6-*O*-(2,3,4-Tri-*O*-benzyl-α-D-glucopyranosyl)-2,3,4-tri-*O*-benzyl-D-glucopyranosyl-*O*-[PNV] (**6**) was prepared from compound **5** (0.197 g) as described for the preparation of compound **4**: yield, 0.160 g (Thr, 0.187 g). The carbonyl absorption at 1730 cm^{-1} is not present in this compound. Assuming quantitative transesterification of compound **5** and taking in account the theoretical decrease in weight of resin, the calculated amount of saccharide attached to resin **6** is 0.32 mmol/g.

Anal. Found: N, 1.58; OCH_3, 2.36.

Release of Saccharide. Resin **6** (0.2 g) was suspended, by stirring, in dioxane (250 ml) and irradiated for 32 hr. The resin (**7**) was filtered off (it had an absorption at 1720 cm^{-1}) and the solution was evaporated *in vacuo* to a third of its original volume. Water (100 ml), acetic acid (1 drop), and a small amount of 10% palladium on charcoal were added and the solution was hydrogenated for 18 hr at room temperature and at 46 psi. The catalyst was then filtered off and the solution evaporated *in vacuo*. The residue was dissolved in water (10 ml) and was checked (40–100-μl samples) by paper chromatography (in the two solvent systems indicated) and was shown to contain a material migrating like isomaltose, a material migrating like glucose, and a few additional spots, probably corresponding to partially benzylated products. A portion of the aqueous solution (1.0 ml) was freeze dried and separated by preparative paper chromatography (system II). The products were eluted from the paper as follows. The bands containing glucose and isomaltose were cut into slices and placed into the outer part of a 5-ml disposable syringe that had been plugged with some washed cotton. The paper was then wet with water and the syringe centrifuged inside a 12-ml conical tube. The last step was repeated several times using a total of about 2.5 ml of water. Finally the yield of products was determined by the phenol–sulfuric acid test: glucose, 0.058 mmol/g (17.7%); isomaltose, 0.041 mmol/g (12.5%). Yields are based on the saccharide content of compound **6**. The yield of free disaccharide, isomaltose, remains similar (10.8%) also when calculated on the basis of the monosaccharide derivative **4**. Isomaltose was obtained on freeze drying. The trimethylsilyl derivative of the synthetic isomaltose was compared to trimethylsilylated isomaltose and gentibiose by glc (22 psi, 178°) and was shown to contain the peaks corresponding to isomaltose at retention time 13.7 and 18.5 min. No evidence for gentibiose (10% contamination should have been detected), retention time 16.8 and 18.5 min, was obtained. The synthetic isomaltose had $[\alpha]^{23}D$ + 108° (*c* 0.14, water) and its purity was evaluated by a method developed for the determination of glucose, isomaltose, and gentibiose in admixture.[13] A solution of the synthetic isomaltose (120 μg/ml, by phenol–sulfuric acid test minus a small amount of contaminating glucose determined with glucose oxidase) was digested by α-glucosidase and the glucose obtained was determined with glucose oxidase (97 μg/ml). When the same was repeated with β-glucosidase, no evidence for digestion was obtained.

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Synthesis and Some Pharmacological Properties of [1-Deamino,9-thioglycine]oxytocin[1,2]

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Abstract: [1-Deamino,9-thioglycine]oxytocin, an analog of deamino-oxytocin in which the C-terminal carboxamide function has been formally replaced by a thiocarboxamide group, has been synthesized by coupling deaminotocinoic acid (the cyclic disulfide of β-mercaptopropionyltyrosylisoleucylglutaminylasparaginylcysteine) with prolylleucylthioglycinamide. The latter peptide was elaborated from thioglycinamide prepared by the treatment of Z-Gly-NH_2 with P_2S_5, followed by removal of the protecting group. The analog was found to possess 46 ± 5 units/mg of oxytocic activity and 14.9 ± 0.3 units/mg of avian vasodepressor activity, or approximately 6 and 1.5%, respectively, of the corresponding activities of crystalline deamino-oxytocin.

During the systematic assessment of the importance of the various functional groups of oxytocin (Figure 1) to its biological activity, it was determined that the C-terminal amide is a structural feature important to the biological activity of the hormone. In these studies the C-terminal $CONH_2$ group of oxytocin, or its highly potent analog deamino-oxytocin, was formally replaced by COOH,[3,4] $CONHCH_3$,[5] $CON(CH_3)_2$,[5] and H.[6] For each of the resulting analogs, the biological activities were found to be drastically lower than those of the parent compounds. In the studies which left the glycine residue intact, all the structural modifications introduced were in the noncarbonyl portion of the amide. In this paper we wish to report an extension of this study, wherein the carbonyl moiety of the C-terminal amide of deamino-oxytocin has been formally replaced by a thiocarbonyl group, thus forming an analog bearing a thioglycinamide residue. The synthesis of this compound, [1-deamino,9-thioglycine]oxytocin ([1-deamino,9-Glyt]-oxytocin),[2] was undertaken not only as a part of this structure–activity study, but also as a test of the feasibility of using the thioamide functional group in the context of polypeptide synthetic chemistry.

Ried and coworkers[7] have reported the preparation of several thioamide containing dipeptides, which have

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(2) The symbol Glyt is used to indicate the thiocarbonyl analog of the glycine residue. This symbol is consistent with the Recommendations (1971) of the IUPAC–IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, 247, 977 (1972), whose symbols for the other amino acid residues were followed. The optically active amino acid residues are of the L configuration.

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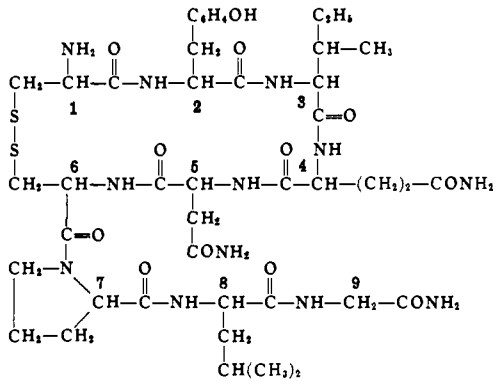

Figure 1. Structure of oxytocin with numbers indicating the position of the individual amino acid residues. In deamino-oxytocin the NH_2 at position 1 is replaced with H.

the general structure: XNHCRHCSNHCR'HCOOH, where X = tosyl or benzyloxycarbonyl, and R and R' represent the side chains of the amino acid residues. These N-protected peptides, termed endothiopeptides according to the nomenclature proposed by Wieland and Bartmann,[8] were synthesized by the reaction under basic conditions of an amino acid with an N-blocked amino acid thionoester. Wieland and Bartmann have reported the synthesis of peptides having a C-terminal thioacid, for which the class name terminal-thiopeptide was proposed. A thorough review of the chemistry of thioamides by Walter and Voss[9] appeared in 1970.

The route chosen to synthesize [1-deamino,9-Glyt]-oxytocin was a condensation of deaminotocinoic acid,[10] which possesses the disulfide ring moiety of deamino-oxytocin, with the tripeptide Pro-Leu-Glyt-NH_2, which represents the "tail" portion of the hormone analog. During the course of this work, we learned from Professor J. Rudinger of a recently completed synthesis of oxytocin and deamino-oxytocin by this "ring plus tail" approach by Professor Rudinger and his colleagues.[11] The use of preformed disulfide rings in peptide fragment condensations had previously been used by Hiskey[12] and by Rittel, *et al.*[13]

The first key intermediate used in our synthesis, deaminotocinoic acid, was prepared by a modification of the procedure used previously.[10] N^{α}-Boc protection was used throughout, and the C terminus was protected as the benzyl ester as in the synthesis of deamino-pressinoic acid.[14] The intermediate peptides were thus the same as those prepared by Mühlemann, *et al.*[11] However, all residues except isoleucine were incorporated by the *p*-nitrophenyl ester method.[15] Boc-isoleucine was coupled in high yield by means of the dicyclohexylcarbodiimide-1-hydroxybenzotriazole (DCCI-HBT) preactivation procedure[16] which was modified by the removal of dicyclohexylurea (DCU) before coupling.

For comparative purposes, it was decided to synthesize deamino-oxytocin by the coupling of deaminotocinoic acid and Pro-Leu-Gly-NH_2, following the procedure of Mühlemann, *et al.*,[11] before proceeding with the synthesis of the thioamide analog of deamino-oxytocin. The deamino-oxytocin so obtained was isolated in an overall yield of 48% in crystalline form after purification by partition chromatography[17,18] and gel filtration.[19] After one recrystallization from H_2O (75% recovery) and overnight drying at 0.04 mm at room temperature, it was compared directly in the oxytocic and avian vasodepressor (AVD) assays[20] with a sample of crystalline deamino-oxytocin that had been synthesized by the stepwise *p*-nitrophenyl ester method,[18] purified, and dried in an identical manner. The potencies of the two samples agreed within experimental error. Both samples had $[\alpha]^{23}D$ 103 ± 1° (*c* 0.5, 1 *N* HOAc).

The synthesis of Pro-Leu-Glyt-NH_2 began with the hydrobromide of thioglycinamide, prepared according to the method of Jakopčić and Hahn.[21] In this method Z-Gly-NH_2 is treated with phosphorus pentasulfide in dioxane to yield Z-Glyt-NH_2, which is deprotected with HBr–HOAc. Reaction of thioglycinamide with Boc-Leu-ONp gave Boc-Leu-Glyt-NH_2, which was deblocked with HCl–HOAc. The product was coupled by the mixed anhydride procedure, as described by Vaughan and Eichler,[22] to Boc-Pro-OH. The protected tripeptide obtained was deblocked with HCl–HOAc to yield Pro-Leu-Glyt-NH_2, and the latter was coupled with deaminotocinoic acid using DCCI–HBT. The resulting [1-deamino,9-Glyt]oxytocin was purified by partition chromatography followed by gel filtration. In each purification system the thio analog behaved quite differently than did deamino-oxytocin in the same system.

The presence of the thioamide group presented no particular problems in the synthesis of the thio tripeptide and the thio analog. The thioamide withstood the conditions of peptide coupling, using both preactivated and *in situ* activated carboxyl groups, as well as conditions normally used for Boc-group removal.

The chromatographically pure [1-deamino,9-Glyt]-

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oxytocin was found to possess 46 ± 5 USP units/mg of oxytocic and 14.9 ± 0.3 USP units/mg of AVD activity when assayed against a synthetic oxytocin standard. Thus the formal substitution of sulfur for oxygen in the C-terminal amide of deamino-oxytocin has given an analog with drastically altered bioactivities. The oxytocic and AVD activities have been reduced to approximately 6 and 1.5%, respectively, of the corresponding activities of crystalline deamino-oxytocin reported by Ferrier, *et al.*,[18] which were 803 ± 36 units/mg (oxytocic) and 975 ± 24 units/mg (AVD) on an anhydrous basis. These results again emphasize the importance of the integrity of the glycinamide residue in the highly potent deamino-oxytocin for full expression of its biological activity.

Experimental Section[23]

Z-Glyt-NH_2 (I). A rapidly stirred suspension of Z-Gly-NH_2 (3.64 g, 17.5 mmol) and phosphorus pentasulfide (1.94 g, 87.5 mmol) in peroxide-free dioxane (30 ml) was heated in an oil bath from room temperature to 95° during a 15-min period. The hot, slightly yellow suspension was stirred for 2 min as boiling began. Strict control of the reaction conditions is necessary. The reaction mixture was poured into stirred 50% saturated NaCl solution. Crystals began to form immediately. The mixture was held at 0° for 30 min, and the crude product was collected, washed with H_2O, and dried *in vacuo*, wt 3.18 g. Recrystallization of the crude product from $CHCl_3$ (40 ml/g) resulted in 51% overall yield: mp 141–143°; homogeneous by tlc (B); uv max (95% EtOH) 265 nm (ϵ 1.39 × 10^4).[24] *Anal.* Calcd for $C_{10}H_{12}N_2O_2S$: C, 53.55; H, 5.39; N, 12.49; S, 14.30. Found: C, 53.64; H, 5.35; N, 12.46; S, 14.23.

HBr-Glyt-NH_2 (II). I (3.48 g, 15.6 mmol) was treated with 40% wt/wt HBr–HOAc (30 ml). Et_2O was added to the slightly yellow suspension after 20 min; the product was collected and washed with Et_2O. The solid, which retained the odor of HBr after drying *in vacuo* over NaOH, was washed with HOAc (20 ml) and with Et_2O and again dried *in vacuo:* wt 2.50 g (95%); mp 167–168° dec (lit.[21] mp 158–159°); uv max (95% EtOH) 264 nm (ϵ 1.16 × 10^4). The compound was used without further purification or characterization.

Boc-Leu-Glyt-NH_2 (III). Boc-Leu-ONp (4.93 g, 14.0 mmol) and II (2.38 g, 14.0 mmol), suspended in EtOAc (70 ml) at 0°, were treated with *N*-methylmorpholine (1.52 ml, 14.0 mmol) and stirred at room temperature for 24 hr. The reaction mixture was partitioned in EtOAc–H_2O (250/250 ml); the EtOAc solution was washed successively with 1 *M* $NaHCO_3$, 50% saturated NaCl solution, and brine. The dried ($MgSO_4$), buff colored solution was reduced in volume (40 ml) and diluted with cyclohexane (200 ml). The tan powder which precipitated slowly at 5° was purified further by chromatography on a column (2.8 × 32 cm) of silica gel (70–325 mesh) packed in $CHCl_3$. A bright yellow impurity was eluted from the column with $CHCl_3$, and the product was eluted with $CHCl_3$–CH_3OH (19:1, v/v). The solvents were removed, and the desired material crystallized at 5° from EtOAc–cyclohexane (1:6, 350 ml). The material was collected, washed with cyclohexane, and dried *in vacuo:* wt 3.07 g (73%); mp 164–166°; $[\alpha]^{23}D$ −5.4° (*c* 1.0, dimethylformamide (DMF)); homogeneous by tlc (A); uv max (95% EtOH) 266 nm (ϵ 1.40 × 10^4). *Anal.* Calcd for $C_{13}H_{25}N_3O_3S$: C, 51.46; H, 8.30; N, 13.85; S, 10.57. Found: C, 51.40; H, 8.26; N, 13.90; S, 10.36.

Boc-Pro-Leu-Glyt-NH_2 (IV). III (303 mg, 1 mmol) was dissolved in 1 *N* HCl–HOAc (5 ml) and stirred for 20 min. The slightly yellow solution was added dropwise to Et_2O to precipitate the dipeptide salt, which was collected, washed with Et_2O, and dried *in vacuo*.

Boc-Pro-OH (215 mg, 1 mmol), dissolved in peroxide-free tetrahydrofuran (THF) (2 ml), was treated with *N*-methylmorpholine (0.11 ml, 1 mmol), cooled to −15°, and reacted with isobutyl chloroformate (0.13 ml, 1 mmol). After 2 min a THF (2 ml) solution of the dipeptide salt at −15° was added, followed immediately by *N*-methylmorpholine (0.11 ml, 1 mmol). The reaction mixture was kept at −10 to 0° for 10 min and then stirred at room temperature for 4 hr.

The solvent was evaporated and the crude product partitioned in EtOAc–H_2O (2:1, 30 ml). The EtOAc layer was washed successively with H_2O, 1 *N* $NaHCO_3$, H_2O, and brine. The dried (Na_2SO_4) solution was reduced to a volume of 1–2 ml; crystallization began quickly. The crystals were collected, washed with EtOAc and cyclohexane, and dried *in vacuo:* wt 217 mg (54%); mp 142–144°; homogeneous by tlc (A). An analytical sample was prepared by recrystallization from EtOAc (5 ml/g), recovery 72%: mp 143.5–145.5°; $[\alpha]^{23}D$ −42.8° (*c* 0.65, DMF); uv max (95% EtOH) 267 nm (ϵ 1.24 × 10^4). *Anal.* Calcd for $C_{18}H_{32}N_4O_4S$: C, 53.97; H, 8.05; N, 13.98; S, 8.00. Found: C, 54.14; H, 7.96; N, 14.13; S, 8.07.

[1-Deamino,9-Glyt]oxytocin (V). IV (39.2 mg, 0.098 mmol), contained in a small conical centrifuge tube, was reacted with 1 *N* HCl–HOAc (0.5 ml) for 20 min at room temperature. Addition of Et_2O (10 ml) precipitated the tripeptide salt, which was spun down. Following removal of the supernatant, the solid was washed three times while still in the centrifuge tube with Et_2O. After the final removal of Et_2O, the white solid was dried over P_2O_5 *in vacuo*.

To the centrifuge tube containing the deprotected tripeptide salt was added deaminotocinoic acid (52.8 mg, 0.07 mmol) and 1-hydroxybenzotriazole (18.8 mg, 0.14 mmol). The viscous solution obtained upon adding 0.70 ml of dimethylformamide containing 0.098 ml of *N*-methylmorpholine was treated at 0° with solid DCCI (17.4 mg, 0.084 mmol). The reaction was stirred at 0° for 2 hr and at room temperature for 24 hr. The solids present were removed by filtration and washed with DMF (2 × 0.1 ml). The filtrate wash was evaporated to a thin oil which was dissolved in the upper phase (1 ml) of the solvent system 1-BuOH–C_6H_6–pyridine–HOAc–H_2O (100:100:3:7:190).[18] The solution was subjected to partition chromatography on a column (2.8 × 63 cm) of Sephadex G-25 (100–200 mesh) at a flow rate of 24 ml/hr. A major peak, detected by the Folin–Lowry method,[25] emerged at R_f 0.72,[26] mostly separated from a much smaller peak at a higher R_f. The material, 67 mg, which was isolated from the major peak by evaporation and lyophilization from aqueous HOAc, was dissolved in 8.5 *N* HOAc (0.7 ml), diluted with 0.2 *N* HOAc (1.2 ml), and subjected to gel filtration at a flow rate of 25 ml/hr on a column (2.8 × 68 cm) of Sephadex G-25 (200–270 mesh) equilibrated to 0.2 *N* HOAc. The [1-deamino,9-Glyt]oxytocin emerged as a sharp symmetrical peak at 96% of the column bed volume:[27] wt 37 mg (53%); $[\alpha]^{24}D$ −76.4° (*c* 0.208, 8.5 *N* HOAc); homogeneous on tlc (C); uv max (8.5 *N* HOAc) 265 nm (ϵ 1.31 × 10^4). Amino acid analysis[28] after a 19-hr hydrolysis in 6 *N* HCl gave the following ratios: Asp, 1.02; Glu, 1.00; Pro, 1.09; Gly, 1.00; Ile, 0.93; Leu, 0.99; Tyr, 0.93. A sample hydrolyzed identically after a performic acid oxidation by the method of Moore[29] had a cysteic acid to Asp ratio of 1.02. *Anal.* Calcd for $C_{43}H_{65}N_{11}O_{11}S_3 \cdot 3H_2O$: C, 48.62; H, 6.74; N, 14.50; S, 9.06. Found: C, 48.75; H, 6.62; N, 14.38; S, 9.21.

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