml each of 95% EtOH, H_2O (two portions), 95% EtOH, and Et_2O, and collected each time by centrifugation: wt 4.0 g (76%); mp 229–231°; $[\alpha]^{24}D$ −19.0° (*c* 0.6, DMF); tlc (B,D) showed slight traces of two contaminants. *Anal.* Calcd for $C_{56}H_{65}N_7O_{11}S$: C, 64.41; H, 6.27; N, 9.39. Found: C, 64.02; H, 6.11; N, 9.18.

Pressinoic Acid. The reaction mixture resulting from condensation of Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-OBzl (1.4 mmol) with Z-Cys(Bzl)-ONp was stirred with EtOH (seven volumes). The resultant protected hexapeptide ester was collected by filtration and washed with H_2O (three 20-ml portions), EtOH (15 ml), Et_2O (two 10-ml portions): wt 1.38 g (78%); mp 243–246°; $[\alpha]^{23}D$ −31.8° (*c* 1, DMF); tlc (C) showed a trace of an immobile contaminant. This product was used successfully for synthesis of pressionic acid and pressinamide.

A stirred solution of this preparation of Z-Cys(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-OBzl (200 mg) in boiling liquid NH_3 (200 ml, freshly distilled from Na) was treated with Na in small amounts until a blue color pervaded the solution for 20 sec. After destruction of excess Na with NH_4Cl, the solution was evaporated to about 30 ml and then lyophilized. The residues from two such reaction procedures were dissolved in 0.1% TFA (800 ml), the pH was adjusted to 7 with NH_4OH, and the solution was oxidized by aeration at room temperature overnight. The Ellman test[24] for SH was negative. The solution was concentrated *in vacuo*, frozen at −20 to −30°, and lyophilized. The residue was dissolved in 10 ml of the upper phase of the solvent system 1-BuOH–10% aqueous pyridine containing 0.1% AcOH (1:1) and subjected to partition chromatography on a column (2.8 × 70 cm) of Sephadex G-25 (100–200 mesh) at a flow rate of 22 ml/hr. Peptide materials, detected by the Folin–Lowry method,[25] emerged in a major peak at R_f 0.13, which was well separated from a smaller peak at R_f 0.29 and several minor peaks. The materials isolated from the two main peaks were identified as pressinoic acid and pressinamide, respectively, by amino acid analysis.[26] The crude pressinoic acid, dissolved in 0.2 *N* AcOH (3 ml) and subjected to gel filtration at a flow rate of 31 ml/hr on a column (2.8 × 63 cm) of Sephadex G-25 (200–270 mesh) equilibrated with 0.2 *N* AcOH, emerged as a single sharp peak at 106% of the total column volume. The lyophilized product isolated from this peak dissolved readily in 0.5 *N* AcOH (3 ml) and began to crystallize in dense clusters of tiny needles within a few minutes: wt 84 mg; mp 202–204°; homogeneous on tlc (E,F). The compound was recrystallized from H_2O: $[\alpha]^{25}D$ −11.4° (*c* 0.5, *N* AcOH). Amino acid analysis following 25-hr hydrolysis in 6 *N* HCl gave the following molar ratios: Asp, 1.0; Glu, 1.0; Cys, 1.9; Tyr, 0.9; Phe, 0.9; NH_3, 1.8. *Anal.* Calcd for $C_{33}H_{42}N_8O_{10}S_2 \cdot H_2O$: C, 49.98; H, 5.59; N, 14.13. Found: C, 49.99; H, 5.50; N, 14.28. Pressinoic acid could also be crystallized from 95% EtOH. The compound exhibited no detectable oxytocic, pressor, or AVD activities and showed no inhibition of the effects of the U.S.P. standard in these systems.

Pressinamide. A solution of Z-Cys(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-OBzl (80 mg) in liquid NH_3 (100 ml, freshly distilled from Na) was refluxed for 4 hr. Subsequent steps for deprotection with Na, ring closure by aeration, isolation, and partition chromatography of the product, were carried out as described previously to give 31 mg of the lyophilized material from the major peak at R_f 0.32. This material was combined with similar fractions from other preparations (total of 138 mg), stirred in 0.2 *N* AcOH (14 ml), filtered, and subjected to gel filtration as described previously. The product was isolated from the major peak at 102% of the total column volume: wt 116 mg; $[\alpha]^{24}D$ −17.5° (*c* 1, *N* AcOH); homogeneous on tlc (E). Amino acid analysis following 22-hr hydrolysis in 6 *N* HCl showed the following molar ratios: Asp, 1.0; Glu, 1.0; Cys, 1.8; Tyr, 0.9; Phe, 1.0; NH_3, 2.8. *Anal.* Calcd for $C_{33}H_{43}N_9O_9S_2 \cdot H_2O$: C, 50.05; H, 5.73; N, 15.92. Found: C, 50.37; H, 5.63; N, 15.79. An oxytocic potency of approximately 0.24 unit/mg was measured. The compound had no pressor or AVD activity and no inhibitory properties.

Deaminopressinoic Acid. After condensation of Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-OBzl (1.0 mmol) with *p*-nitrophenyl *S*-Bzl-β-mercaptopropionate, the reaction mixture was filtered and the precipitate washed with EtOH (eight 5-ml portions): wt 0.88 g (79%); mp 235–238°; $[\alpha]^{23}D$ −27.7° (*c* 1, DMF); tlc (B,G) showed slight traces of impurities, but this preparation of *S*-Bzl-β-mercaptopropionyl-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-OBzl was found to give satisfactory results in the synthesis of deaminopressinoic acid and deaminopressinamide.

A solution of the above compound (200 mg, 0.18 mmol) in liquid NH_3 was treated with Na as described previously. The lyophilized products from this and an identical reaction mixture were dissolved in H_2O–acetone (350 ml:330 ml). A solution of freshly recrystallized ICH_2CH_2I (100 mg, 0.35 mmol) in acetone (20 ml) was added over a period of 1 min. Oxidation was complete in 5 hr, as judged by the Ellman test. The product was isolated by concentration and lyophilization, and treated with a mixture of upper phase (15 ml) and lower phase (2.5 ml) of the solvent system 1-BuOH–benzene–3.5% AcOH in 1.5% aqueous pyridine (2:1:3). Material soluble in this mixture was subjected to partition chromatography on a column (2.8 × 64 cm) of Sephadex G-25 (100–200 mesh) at a flow rate of 31 ml/hr, and the separated products were detected, isolated, and identified as described previously: deaminopressinoic acid (R_f 0.12), 168 mg; deaminopressinamide (R_f 0.29), 10 mg. A portion of the deaminopressinoic acid (50 mg) was subjected to gel filtration in 0.2 *N* AcOH and eluted at 130% of column volume: wt 32 mg; $[\alpha]^{24}D$ −39.9° (*c* 0.5, *N* AcOH); homogeneous on tlc (E,H). Amino acid analysis following 23-hr hydrolysis in 6 *N* HCl showed the following ratios: Asp, 1.0; Glu, 1.0; Tyr, 0.9; Phe, 1.0; NH_3, 2.0; Cys, 0.3; mixed disulfide of cysteine and β-mercaptopropionic acid, 0.6. *Anal.* Calcd for $C_{33}H_{41}N_7O_{10}S_2$: C, 52.29; H, 5.44; N, 12.90. Found: C, 51.98; H, 5.53; N, 12.77. An oxytocic potency of approximately 0.05 unit/mg was measured. The compound has no pressor or AVD activity and no inhibitory properties.

Deaminopressinamide. The procedures used for synthesis and purification of this compound were exactly the same as for deaminopressinoic acid except that the two solutions of the protected peptide precursor (200 mg in each) in liquid NH_3 were refluxed for 5.5 hr prior to the reduction with Na. Partition chromatography of the combined lots showed one main peak (R_f 0.30) from which 107 mg of product was isolated. After gel filtration (elution at 140% of column volume) the product was homogeneous in tlc (E,H). Amino acid analysis following 23-hr hydrolysis in 6 *N* HCl at 110° gave the following molar ratios: Asp, 1.0; Glu, 1.0; Tyr, 0.8; Phe, 0.9; NH_3, 2.9; Cys, 0.4; mixed disulfide of cysteine and β-mercaptopropionic acid, 0.6. *Anal.* Calcd for $C_{33}H_{42}N_8O_9S_2$: C, 52.23; H, 5.57; N, 14.77. Found: C, 52.46; H, 5.83; N, 14.55. This compound (26 mg) crystallized from H_2O (1 ml) after standing for 2 days at room temperature and could be recrystallized easily thereafter; $[\alpha]^{23}D$ −49.3° (*c* 0.5, *N* AcOH). An oxytocic potency of approximately 0.5 unit/mg was measured. The compound had no pressor or AVD activity and no inhibitory properties.

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