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Four Cyclic Disulfide Pentapeptides Possessing the Ring of Isotocin and Glumitocin†

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[4-Serine]tocinoic acid and [4-serine]tocinamide (the 20-membered disulfide pentapeptide and pentapeptide amide ring of isotocin and glumitocin), as well as the two deamino analogs [4-serine]deaminotocinoic acid and [4-serine]deaminotocinamide, were synthesized from four protected polypeptide precursors which had been prepared by the stepwise active ester method. All four precursors were prepared from the same intermediate Boc-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl (1). For the preparation of the two-ring compounds containing C-terminal amides, 1 was treated with MeOH saturated with NH₃ *prior* to condensation with either Boc-Cys(Bzl)-ONSu or β-Mpa(Bzl)-ONp. For the preparation of [4-serine]tocinoic acid and [4-serine]deaminotocinoic acid, 1 was condensed with either Z-Cys(Bzl)-ONp or β-Mpa(Bzl)-ONp. The resulting four protected precursors were then converted to the corresponding ring compounds by deprotection with Na in NH₃, followed by oxidative cyclization. None of the ring compounds showed any significant oxytocic, avian vasodepressor, or rat pressor activity. All showed a slight degree of antioxytocic and antiavian vasodepressor activity but no antipressor activity. Both [4-serine]tocinoic acid and [4-serine]tocinamide showed some milk-ejecting activity.

At the present time nine chemically related polypeptide hormones have been isolated from the posterior pituitary gland of mammals, birds, amphibians, and fish: oxytocin,[2] arginine-vasopressin,[3] lysine-vasopressin,[4] vasotocin,[5] mesotocin,[6] isotocin,[7] glumitocin,[8] valitocin,[9] and aspartocin.[9] All of these hormones contain a 20-membered disulfide ring.

The four cyclic polypeptides corresponding to the ring moiety of arginine- and lysine-vasopressin

(Cys—Tyr—Phe—Gln—Asn—Cys),
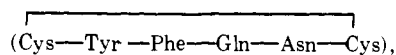
pressinoic acid[10] and pressinamide,[10-13] and their deamino analogs, deaminopressinoic acid,[10] and deaminopressinamide,[10,14] have been synthesized. None of these four

compounds showed any pressor[10,13,14] or avian vasodepressor (AVD)[10] activities. Ferger, *et al.*, found that all except pressinoic acid showed a slight degree of oxytocic activity in the range of 0.05–0.5 unit/mg.[10] Under comparable conditions of oxytocic assay, Zaoral and Flegel[13,14] found 0.26 and 1.03 units/mg for pressinamide and deaminopressinamide, respectively. They also reported antidiuretic and milk-ejecting activity for both compounds.

Four polypeptides corresponding to the ring moiety of oxytocin, vasotocin, valitocin, and mesotocin

(Cys—Tyr—Ile—Gln—Asn—Cys)
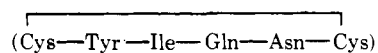
have also been synthesized. Tocinoic acid[15] and tocinamide[13,16-18] have oxytocic potencies of 0.2–0.3 unit/mg[15] and about 3 units/mg,[13,16,18] respectively. Deaminotocinoic acid[15] has an oxytocic potency of about 3.7 units/mg, while deaminotocinamide was found by Hruby, *et al.*,[18] to have 34 units/mg and by Zaoral and Flegel[14] to have 16 units/mg of this activity. Zaoral and Flegel have also re-
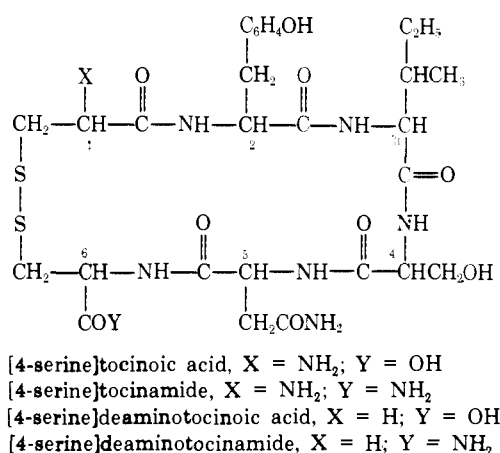
isotocin, $R = CH(CH_3)CH_2CH_3$
glumitocin, $R = CH_2CH_2CONH_2$
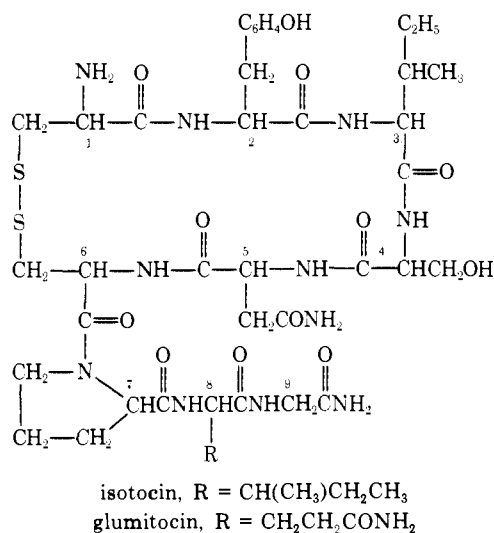
Figure 1. Structure of isotocin and glumitocin with numbers indicating the position of the individual amino acid residues.

[4-serine]tocinoic acid, $X = NH_2$; $Y = OH$
[4-serine]tocinamide, $X = NH_2$; $Y = NH_2$
[4-serine]deaminotocinoic acid, $X = H$; $Y = OH$
[4-serine]deaminotocinamide, $X = H$; $Y = NH_2$

Figure 2. Structure of [4-serine]tocin ring compounds with numbers indicating the position of the individual amino acid residues.

ported milk-ejecting activity for tocinamide[13] and antidiuretic potencies for tocinamide[13,14] and deaminotocinamide.[14]

Until the recent isolation of aspartocin[9] ([4-asparagine]oxytocin), the only known natural hormones with a ring structure different from those of oxytocin and vasopressin were isotocin ([4-serine,8-isoleucine]oxytocin), found in some teleosts, and glumitocin ([4-serine,8-glutamine]oxytocin), found in some elasmobranches. Both of these hormones (Figure 1) have a serine residue in the 4 position replacing the glutamine residue of oxytocin. Detailed pharmacological studies on the first synthetic isotocin[19] and glumitocin[20,21] gave results comparable to those found for the isolated natural products.[7,8] Isotocin was found to have oxytocic, AVD, and rabbit milk-ejecting potencies of 150, 320, and 300 units/mg and antidiuretic and rat pressor potencies of 0.18 and 0.06 unit/mg, respectively. Synthetic glumitocin has oxytocic, rabbit milk-ejecting, antidiuretic, and pressor potencies of 8–10, 53, 0.41 and 0.35 units/mg, respectively. We report here the synthesis and some of the pharmacological properties of four cyclic peptides, [4-serine]tocinoic acid, [4-serine]deaminotocinoic acid, [4-serine]tocinamide, and [4-serine]deaminotocinamide (Figure 2), corresponding to the ring moiety of isotocin and glumitocin.

All of the [4-serine]tocin compounds were successfully synthesized from the common intermediate, Boc-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl (1). This protected pentapeptide was prepared from suitably protected amino acid p-nitrophenyl esters[22] or N-hydroxysuccinimide esters[23] using the stepwise method of peptide synthesis as developed in a synthesis of oxytocin.[24] [4-Serine]tocinoic acid and [4-serine]deaminotocinoic acid were prepared from Z-Cys(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl (2) and β-Mpa(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl (3), respectively. Compounds 2 and 3 were prepared from 1 by continuing the stepwise synthesis with either Z-Cys(Bzl)-ONp or β-Mpa(Bzl)-ONp.

A portion of 1 was converted into its corresponding C-terminal carboxamide by treatment with anhydrous MeOH saturated with anhydrous NH_3.[25] Boc-Cys(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-NH_2 (4) and β-Mpa(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-NH_2 (5) were then prepared by condensing the amide of 1 with Boc-Cys(Bzl)-ONSu or β-Mpa(Bzl)-ONp, respectively. The N-terminal protecting groups of 2 and 4 were re-

moved prior to treatment with sodium in anhydrous liquid NH_3[26] to increase the solubility of the peptides. Compounds 3 and 5, because of their low solubilities in liquid NH_3, had to be ground into very fine powders prior to the reaction with Na and required longer reaction times. In the case of [4-serine]tocinoic acid and [4-serine]tocinamide, the disulfide bond was formed by oxidation with 0.01 N $K_3Fe(CN)_6$.[27] The disulfide bond of the two deamino analogs was formed by oxidation with diiodoethane.[28] With the exception of [4-serine]deaminotocinamide, the compounds were purified by partition chromatography on Sephadex G-25[29] in various solvent systems, followed by gel filtration.[30] [4-Serine]deaminotocinamide proved to be quite insoluble in any useful combination of solvents for partition chromatography on Sephadex. It was, therefore, purified by gel filtration on Sephadex G-15 in 50% aqueous AcOH[31] followed by reprecipitation from 30% AcOH and a second gel filtration on Sephadex G-15 in 50% AcOH.

The highly purified compounds were tested for oxytocic, avian vasodepressor, and rat pressor activities. U.S.P. posterior pituitary reference standard was used throughout. Oxytocic assays were performed on isolated uteri from rats in natural estrus according to the method of Holton,[32] as modified by Munsick,[33] with the use of magnesium-free van Dyke–Hastings solution as the bathing fluid. Avian vasodepressor assays were performed by the method of Coon,[34] as described in the U. S. Pharmacopeia,[35] using conscious chickens as suggested by Munsick, Sawyer, and van Dyke.[36] Pressor assays were carried out on anesthetized male rats as described in the U. S. Pharmacopeia.[37] None of the four compounds showed any significant activity when tested for oxytocic, avian vasodepressor, or rat pressor responses in solutions of up to 0.3–0.4 mg/ml. However, all four showed slight or very slight inhibition of the oxytocic and avian vasodepressor response to oxytocin, with [4-serine]tocinamide and [4-serine]deaminotocinamide being approximately $\frac{1}{30}$ as effective as deaminopenicillamine-oxytocin[38,39] in these assays and the other compounds less potent. None of the compounds exhibited antipressor activity.

[4-Serine]tocinoic acid and [4-serine]tocinamide gave responses equivalent to those of oxytocin and deaminooxytocin in the milk ejection assay[40]‡ although their potencies were only $\frac{1}{14,000}$ and $\frac{1}{7000}$, respectively, of the potency of deamino-oxytocin.

In summary, the [4-serine]tocin ring compounds have

‡M. E. Hadley and V. J. Hruby, manuscript in preparation.

been shown to be devoid of even a small fraction of the oxytocic, AVD, and rat pressor activities possessed by the 4-serine-containing natural hormones isotocin and glumitocin. However, the ring compounds do retain slight milk-ejecting activity. Similarly, previous studies[13,15] have shown that the tocin and pressin rings are devoid of AVD[10,15,16,18] and rat pressor[10,13] activities and possess slight milk-ejecting[13,16] activity. The tocin and pressin rings differ from the [4-serine]tocin ring compounds in that they retain a significant fraction of the oxytocic[10,11,13,15,16,18] activity of their parent hormones, while the [4-serine]tocin ring compounds do not. It is apparent from the slight antioxytocic and anti-AVD activities of the [4-serine]tocin ring compounds that sufficient structural and conformational integrity is retained by these rings for some interaction with the receptors responsible for these activities. The absence of agonist or antagonist activity in the pressor assay may not be significant since the activities of the natural hormones in this assay are so low that a reduced activity of the ring moiety may not be detectable.

Experimental Section§

Boc-Ser(Bzl)-Asn-Cys(Bzl)-OBzl (6). A solution of 5.65 g (11 mmol) of Boc-Asn-Cys(Bzl)-OBzl[10] and 2 ml of anisole in 20 ml of trifluoroacetic acid (TFA) was stirred for 30 min at room temperature and the TFA and anisole were removed by rotary evaporation at 1 mm and 30°. The oily residue was triturated with Et_2O, evaporated to dryness and triturated again under Et_2O until it solidified, collected, washed with Et_2O, and dried over KOH *in vacuo.* The product was dissolved in 15 ml of DMF and neutralized to pH 7 (Fisher indicator solution) with *N*-methylmorpholine. The solution was treated with 5.0 g (12.4 mmol) of Boc-Ser(Bzl)-ONSu for 40 hr. The product was precipitated with 100 ml of H_2O, collected, washed with H_2O (two 30-ml portions), EtOAc (two 30-ml portions), and Et_2O (three 30-ml portions), and dried *in vacuo:* 5.94 g (78%); mp 144–145°; $[\alpha]^{25}_{546}$ −22.7° (*c* 0.5, DMSO). *Anal.* $(C_{36}H_{44}N_4O_8S)$ C, H, N.

Boc-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl (7). A solution of 6 (3.22 g, 4.65 mmol) in 15 ml of TFA and 1.5 ml of anisole was stirred 30 min. The product was precipitated with 300 ml of anhydrous Et_2O, collected, washed with Et_2O, and dried *in vacuo.* This product was dissolved in 20 ml of DMF, neutralized to pH 7 with *N*-methylmorpholine, and treated for 40 hr with Boc-Ile-ONSu (1.70 g, 5.2 mmol). The product was precipitated, collected, washed, and dried as before: 3.43 g (92%); mp 197–199°; $[\alpha]^{25}_{546}$ −25.1° (*c* 0.5, DMSO). *Anal.* $(C_{42}H_{55}N_5O_9S)$ C, H, N.

Boc-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl (1). This protected polypeptide ester was prepared from 7 (5.6 g, 6.8 mmol) and Boc-Tyr(Bzl)-ONSu (3.44 g, 7.5 mmol) in the manner described in the previous section: 6.51 g (90%); mp 219–220°; $[\alpha]^{25}_{546}$ −15.1° (*c* 0.5, DMSO). *Anal.* $(C_{58}H_{70}N_6O_{11}S)$ C, H, N.

Z-Cys(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl (2). This product was prepared by treatment of 1 (0.95 g, 0.90 mmol) with TFA–10% anisole, followed by neutralization in the usual manner, and then reaction with Z-Cys(Bzl)-ONp (0.45 g, 0.97 mmol) for 20 hr. An additional equivalent of *N*-methylmorpholine was added and the reaction continued for 6 hr longer. The product was precipitated with EtOAc–H_2O (30:1), collected, washed with EtOH, EtOH–H_2O (1:1), EtOH, and Et_2O, and dried *in vacuo:* 0.94 g (81%); mp 238–240.5°; $[\alpha]^{25}_{546}$ −26.1° (*c* 0.5, DMSO). *Anal.* $(C_{71}H_{79}N_7O_{12}S_2)$ C, H, N.

[4-Serine]tocinoic Acid. A 200-mg (0.15 mmol) portion of 2 was treated for 50 min with 16 ml of anhydrous 2.6 *N* HBr–AcOH. The resulting HBr salt was precipitated with anhydrous Et_2O, collected, washed with Et_2O, and dried *in vacuo.* This product was added to refluxing anhydrous liquid NH_3 (100 ml freshly distilled from Na) containing excess Na in solution. The solution was stirred and the blue color was allowed to persist for 4 min. The excess Na was allowed to react with a few drops of AcOH,

and the NH_3 was removed by evaporation and lyophilization. The residue was dissolved in 400 ml of deaerated 0.1% aqueous AcOH. The pH of the solution was adjusted to 8.5 with 3 *N* NH_4OH, and the sulfhydryl compound was oxidized with 30 ml of 0.01 *N* $K_3Fe(CN)_6$. After 15 min the pH was adjusted to 4 with AcOH and 4 ml of Rexyn 203 (Cl⁻ cycle) (Fisher Scientific Co.) was added to remove ferro- and excess ferricyanide ions. The suspension was stirred for 15 min; the resin was filtered off and washed three times with 20 ml of 10% aqueous AcOH. Redistilled 1-butanol (40 ml) was added to the combined filtrates to prevent bumping when the total volume was reduced to 150 ml by rotary evaporation. The remaining solution was lyophilized. The residue was dissolved in 5 ml of the upper phase and 3 ml of the lower phase of the system 1-BuOH–AcOH–H_2O (4:1:4) and subjected to partition chromatography by the method of Yamashiro[29] on a 2.9 × 61 cm column of Sephadex G-25 (100–200 mesh). Fractions of 9.0 ml each were collected. The peptide material was detected by reading the absorbance of the eluate at 280 nm, and the symmetrical major peak at R_f 0.21 (fractions 42–57) was isolated by evaporation and lyophilization: 53.0 mg. The product was well separated from impurities which were found in fractions 13–24. The product was dissolved in 4 ml of 20% aqueous AcOH and further purified by gel filtration on a 2.9 × 65 cm column of Sephadex G-25 (200–270 mesh) equilibrated with 20% AcOH; 4.0-ml fractions were collected. The compound, preceded by a small amount (<5%) of impurity (fractions 67–71), emerged as a sharp peak at 75% of the column volume (fractions 73–81) and was isolated by lyophilization: 30.1 mg (29%); $[\alpha]^{25}_{546}$ −34.5° (*c* 0.5, DMSO). The peptide gave a single symmetrical spot on tlc. Amino acid analysis[41] following 36-hr hydrolysis in 6 *N* HCl at 110° gave the following molar ratios: Asp, 1.1; Ser, 1.0; Cys, 1.8; Ile, 1.0; Tyr, 0.80; NH_3, 1.3. *Anal.* $(C_{28}H_{41}N_7O_{10}S_2 \cdot H_2O)$ C, H, N.

β-Mpa(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl (3). This compound was prepared from 1 (1.0 g, 0.94 mmol) and β-Mpa(Bzl)-ONp (0.32 g, 1.0 mmol) in the same manner as described for 2: 0.85 g (79%); mp 242–244°; $[\alpha]^{25}_{546}$ −15.9° (*c* 0.5, DMSO). *Anal.* $(C_{63}H_{72}N_6O_{10}S_2)$ C, H, N.

[4-Serine]deaminotocinoic Acid. A finely ground sample of 3 (0.22 g, 0.185 mmol) was treated in 100 ml of anhydrous liquid NH_3 with excess Na for 10 min as described previously. The lyophilized product was dissolved in 220 ml of deaerated, deionized H_2O and 205 ml of deaerated acetone. Diiodoethane (54.5 mg, 0.191 mmol, freshly recrystallized two times from Et_2O) dissolved in 15 ml of acetone was added. The reaction was followed by the Ellman test[42] and judged complete after 15 min. AcOH (5 ml) was added, the acetone was removed by rotary evaporation, and the remainder was lyophilized to dryness. The residue was dissolved in 6 ml of the upper phase and 3 ml of the lower phase of the system 1-BuOH–EtOH–pyridine–AcOH–H_2O (5:1:1:0.15:7) and subjected to partition chromatography on a 2.9 × 64 cm column of Sephadex G-25 (100–200 mesh); 5.5-ml fractions were collected. The peptide material was detected by the Folin–Lowry method[43] and the symmetrical major peak at R_f 0.41 (fractions 40–55) was isolated by evaporation and lyophilization: 93.6 mg. Minor impurities (fractions 24–30) were well separated from the major product. The product was dissolved in 8 ml of AcOH–H_2O (4:1) and further purified by gel filtration on a 2.9 × 64 cm column of Sephadex G-25 (100–200 mesh) equilibrated with 50% aqueous AcOH; 4-ml fractions were collected. The compound emerged as a single sharp peak at 67% of the column volume: 67.8 mg (54%); $[\alpha]^{25}_{546}$ −50.1° (*c* 0.5, DMSO). The peptide gave a single symmetrical spot on tlc. Amino acid analysis following 36-hr hydrolysis in 6 *N* HCl at 110° gave the following molar ratios: Asp, 1.1; Ser, 0.90; Ile, 1.0; Tyr, 0.91; NH_3, 1.2; Cys, 0.48; mixed disulfide of Cys and β-Mpa, 0.59. *Anal.* $(C_{28}H_{40}N_6O_{10}S_2)$ C, H, N.

Boc-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-NH₂ (8). A 250-ml round-bottom flask containing a finely ground suspension of 1 (1.13 g, 1.07 mmol) in 100 ml of anhydrous MeOH saturated with anhydrous NH_3 at 0° was sealed and placed in a desiccator at room temperature for 3 days with constant stirring. The MeOH and NH_3 were removed by rotary evaporation. The product was then triturated with Et_2O, collected, and washed with Et_2O. After drying *in vacuo* it was dissolved in DMF, precipitated with H_2O, collected, washed with H_2O and Et_2O, and dried *in vacuo:* 0.85 g (86%); mp 245–248° dec; $[\alpha]^{25}_{546}$ −19.5° (*c* 0.5, DMSO). *Anal.* $(C_{51}H_{65}N_7O_{10}S \cdot H_2O)$ C, H, N.

Boc-Cys(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-NH₂ (4). This protected hexapeptide was prepared from 8 (0.85 g, 0.86

mmol) and Boc-Cys(Bzl)-ONSu (0.41 g, 1.0 mmol) as described previously. The product was precipitated with H_2O, collected, and washed with H_2O, EtOH, and Et_2O. It was dried *in vacuo*: 1.00 g (97%); mp 260-262° dec; $[\alpha]^{25}_{546}$ -33.6° (*c* 0.5, DMSO). *Anal.* ($C_{61}H_{76}N_8O_{11}S_2$) C, H, N.

[4-Serine]tocinamide. The Boc group of 4 (243 mg, 0.209 mmol) was removed with TFA-10% anisole. The TFA salt obtained was treated with Na in liquid NH_3, and the disulfide bond was formed by oxidation with $K_3Fe(CN)_6$ as described for the synthesis of [4-serine]tocinoic acid. The crude product was dissolved in 6 ml of the upper phase and 3 ml of the lower phase of the system 1-BuOH-EtOH-pyridine-AcOH-H_2O (4:1:1:0.4:6.4) and subjected to partition chromatography on a 2.9 × 60 cm column of Sephadex G-25 (100-200 mesh); 5.1-ml fractions were collected. The peptide material was detected by the Folin-Lowry method, and the symmetrical major peak at R_f 0.33 (fractions 36-60) was isolated by evaporation and lyophilization: 101 mg. This product was dissolved in 4 ml of 50% aqueous AcOH and further purified by gel filtration on a 2.2 × 111 cm column of Sephadex G-15 (40-120 μ beads) equilibrated with 50% AcOH; 3.6-ml fractions were collected. The peptide, preceded by a small amount of impurity (fractions 35-43), emerged as a sharp symmetrical peak at 45% of the column volume (fractions 48-61): 67.8 mg (48%); $[\alpha]^{25}_{546}$ -75.5° (*c* 0.5, DMSO). The peptide showed a single symmetrical spot on tlc. Amino acid analysis following 36-hr hydrolysis in 6 N HCl at 110° gave the following molar ratios: Asp, 1.1; Ser, 1.0; Cys, 2.0; Ile, 1.1; Tyr, 0.81; NH_3, 2.0. *Anal.* ($C_{28}H_{42}N_8O_9S_2$) C, H, N.

β-Mpa(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-NH_2 (5). This polypeptide was prepared from 8 (0.65 g, 0.66 mmol) and β-Mpa(Bzl)-ONp (0.24 g, 0.75 mmol) in the manner described for the preparation of 3: 0.57 g (81%); mp 262-263° dec; $[\alpha]^{25}_{546}$ -25.5° (*c* 0.5, DMSO). *Anal.* ($C_{56}H_{67}N_7O_9S_2 \cdot H_2O$) C, H, N.

[4-Serine]deaminotocinamide. A 225-mg (0.21 mmol) portion of finely ground 5 was treated as described for the preparation of [4-serine]deaminotocinoic acid. The crude product was dissolved in 5 ml of AcOH-H_2O (4:1) and subjected to gel filtration on a column (2.2 × 111 cm) of Sephadex G-15; 3.6-ml fractions were collected. The peptide material was detected by reading the absorbance of the eluate at 280 nm. The major peak emerged as a sharp peak at 57% of the column volume (fractions 63-75): 256 mg. This crude product, containing sodium acetate, was precipitated from 30 ml of 30% aqueous AcOH and subjected to a second gel filtration as described above. The product emerged as a single symmetrical peak at 58% of the column volume: 29.8 mg (21%); $[\alpha]^{25}_{546}$ -96.5° (*c* 0.5, DMSO). The peptide gave a single symmetrical spot on tlc. Amino acid analysis after 36-hr hydrolysis in 6 N HCl at 110° gave the following molar ratios: Asp, 1.1; Ser, 1.0; Ile, 0.97; Tyr, 0.79; Cys, 0.51; mixed disulfide of Cys and β-Mpa, 0.62. *Anal.* ($C_{28}H_{41}N_7O_9S_2$) C, H, N.

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