Effect of an Amide Group in Place of the Disulfide Bridge in Deamino-oxytocin†‡

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Synthesis and biological properties are reported of an analog of oxytocin, in which the cysteine residues in positions 1 and 6 are replaced by β-alanine and aspartic acid residues, respectively. The most significant feature of this analog ([*cyclo*-1-β-alanine-6-aspartic acid]oxytocin) is the formal substitution of the disulfide bond encountered in oxytocin by an amide linkage. This analog was found to possess 2.93 ± 0.08 unit/mg of milk-ejecting activity in rabbit, 1.13 ± 0.04 unit/mg of rat oxytocic activity, approximately 0.01 unit/mg of antidiuretic activity in rat, and no detectable pressor activity in rat. In the avian vasodepressor assay the analog is a weak inhibitor of oxytocin and lysine-vasopressin.

The disulfide bridge is a conspicuous structural component of neurohypophyseal hormones. However, because of synthetic difficulties, few analogs have been prepared which, while retaining the 20-membered ring of hormones, bear directly on the question of the steric and electronic function of the disulfide group. To date, analogs have been investigated in which one sulfur atom is replaced by a methylene group,[1-3] both sulfur atoms by an ethylene bridge,[2-7] or one or both sulfur atoms by selenium.[8-10] All of these analogs retain a high percentage of the biological activities of the parent hormone, as most convincingly shown with those analogs which have been secured as crystalline material.[11,12]

In this communication we report on the synthesis and biological properties of an analog of oxytocin in which the terminal amino group is replaced by a hydrogen atom and the disulfide bridge by an amide group. Of the two possible peptide isomers the cyclic lactam **11** shown in Figure 1 is described. This analog has also been prepared by Jošt, *et al.* Unfortunately, neither the mode of synthesis nor a detailed description of the biological activities has been reported, however, it has been mentioned that the material obtained was completely inactive.[13]

For the synthesis of **11** in our laboratories, the unprotected crystalline tripeptide amide, H-Pro-Leu-Gly-NH_2,[14] was acylated with Z-Asp(OBu^t)-OSu,[15,16] which places an Asp residue in place of a Cys residue in position 6 of oxytocin (see Figure 2). After removal of the N-terminal amino group the tetrapeptide was elongated in a stepwise fashion[17] with activated esters of appropriately protected Asn, Gln, Ile, and Tyr to yield the partially protected octapeptide **8**. In order to replace the Cys residue in position 1 of oxytocin by β-Ala, the N-terminal amino group of **8** was liberated, and the amine was acylated with Aoc-β-Ala-OSu to yield **9**. This peptide was converted to the activated ester **10**, from which the *N*-Aoc group was removed by treatment with CF_3COOH, and which then cyclized to the O-protected lactam at high dilution in pyridine.[18] The analog **11** was then obtained after dealkylation by hydrogenolysis and purification by gel filtration on Sephadex.

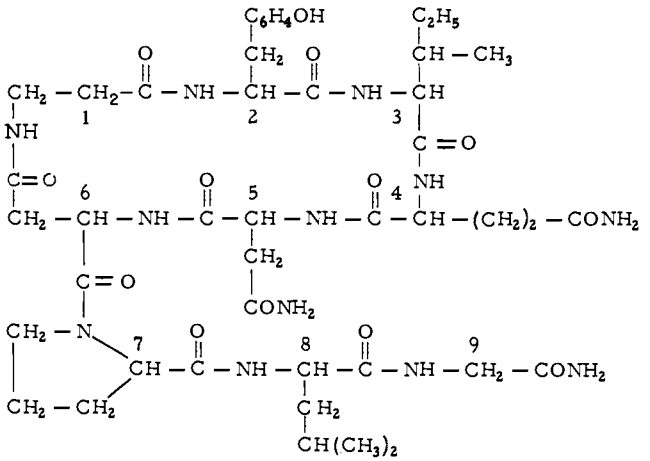

Figure 1. Oxytocin analog in which the cysteine residues in positions 1 and 6 of the hormone have been replaced respectively by β-alanine and aspartic acid residues.

Bioassay of [*cyclo*-1-β-alanine-6-aspartic acid]oxytocin revealed this analog to be a weak agonist of certain activities characteristic for neurohypophyseal hormones as well as a weak antagonist of these hormones in some other systems. The analog possesses 2.93 ± 0.08 (SEM) unit/mg of milk-ejecting activity in the lactating rabbit and 1.13 ± 0.04 (SEM) unit/mg of oxytocic activity on the isolated rat uterus. While the analog is ineffective in reducing the blood pressure in the chicken, it is a weak inhibitor of the avian vasodepressor response when injected 1–2 min prior to the standard dose of oxytocin or lysine-vasopressin. At a weight ratio of analog: oxytocin of 833:1 the response was inhibited by approximately 13%, which increased to approximately 25 and 62.5% at ratios of 1000:1 and 2000:1, respectively. The lysine-vasopressin-induced avian vasodepressor response was inhibited by *ca.* 25% at a 200:1 ratio of analog to hormone. Compound **11** exhibits an approximate antidiuretic activity of 0.01 unit/mg in rat, but fails to increase the blood pressure in this animal, even when tested in amounts as high as 1 mg of analog per injection.

Experimental Procedures

All chemical reactions were carried out at room temp, and intermediates and products were dried at room temp over P_2O_5 *in vacuo* unless otherwise noted. Organic solvents used for extraction were dried over anhydrous Na_2SO_4 and removed under reduced pressure. All melting points were detd with a Shibata capillary melting point apparatus and are not corrected. Samples used for

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‡Abbreviations used here are in accordance with the IUPAC-IUB Tentative Rules on Biochemical Nomenclature, *Biochemistry*, 5, 1445, 2485 (1966), and with suggestions in *J. Med. Chem.*, 15, 6A (Jan 1972). Other abbreviations used are: Aoc = *tert*-amyloxycarbonyl; –OSu = *N*-hydroxysuccinimide ester. The amino acids (except Gly and β-Ala) are of the L configuration.

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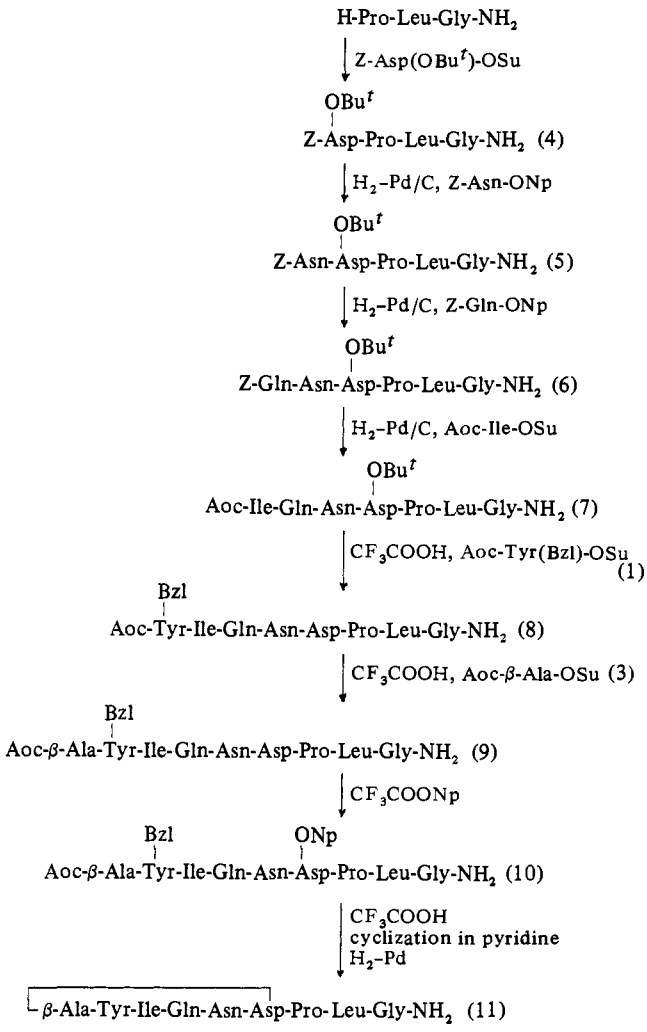

Figure 2. Scheme of the synthesis of [*cyclo*-1-β-alanine-6-aspartic acid]oxytocin.

elementary analysis and optical rotation detns were dried a second time under the conditions specified.

Aoc-Tyr(Bzl)-OSu (I). A soln of Aoc-Tyr(Bzl)-OH·DCHA[19] (5.67 g, 10 mmoles) in EtOAc was shaken with 1 *N* H_2SO_4, the organic layer was washed with H_2O, dried, and evapd. The oily residue was dissolved in a mixt of dioxane (10 ml) and EtOAc (10 ml) together with *N*-hydroxysuccinimide (1.26 g, 11 mmoles). A soln of DCC (2.17 g, 10.5 mmoles) in EtOAc (15 ml) was stirred into the mixt at 0°. Three hr later a few drops of AcOH were added to the reaction mixt. The dicyclohexylurea formed was filtered off 30 min later. The filtrate was evapd to an oily residue which was crystd from *i*-PrOH and the resulting material recrystd from the same solvent: 3.35 g (69.4%); mp 113–115°; $[\alpha]^{18}D$ −39.0° (*c* 2.2, DMF). The second crop was obtained from the mother liquor of the recrystn: 0.80 g (16.6%); mp 112–114°. *Anal.* ($C_{25}H_{30}O_7N_2$). Calcd: C, 64.7; H, 6.27; N, 5.81%. Found: C, 65.0; H, 6.20; N, 5.93%.

Aoc-β-Ala-OH·DCHA (2). To a soln of H-β-Ala-OH (1.78 g, 20 mmoles) in a mixt of dioxane (10 ml) and H_2O (5 ml), Aoc-N_3[19] (5 g, 32 mmoles) and Et_3N (5.6 ml, 40 mmoles) were added, and the mixt was stirred at 30° for 2 days. The reaction mixt was acidified to pH 1 with 1 *N* HCl, and pptd oily material was extd with EtOAc. The organic phase was washed with H_2O, and the product was extd with 5% $NaHCO_3$ soln. The $NaHCO_3$ ext was acidified with 2 *N* HCl and extd with EtOAc. The organic layer was washed with H_2O, dried, and concd. The oily residue was dissolved in a mixt of Et_2O and *n*-hexane and then neutralized with DCHA (4 ml, 0.02 mole) which induced crystn. Recrystn from Et_2O contg several drops of MeOH gave columns: 5.59 g (72.7%); mp 125–126°. A sample for analysis was dried for 23 hr at 80°. *Anal.* ($C_{21}H_{40}O_4N_2 \cdot 0.33H_2O$) Calcd: C, 64.6; H, 10.5; N, 7.17%. Found: C, 64.7; H, 10.6; N, 7.09%.

Aoc-β-Ala-OSu (3). Compd 2 (1.92 g, 5 mmoles) was converted to the free acid, Aoc-β-Ala-OH, as described for compd 1. The oily compd was dissolved in dry pyridine (3 ml), and CF_3COOSu[20] (10 mmoles) was added. After 30 min, a large amount of H_2O was added to the reaction mixt to ppt the product, which was extd with EtOAc the organic layer was washed repeatedly with 1 *N* HCl and then with H_2O, dried, and concd to dryness. The product was crystd from *n*-hexane and recrystd from Et_2O-*n*-hexane to yield needles: 1.15 g (76.7%); mp 83–84°. *Anal.* ($C_{13}H_{20}O_6N_2$) Calcd: C, 52.0; H, 6.71; N, 9.33%. Found: C, 51.6; H, 6.62; N, 9.22%.

Z-Asp(OBu^t)-Pro-Leu-Gly-NH$_2$ (4). Z-Asp(OBu^t)-OSu[15,16] (16.81 g, 40 mmoles) was added to a soln of H-Pro-Leu-Gly-NH$_2$[14] (11.37 g, 40 mmoles) in DMF (40 ml). The mixt was allowed to react for 2 days. A large amount of H_2O was added to give an oily ppt which was extd with EtOAc. To the organic layer dimethylaminopropylamine[21] (2 ml) was added. The soln was washed 30 min later successively with 1 *N* HCl, H_2O, 5% $NaHCO_3$, and H_2O, dried, and concd to dryness: 21.4 g (90.7%). This material was used in the following reaction without further purification. A sample for analysis was dried for 18 hr at 80°: $[\alpha]^{17}D$ −56.8° (*c* 3.1, DMF). *Anal.* ($C_{29}H_{43}O_8N_5$) Calcd: C, 59.1; H, 7.35; N, 11.9%. Found: C, 58.9; H, 7.60; N, 11.8%.

Z-Asn-Asp(OBu^t)-Pro-Leu-Gly-NH$_2$ (5). Compd 4 (11.79 g, 20 mmoles) was subjected to catalytic hydrogenolysis for 5 hr in EtOH (120 ml) using 5% Pd/C (1 g) as catalyst. The catalyst was removed by filtration and the solvent was evapd. The resulting solid was dissolved in DMF (30 ml), together with Z-Asn-ONp[17] (9.30 g, 24 mmoles), and the mixt was kept for 1 day. The reaction mixt was diluted with EtOAc (300 ml) and stored at 4° for several hr. The ppt formed was filtered, washed with EtOAc, and dried: wt 12.8 g. Recrystn from EtOH gave needles: 10.57 g (75.0%); mp 173–175°. A sample for analysis was dried for 18 hr at 100°: $[\alpha]^{17}D$ −60.8° (*c* 2.2, DMF). *Anal.* ($C_{33}H_{49}O_{10}N_9 \cdot 0.2H_2O$) Calcd: C, 56.0; H, 7.04; N, 13.9%. Found: C, 56.0; H, 7.13; N, 13.9%.

Z-Gln-Asn-Asp(OBu^t)-Pro-Leu-Gly-NH$_2$ (6). Compd 5 (10.56 g, 15 mmoles) was subjected to catalytic hydrogenolysis for 5 hr in a mixt of EtOH (120 ml) and H_2O (60 ml) using 5% Pd/C (1 g), as described above. The deprotected peptide was dissolved in DMF (60 ml) together with Z-Gln-ONp[17] (7.23 g, 18 mmoles), and the mixt was allowed to react for 3 days. The reaction mixt was diluted with EtOAc (400 ml) to give crude product as ppt which was filtered, washed with EtOAc, and dried. Repptn from 90% EtOH gave the product: 10.74 g (86.1%); mp 212–214° dec. A sample for analysis was dried for 16 hr at 80°: $[\alpha]^{18}D$ −55.6° (*c* 2.2, DMF). *Anal.* ($C_{38}H_{57}O_{12}N_8$) Calcd: C, 54.9; H, 6.91; N, 15.2%. Found: C, 54.7; H, 6.94; N, 15.1%.

Aoc-Ile-Gln-Asn-Asp(OBu^t)-Pro-Leu-Gly-NH$_2$ (7). Compd 6 (2.50 g, 3 mmoles) was subjected to catalytic hydrogenolysis in a mixt of EtOH (50 ml) and H_2O (30 ml) for 4 hr as described in the synthesis of compd 5. The deprotected peptide was dissolved in DMF (25 ml) together with Aoc-Ile-OSu[7] (1.23 g, 3.6 mmoles), and the mixt was kept for 16 hr. The reaction mixt was diluted with EtOAc (100 ml), cooled to 4°, and stored for several hr. Subsequently the pptd material was filtered, washed with EtOAc, and dried. Repptn from 95% EtOH gave the final product: 2.19 g (79.0%); mp 202–204° dec. A sample for analysis was dried for 16 hr at 80°: $[\alpha]^{18}D$ −53.7° (*c* 2.0, DMF). *Anal.* ($C_{42}H_{72}O_{13}N_{10}$) Calcd: C, 54.5; H, 7.85; N, 15.1%. Found: C, 54.3; H, 8.05; N, 15.1%.

Aoc-Tyr(Bzl)-Ile-Gln-Asn-Asp-Pro-Leu-Gly-NH$_2$ (8). Compd 7 (2.03 g, 2.2 mmoles) was dissolved in CF_3COOH (10 ml), and the soln was kept for 90 min. Volatile components were evapd, and the residual oil was triturated with Et_2O to give solid material. The ppt was filtered, washed with Et_2O, and dried over NaOH. The free peptide trifluoroacetate was dissolved in DMA (10 ml) together with compd 1 (1.27 g, 2.64 mmoles) and *N*-ethylmorpholine (0.3 ml) was added. While being stored for 24 hr the mixt solidified; EtOAc was added, and the solid material was filtered, washed with EtOAc, and dried. The crude product (2.45 g), suspended in 90% EtOH (100 ml), was refluxed for 30 min, and the insoluble material was isolated by filtration, washed with 90% EtOH, and dried: 2.00 g (80.9%); mp 225–227° dec. A sample for analysis was dried for 16 hr at 80°: $[\alpha]^{18}D$ −41.0° (*c* 1.1, DMSO). *Anal.* ($C_{54}H_{79}O_{15}N_{11}$) Calcd: C, 57.8; N, 7.10; N, 13.7%. Found: C, 57.5; H, 7.30; N, 13.8%.

Aoc-β-Ala-Tyr(Bzl)-Ile-Gln-Asn-Asp-Pro-Leu-Gly-NH$_2$ (9). Compd 8 (0.90 g, 0.8 mmole) was treated with CF_3COOH (8 ml) for 35 min and worked up as described above. The trifluoroacetate was dissolved in DMA (6 ml) together with compd 3 (0.30 g, 1 mmole), and *N*-ethylmorpholine (0.21 ml, 1.6 mmoles) was added. One day later the product was isolated as described: 0.90 g (93.8%); mp 234–236° dec. A sample for analysis was dried for 18 hr at 100°:

$[\alpha]^{18}D$ −41.2° (*c* 1.2, DMSO). *Anal.* ($C_{57}H_{84}O_{16}N_{12}\cdot 0.5H_2O$) Calcd: C, 56.9; H, 7.13; N, 14.0%. Found: C, 56.9; H, 7.28; N, 13.8%.

Aoc-β-Ala-Tyr(Bzl)-Ile-Gln-Asn-Asp(ONp)-Pro-Leu-Gly-NH_2 (10). CF_3COONp[4,20] (590 mg, 2.5 mmoles) was added to a suspension of compd 9 (600 mg, 0.5 mmole) in DMA (5 ml) contg pyridine (5 ml). The mixt was stirred for 3 hr at 50°. A large excess of Et_2O was added to the reaction mixt, and the resulting ppt was filtered, washed with Et_2O, and dried, wt 590 mg. Repptn from DMA (60 ml)–Et_2O (300 ml) gave the product: 466 mg (70%); mp 226–230° dec. A sample for analysis was dried for 18 hr at 100°: $[\alpha]^{18}D$ −39.1° (*c* 1.1, DMSO). *Anal.* ($C_{63}H_{87}O_{18}N_{13}\cdot H_2O$) Calcd: C, 56.8; H, 6.73; N, 13.7%. Found: C, 56.4; H, 7.04; N, 13.9%.

[β-Ala-Tyr-Ile-Gln-Asn-Asp-Pro-Leu-Gly-NH_2 (11). Compd 10 (394 mg, 0.296 mmole) was dissolved in CF_3COOH (3 ml), and the soln was allowed to react for 60 min. The soln was concd to an oily residue which solidified upon trituration with Et_2O. The powder was isolated by filtration, washed with Et_2O, and dried over NaOH. The *p*-nitrophenyl ester of *O*-Bzl-nonapeptide amide trifluoroacetate (390 mg) thus obtained was suspended in DMA (12 ml), and the suspension was added slowly to pyridine (300 ml) at 50° under vigorous stirring. Stirring was continued for an additional 5 hr at the same temp. The reaction mixt was then concd to a small volume and diluted with a large excess of Et_2O. The pptd material was filtered, washed with Et_2O, and dried: wt 346 mg. This material was dissolved in 100 ml of a H_2O–EtOH mixt (1:1), a small amt of insoluble material was removed by filtration, and the filtrate was successively passed through columns of CM-Sephadex (H^+ form, 2 × 8 cm) and Amberlite IR-45 (OH^- form, 0.9 × 20 cm). The columns were eluted and washed with the same solvent. The final effluent and washings were combined and concd to about 40 ml. The clear soln was subjected to catalytic hydrogenolysis for 15 hr using Pd Black as catalyst. The catalyst was removed by filtration and the filtrate was evapd to dryness. The material was dissolved in H_2O and lyophilized to yield 126 mg of powder. It was redissolved in 0.1 *N* AcOH (2 ml) and applied to a column of Sephadex G-25 (2 × 138 cm), which was developed with 0.1 *N* AcOH. The fractions contg product, as detected by measurement of uv absorption at 280 nm, were pooled, lyophilized, and dried: yield 61 mg (21% based on compd 10). Paper chromatography of the product (Toyo Roshi No. 51) with the solvent system *n*-BuOH–AcOH–H_2O (4:1:1) showed only one spot with an R_f of 0.56 (Tyr had an R_f of 0.39) using Pauly reagent;[22] in the solvent system *n*-BuOH–C_6H_5N–H_2O (4:1:1) again only one spot (R_f 0.45; Tyr R_f 0.25) was found. In both chromatograms no ninhydrin-active component was detectable. Similarly, the product was homogeneous upon paper electrophoresis (Toyo Roshi no. 514, pH 4.8, 0.2 *M* C_5H_5N–AcOH). A sample for analysis was dried for 24 hr at 100°; $[\alpha]^{17}D$ −47.5° (*c* 0.38, H_2O). Loss of wt upon drying was 6.1%. *Anal.* ($C_{44}H_{66}O_{13}N_{12}\cdot H_2O$) Calcd: C, 53.4; H, 6.93; N, 17.0%. Found: C, 53.5; H, 6.83; N, 16.8%. Mol wt, calcd: 989. Found: 999.

A sample was hydrolyzed with 6 *N* HCl in the presence of phenol for 48 hr at 105° and subjected to amino acid analysis;[23] the following molar ratios were obtained with Leu taken as 1.00: β-Ala, 1.00; Tyr, 0.97; Ile, 1.00; Glu, 1.00; Asp, 2.00; Pro, 0.97; Leu, 1.00; Gly, 0.97.

Bioassay Methods. At least 3 animals were used for the determination of the activity of compd 11 in the different assay systems. For bioassays a four-point design or matches were used, generally employing USP Posterior Pituitary Reference Standard. When compd 11 was tested for its inhibitory properties, oxytocin or lysine-vasopressin served as standards. A minimum of 3 animals was used for testing the activities characteristic of oxytocin, and no less than 6 animals for the determination of activities characteristic of vasopressin. Milk-ejecting activity was measured in the urethane-anesthetized, lactating rabbit, as described by Chan.[24] Determinations of oxytocic activities were performed on isolated uterine horns from rats in natural estrus, determined on the morning of the assay by vaginal smear by the method of Holton[25] as modified by Munsick,[26] utilizing Mg^{2+}-free van Dyke–Hastings soln. Avian vasodepressor assays were performed on conscious chickens according to the procedure of Munsick, *et al.*[27] The antidiuretic activity was examined in inactin- and ethanol-anesthetized, hydrated male Sprague–Dawley rats, according to the method of Jeffers, *et al.*,[28] with some modifications. Pressor activity was deternd in atropinized, urethane-anesthetized male rats following the procedures in the U. S. Pharmacopeia.[29]

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