Synthesis and Some Pharmacological Properties of 8-L-Methionine-oxytocin

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Synthesis of 8-L-methionine-oxytocin from *N*-[(*tert*-butyloxy)carbonyl]tocinoic acid and L-prolyl-L-methionylglycinamide is described. Removal of the NH_2 protecting group by acidolysis from the partially protected nonapeptide, followed by purification by chromatography, produced the hormone analogue in homogeneous form. It exhibits the pharmacological activities characteristic of the parent hormone, oxytocin. The new analogue, 8-L-methionine-oxytocin, possesses 200 ± 4 units per mg of oxytocic activity determined on the isolated rat uterus, 193 ± 7 units per mg of avian depressor activity, 27 ± 0.8 units per mg of rat pressor activity, and 0.4 ± 0.2 unit per mg of rat antidiuretic activity.

Methionine and leucine can replace each other in natural analogues of several biologically active peptides. For instance, position 5 in gastrin from porcine tissues[2a] is occupied by methionine, while leucine occurs in the same position in human gastrin.[2b] A further illustration of this point is provided by the enkephalins,[3] one with leucine and the other with methionine at the C terminus. The similarity between leucine and methionine in their physical properties, such as solubility, is also suggestive. Samples of L-leucine prepared by isolation from natural sources were often contaminated by L-methionine; in the separation of methionine from other amino acids present in protein hydrolysates, it crystallizes together with leucine[4] when the solution is saturated with NaCl. For such reasons we expected that replacement of leucine by methionine in oxytocin should result in a hormonally active analogue. Also, the possibility that 8-L-methionine-oxytocin, the result of a single-base mutation, might be found sooner or later in some animal species seemed not too unrealistic.

Synthesis of 8-L-methionine-oxytocin was motivated also by the attempts toward the preparation of a biologically important segment of the carboxyl carrier protein of the transcarboxylase[5] from *Propionibacterium shermanii*. In this segment, biotin (a thioether) is attached to the ε-amino group of a lysine residue that lies between two methionine moieties. Therefore, for an unequivocal synthesis of this segment, a study of conditions which could suppress the side reactions involving the thioether in the methionine side chains was certainly indicated. The 8-L-methionine analogue of oxytocin[6] was selected as a model compound for such studies, since in its stepwise[7] synthesis the methionine residue is incorporated in the chain already in the first coupling step and thus can suffer side reactions throughout the chain-lengthening process. The stepwise approach, however, produced, in this case, intermediates which were not pure enough to be used in the preparation of the desired final product. The new oxytocin analogue, therefore, was secured via a scheme developed in the laboratory of V. du Vigneaud[8] and, independently, by the group led by Schwyzer and Rudinger[9] for the synthesis of oxytocin. This scheme is based on the coupling of the hexapeptide derivative tocinoic acid with the C-terminal tripeptide portion of the molecule. This coupling was carried out under conditions which keep racemization at a minimum. For the synthesis of 8-L-methionine-oxytocin, a partially protected tocinoic acid derivative was prepared according to the route known from the literature,[8,9] with some modifications. Thus, in the coupling steps, *p*-nitrophenyl esters[10] were used and their reactions were catalyzed[11] by 1-hydroxybenzotriazole. This led to improvements in yields. Some simplifications in the coupling of the two segments and in the purification of both the tocinoic acid derivative and the final nonapeptide amide are also described under the Experimental Section. Perhaps more important is the application of the scavenger acetyl-DL-methionine *n*-butyl ester[12] added for the suppression of S-alkylation of the methionine residue. Moreover, the synthesis was used for testing the newly developed method of prevention of O-acylation[13] with additives such as 2,4-dinitrophenol or pentachlorophenol. Accordingly, the tyrosine residue was incorporated into the peptide chain with its phenolic hydroxyl group unprotected. No O-acylation was observed in the presence of these additives. Other details of the synthesis are summarized in Chart I.

The new analogue, 8-L-methionine-oxytocin, was obtained practically pure simply by deprotection of the *N*-[(*tert*-butyloxy)carbonyl] derivative (XI) with trifluoroacetic acid. Final purification was achieved by chromatography on a column of silica gel in a system of ethyl acetate–pyridine–acetic acid–water. The purified material was homogeneous on thin-layer chromatography and paper chromatography, as well as on high-pressure liquid chromatography, and gave satisfactory amino acid analysis.

A comparison of the CD spectra of oxytocin and 8-L-methionine-oxytocin in 0.01 N HCl (Figure 1) suggests that the conformation of the new analogue is similar to that of its parent hormone. When tested on the isolated horns of uteri from virgin rats,[14] 8-L-methionine-oxytocin possessed 200 ± 4 (SEM) units per mg of oxytocic activity. In the avian depressor assay,[15] a potency of 193 ± 7 units per mg was determined. The pressor activity[16] was found to be 27 ± 0.8 units per mg, and the antidiuretic effect[17] was 0.4 ± 0.2 unit per mg. These pharmacological properties show that 8-L-methionine-oxytocin is a potent analogue of oxytocin.

Experimental Section

Uncorrected capillary melting points are reported. The solvents used were reagent grade; dimethylformamide (DMF) was dried over a 4Å molecular sieve (Davison). For amino acid analysis, samples were hydrolyzed with constant-boiling hydrochloric acid in evacuated sealed ampules at 110 °C for 16 h and analyzed by the Spackman–Stein–Moore method on a Beckman Spinco 120C amino acid analyzer. Thin-layer chromatograms (TLC) were run on glass plates precoated with silica gel (Brinkman) in the following solvent systems: A, $CHCl_3$–MeOH (9:1); B, EtOAc–pyridine–AcOH–H_2O (60:30:6:11); C, $CHCl_3$; D, *n*-BuOH–AcOH–H_2O (4:1:1); E, $CHCl_3$–MeOH (8:2); F, $CHCl_3$–AcOH–MeOH (18:1:2); G, *n*-BuOH–AcOH–H_2O (100:15:35); H, *n*-BuOH–EtOAc–AcOH–H_2O (1:1:1:1). Abbreviations used are: DCC, dicyclohexylcarbodiimide; LC, liquid chromatography.

Boc-L-Met-Gly-NH_2 (I). Boc-Met (Bachem) (9.25 g, 37.5 mmol) and *N*-methylmorpholine (NMM; 3.75 g, 37.5 mmol) were dissolved in DMF (60 mL) and the solution was cooled to −15 °C.

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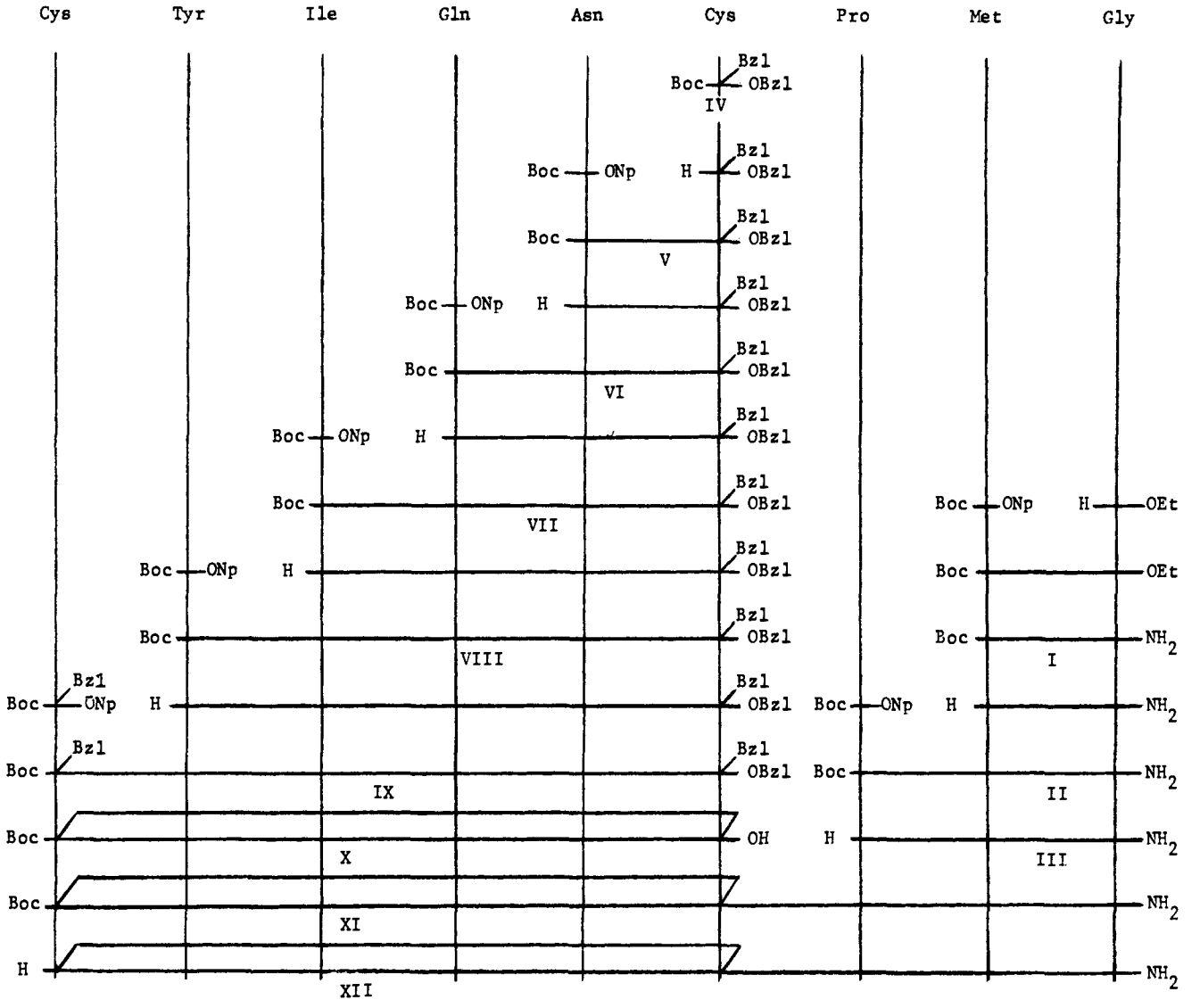
Chart I



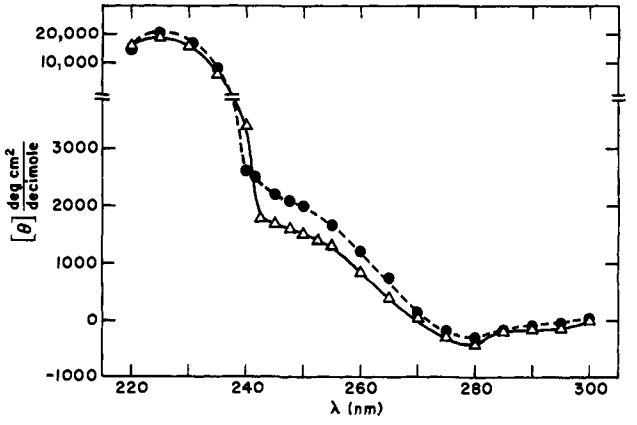


Figure 1. CD spectra (in 0.01 N HCl) of oxytocin (1.96 mg/mL) (●) and of 8-L-methionine-oxytocin (1.84 mg/mL) (△).

Isobutylchloroformate (4.2 mL, 35 mmol) was added and the reaction mixture stirred at −15 °C. After 2 min, a solution of Gly-OEt·HCl (Aldrich) (3.5 g, 25 mmol) and NMM (5 g, 50 mmol) in DMF (20 mL) was added. The reaction mixture was maintained basic by periodic addition of NMM (a total of 2 mL) during the first 0.5 h. The acylation was complete after about 2.5 h (TLC, negative ninhydrin reaction). A 2 M solution of $KHCO_3$ (40 mL) was added, and the mixture was vigorously stirred for 0.5 h when it was diluted with H_2O (300 mL). The precipitate was filtered, washed with water, and dried over P_2O_5. It weighed 8.0 g (96%): mp 92–94 °C; homogeneous on TLC, R_f (A) 0.6.

The ethyl ester (8.0 g) was dissolved in methanol (100 mL), and the solution was cooled in an ice–water bath, saturated with dry NH_3 gas, and allowed to stand overnight at room temperature. The product was secured by evaporation and trituration of the residue with ether. The amide was filtered, washed well with ether, and dried to yield 7.0 g (95%), mp 89–93 °C. A small sample was recrystallized from a mixture of EtOAc–hexane: mp 93–94 °C dec; $[\alpha]^{25}_D$ −12.5° (*c* 2, DMF); R_f (A) 0.4. Anal. ($C_{12}H_{23}N_3O_4S$) C, H, N.

Boc-L-Pro-L-Met-Gly-NH_2 (II). A sample of compound I (3.9 g, 13 mmol) was treated with TFA (50 mL) containing acetyl-DL-methionine *n*-butyl ester (16.0 g, 65 mmol). After the mixture was stored for 0.5 h at room temperature, the TFA was removed under reduced pressure and the viscous residue triturated several times with ether. The oily trifluoroacetate salt (still containing a trace of the scavenger) was dried in a desiccator over NaOH. It weighed 3.0 g.

An aliquot of this material (2.0 g, 7 mmol) was coupled with Boc-Pro (Bachem, 2.1 g, 10.5 mmol) by the mixed anhydride procedure as described for I. The acylation was complete in 3 h. After the addition of 2 M $KHCO_3$ (15 mL) and stirring for 0.5 h, the mixture was diluted with water (100 mL). The solution was saturated with NaCl and extracted with EtOAc (8 × 30 mL). The organic layer was washed with 10% citric acid solution (2 × 5 mL) and finally with water (2 × 5 mL). The organic layer was dried over $MgSO_4$ and concentrated. When dried over P_2O_5, it formed a viscous liquid (3.0 g). According to TLC (system A), the product was contaminated with a small amount of the scavenger. For characterization, a sample was chromatographed

on silica gel with $CHCl_3$–CH_3OH gradient and the product isolated as a solid: mp 113–115 °C; $[\alpha]^{25}_D$ −47.4° (*c* 1, DMF). It was homogeneous on TLC, R_f (A) 0.25. Anal. ($C_{17}H_{25}N_4O_5S$) C, H, N. Amino acid analysis: Pro, 0.92; Gly, 1.07; Met, 0.96. The crude product was used for the next step.

L-Pro-L-Met-Gly-NH_2 (III). The protected tripeptide amide II (1.0 g, 2.5 mmol) was treated with TFA (20 mL) containing acetyl-DL-methionine *n*-butyl ester (3.25 g, 12.5 mmol). After the mixture was stored for 0.5 h, most of the TFA was removed, and the residue was triturated several times with ether and then kept over NaOH in vacuo. The glassy hygroscopic solid (0.8 g, R_f (B) 0.25) was dissolved in MeOH and the solution passed through a column of IRA 400 ion-exchange resin (10 g) to produce the free base, L-Pro-L-Met-Gly-NH_2 (III). The oily material (0.58 g) was homogeneous on TLC: R_f (B) 0.25, R_f (D) 0.30.

Boc-L-Cys(Bzl)-OBzl (IV). A solution of the protected amino acid Boc-L-Cys(Bzl)OH (Bachem, 15.5 g, 50 mmol) in 95% EtOH–H_2O (9:1, v/v) was treated with Cs_2CO_3[18,19] to a pH 6.5 ± 0.2. The solvent was removed in vacuo, and the residue was dried by repeated evaporations with EtOH and benzene and finally in a desiccator over P_2O_5 *overnight*.

Benzyl bromide (6 g, 35 mmol) was added to the solution of the cesium salt (12 g, 27 mmol) in DMF (100 mL) and the material kept *overnight* at room temperature. The solvent was removed in vacuo, and the residue was dissolved in EtOAc, filtered, and evaporated to dryness. Trituration of the resulting solid with ether–petroleum ether (40–60 °C) produced white crystals, which were collected by filtration: 9.0 g (83%); mp 84–86 °C; $[\alpha]^{27}_D$ −40.4° (*c* 2, DMF). On TLC, a single spot was observed by UV and by charring, R_f (C) 0.50. Anal. ($C_{22}H_{27}NO_4S$) C, H, N.

Boc-L-Asn-L-Cys(Bzl)-OBzl (V). A sample of compound IV (1.68 g, 4.2 mmol) was dissolved in TFA (5 mL). After the mixture was stored for 30 min at room temperature, the TFA was removed in vacuo and the residue dried over KOH pellets. It was dissolved in DMF (5 mL), cooled in an ice–water bath, and treated with diisopropylethylamine[20] (DIEA, 0.7 mL), 1-hydroxybenzotriazole[11] (HOBt, 675 mg), DIEA (0.7 mL), and Boc-L-Asn-ONp (Bachem, 1.77 g, 5 mmol). After the mixture was stored for 0.5 h at 0 °C and overnight at room temperature, the solvent was removed and the residue chromatographed on a column of silica gel (Baker, 100 g) with a 95:5 (v/v) mixture of $CHCl_3$ and MeOH as eluent. The fractions containing compound V were pooled, the solvent was evaporated, and the residue was washed with ether: yield 1.9 g (88%); mp 146–149 °C; $[\alpha]^{20}_D$ −37.5° (*c* 1, DMF); lit.[8] mp 145–147 °C; $[\alpha]^{23}_D$ −37.7° (*c* 1, DMF); lit.[9] mp 146–149 °C, $[\alpha]^{25}_D$ −37.2° (*c* 1, DMF); single spot on TLC, R_f (A) 0.50.

Boc-L-Gln-L-Asn-L-Cys(Bzl)-OBzl (VI). A solution of compound V (2.63 g, 5.1 mmol) in TFA (5 mL) was kept at room temperature for 3.4 h. Removal of TFA in vacuo at room temperature left an oil which solidified under ether. The crystalline salt was dissolved in DMF (10 mL) and cooled at 0 °C, and DIEA (1.7 mL, 10.5 mmol), HOBt (680 mg, 5 mmol), and Boc-L-Gln-ONp (Bachem, 2.2 g, 6 mmol) were added to the solution. After 0.5 h at 0 °C and overnight at room temperature, the mixture was diluted with EtOAc (80 mL) and cooled, and the precipitate was filtered, washed with EtOAc and then with ether, and dried. The product weighed 3.02 g (92%): mp 198–200 °C; $[\alpha]^{26}_D$ −24.6° (*c* 1, DMF); lit.[8] mp 199–201 °C, $[\alpha]^{26}_D$ −25.0° (*c* 1, DMF); lit.[9] mp 197–199 °C, $[\alpha]^{25}_D$ −25.2° (*c* 1, DMF). It gave a single spot on TLC, R_f (A) 0.40, R_f (B) 0.70.

Boc-L-Ile-L-Gln-L-Asn-L-Cys(Bzl)-OBzl (VII). A sample of compound VI (2.96 g, 4.6 mmol) was partially deprotected with TFA as described for compound VI. The trifluoroacetate salt gave a single spot on TLC, R_f (E) 0.25. It was dissolved in DMF (20 mL) and treated at 0 °C with DIEA (1.6 mL, 10 mmol), HOBt (675 mg, 5 mmol), and Boc-L-Ile-ONp (Bachem, 2.11 g, 6 mmol), and the mixture was stored overnight at room temperature. The semisolid mixture was diluted with EtOAc (150 mL), stirred, and filtered. The precipitate was washed with EtOAc and ether and dried to yield 3.38 g (97%): mp 234–235 °C dec; $[\alpha]^{20}_D$ −24.3° (*c* 1, DMF); lit.[9] mp 238–240 °C dec, $[\alpha]^{25}_D$ −23.9° (*c* 1, DMF). On TLC, a single spot was observed: R_f (D) 0.65, R_f (E) 0.60.

Boc-L-Tyr-L-Ile-L-Gln-L-Asn-L-Cys(Bzl)-OBzl (VIII). The protected tetrapeptide ester VII (3.8 g, 5 mmol) was partially deblocked with TFA (10 mL). The trifluoroacetate salt was dissolved in DMF (20 mL) and treated at 0 °C with DIEA (1.6 mL, 10 mmol), HOBt (675 mg, 5 mmol), 2,4-dinitrophenol (920 mg, 5 mmol), and Boc-L-Tyr-ONp (Bachem, 221 mg, 5.5 mmol). After the mixture was stored overnight, the solvent was removed in vacuo, and the semisolid residue was triturated with EtOAc, filtered, washed with EtOAc, and dried in a desiccator in vacuo: yield 4.23 g (88%); mp 230–231 °C dec; $[\alpha]^{27}_D$ −20.8° (*c* 0.6, DMF). The sample gave a single spot on TLC: R_f (D) 0.40, R_f (F) 0.30, R_f (G) 0.45. Anal. ($C_{46}H_{61}N_7O_{11}S\cdot 2H_2O$) C, H, N.

The same experiment was carried out with pentachlorophenol rather than 2,4-dinitrophenol as the additive against O-acylation. The results were exactly the same as above. In a third experiment, with HOBt as the sole additive, the yield was 85%, but a weak spot with R_f (F) 0.40 appeared on TLC, presumably the O-acylated derivative. The melting point, 226–229 °C dec, also indicated the presence of a contaminant.

Boc-L-Cys(Bzl)-L-Tyr-L-Ile-L-Gln-L-Asn-L-Cys(Bzl)-OBzl (IX). Removal of the Boc group from compound VIII (4.2 g, 4.4 mmol) was carried out as described in previous paragraphs. The trifluoroacetate salt was dissolved in DMF (40 mL), cooled in an ice–water bath, and treated with DIEA (1.42 mL, 8.9 mmol), HOBt (594 mg, 4.4 mmol), 2,4-dinitrophenol (809 mg, 6.6 mmol), and Boc-L-Cys(Bzl)-ONp[21] (2.12 g, 4.9 mmol). After the mixture was stored for 0.5 h at 0 °C and overnight at room temperature, the solvent was removed in vacuo, and the residue was triturated with EtOAc, filtered, washed with EtOAc and then with ether, and dried. The product (4.77 g, 96%) melted at 242–245 °C dec; $[\alpha]^{27}_D$ −20.7° (*c* 1, DMF). It gave a single spot on TLC, R_f (E) 0.30, R_f (C) 0.45. Anal. ($C_{56}H_{72}O_{12}N_8S_2\cdot 1.5H_2O$) C, H, N.

Boc-L-Cys-L-Tyr-L-Ile-L-Gln-L-Asn-L-Cys-OH (X). A sample of compound IX (493 mg, 0.44 mmol) was dissolved in liquid NH_3 (1 L) and treated with Na until a blue color persisted throughout the solution for 1 min. The color was discharged with NH_4Cl, more NH_4Cl (100 mg) was added, and ammonia was allowed to evaporate. The residue was dissolved in deaerated H_2O (1.5 L) and the pH to about 7 with a few drops of AcOH; MeOH (300 mL) and 1,2-diiodoethane[22] (140 mg, freshly recrystallized in EtOH) were added. The mixture was stored under N_2 for 5 h. After concentration in vacuo, the solution was filtered and lyophilized. The residue was purified by countercurrent distribution in a Craig apparatus in *n*-BuOH–2.5% AcOH (1:1, 40-mL phases) through 15 transfers. The Boc derivative of tocinoic acid[23] was secured from tubes 12–15: mp 195–198 °C, $[\alpha]^{20}_D$ −66.6° (*c* 0.5, EtOH); lit.[9] mp 195–198 °C, $[\alpha]^{25}_D$ −67.3° (*c* 0.5, MeOH). Amino acid analysis: Asp, 0.98; Glu, 1.02; $^1/_2$-Cys, 1.97; Ile, 1.00; Tyr, 0.86.

Boc-L-Cys-L-Tyr-L-Ile-L-Gln-L-Asn-L-Cys-L-Pro-L-Met-Gly-NH_2 (XI). The tripeptide amide III (75 mg, 0.25 mmol) and the protected hexapeptide X (100 mg, 0.12 mmol) were dissolved in DMF (1 mL); a solution of DCC (31 mg, 0.15 mmol) in DMF (1 mL) was added, followed by HOBt (23 mg, 0.15 mmol). The reaction mixture was stirred in an ice bath for 4 h and then left at room temperature for 20 h. Completion of the reaction was monitored by the disappearance of the hexapeptide (TLC in system B). After the mixture was stored for about 24 h, the *N,N*-dicyclohexylurea was removed and washed with small quantities of DMF, and the filtrate was concentrated under reduced pressure. The residue was triturated with EtOAc in a centrifuge tube and the precipitate washed with EtOAc (6 × 5 mL). The product, a white solid (135 mg), was dried over P_2O_5. It was applied to a column of silica gel (10 g) and eluted with a mixture of EtOAc–pyridine–AcOH–H_2O (60:25:6:11). Fractions of 3 mL were collected. The desired protected nonapeptide (65 mg, 50%)[24] was obtained from fractions 3–6: mp 205–210 °C dec; $[\alpha]^{25}_D$ −56.5° (*c* 1, DMF). It was homogeneous on TLC, R_f (B) 0.7. Anal. ($C_{47}H_{68}N_{12}O_{14}S_3$) C, H, N.

8-L-Methionine-oxytocin (XII). A sample of compound XI (24 mg) was treated with TFA (3 mL) containing acetyl-DL-methionine *n*-butyl ester (100 mg, 0.4 mmol) for 0.5 h. The TFA was removed in vacuo and the residue triturated with EtOAc when a white precipitate formed. It was washed by centrifugation with EtOAc (6 × 4 mL). The solid, after drying over P_2O_5, weighed 22 mg (91%), R_f (B) 0.35, with trace amounts of impurities.

The deprotected material from two batches (30 mg) was chromatographed on silica gel (10 g) with EtOAc–pyridine–AcOH–H_2O (60:25:6:11) as eluent. Fractions of 3 mL were

collected. The pure peptide (16 mg) was secured from fractions 7–13. Subsequent fractions yielded 8 mg less pure material. The pure product was homogeneous on TLC, R_f (B) 0.35. On descending paper chromatograms in the freshly prepared solvent system *n*-BuOH–AcOH–H_2O (4:1:5, upper layer), 8-L-methionine-oxytocin moved as a single spot with R_f 0.54. Oxytocin used as reference traveled with R_f 0.61. Amino acid analysis: Asp, 1.08; Glu, 1.05; Pro, 0.96; Gly, 1.01; $^1/_2$-Cys, 1.97; Met, 0.95; Ile, 1.08; Tyr, 0.85. From the recovery, a peptide content of 98% was calculated. This value was confirmed by the absorption at 278 nm of a sample of XII in H_2O. This material was used in the biological assays.

A sample of XII (purified by partition chromatography, cf. above) was subjected to LC on a DuPont 850 liquid chromatograph with a Zorbax ODS 4.6 × 25 mm column and 0.01 N NH_4OAc (pH 4)–CH_3CN system as eluent. The elution was monitored by absorption at 275 nm. A flow rate of 2.5 mL/min was maintained. A solvent gradient of 25–50% CH_3CN in NH_4OAc was used. A single peak was recorded at 39% CH_3CN composition (6 min).

Pharmacological Assays. Rat uterine assays were performed on isolated horns from virgin rats in natural estrus according to the method of Holton,[14a] as modified by Munsick,[14b] with the use of Mg^{2+}-free van Dyke-Hastings solution as bathing fluid. Avian vasodepressor assays were performed on conscious chickens by the method of Coon,[15a] as described in the U.S. Pharmacopeia,[15b] as modified by Munsick et al.[15c] Pressor assays were carried out on anesthetized male rats as described in the U.S. Pharmacopeia.[16] Antidiuretic assays were performed on anesthetized male rats according to the method of Jeffers et al.,[17a] as modified by Sawyer.[17b] The four-point assay design of Schild[25] was used to obtain specific activities as compared to U.S. Pharmacopeia posterior pituitary reference standard.

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Prodrugs of 9-(β-D-Arabinofuranosyl)adenine 2. Synthesis and Evaluation of a Number of 2′,3′- and 3′,5′-Di-*O*-acyl Derivatives[1]

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A number of 2′,3′- and 3′,5′-di-*O*-acyl derivatives of 9-β-D-arabinofuranosyladenine (1) were prepared and evaluated as antivirals. These compounds, designed as prodrugs of 1, offer a range of solubilities and lipophilicities, as well as a resistance to adenosine deaminase, that render some as being attractive as possibly useful antiviral agents. Of particular note is 9-(2,3-di-*O*-acetyl-β-D-arabinofuranosyl)adenine that was found to be effective as a topical agent in a guinea pig model of genital herpes.

In the preceding paper,[2] a series of prodrugs of the potent antiviral agent 9-β-D-arabinofuranosyladenine (VIRA-A,® vidarabine, *ara*-A, 1) was described. These compounds, which are stable, 5′-*O*-acyl derivatives of 1,

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