BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 41 2772—2776 (1968)

Analogs of Oxytocin Containing Leucinamide and Glycylglycinamide in Place of Glycinamide[1]

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(Received June 3, 1968)

Two analogs of oxytocin, [9-leucine]-oxytocin and oxytocinoyl-glycinamide, in which the glycinamide residue at position 9 in oxytocin has been replaced by a leucinamide and glycylglycinamide, respectively, have been synthesized and tested for some of the pharmacological properties characteristic of oxytocin. The protected nona- and decapeptide intermediates were prepared by use of the azide method in a fashion of the fragment condensation. After removal of the protecting groups from the intermediates by means of either sodium in liquid ammonia or anhydrous hydrogen fluoride, the dithiols thus obtained were oxidized to the required analogs. It was found that [9-leucine]-oxytocin possessed approximately 8% oxytocic potency, and oxytocinoyl-glycinamide 0.5% potency compared with oxytocin preparation.

The synthetic approach to the study of structure-activity relationships in oxytocin hormone opens up a wide choice of structural variations.[5] Among the numerous analogs of oxytocin which have been synthesized, very few have involved modifications of the glycinamide residue in position 9; du Vigneaud *et al.* reported the properties of [9-sarcosine]-oxytocin[6] and 9-deamidooxytocin[7] in which the glycinamide residue was replaced by that of sarcosinamide and glycine, respectively. The present paper reports the syntheses and pharmacological properties of two analogs of oxytocin, [9-leucine]-oxytocin[8] and oxytocinoyl-glycinamide[8] in which leucinamide and glycylglycinamide replace the glycinamide residue, respectively. As a peptide for the comparison with these two analogs, oxytocin itself was also prepared *via* the similar reaction-sequence employed for the syntheses of the analogs.

The sequence of reaction employed for the syntheses of oxytocin and its analogs is shown in Fig. 1. For the preparation of the protected nona- and decapeptides for the syntheses of the desired peptides (XV-A, B and C), benzyloxycarbonyl-tripeptide azide which was derived from the corresponding hydrazide (XIII) was allowed to react with hexa- (XI-A and C) or heptapeptide amide (XI-B) in a manner similar to that used by Boissonnas *et al.*[9] The required hexa- or heptapeptide amide (XI-A, B or C) was itself prepared from benzyloxycarbonyl-tripeptide hydrazide (IX) and tri- or tetrapeptide amide (V-A, B or C) followed by removal of the benzyloxycarbonyl group.

The protecting groups of the nona- and decapeptide intermediates (XIV-A, B and C) were removed by treatment with sodium in liquid ammonia by the method of du Vigneaud *et al.*[10] The removal of the protecting groups of the intermediates was also performed by acidolysis with anhydrous hydrogen fluoride as was reported by Sakakibara and Shimonishi.[11] The dithiols so obtained were oxidized in neutral solution by aeration, and the hormonal activities of the products in the solutions were determined by the rat-uterine contracting and avian depressor test.

Total activities of the preparations derived from 0.06 mmol (about 80 mg) of the intermediates are shown in Table 1. The oxytocinoyl-glycinamide (XV-B) possesses a weak activity such as some 0.5% oxytocic potency compared with oxytocin preparation. The relative activities of the analogs XV-B as well as [9-leucine]-oxytocin (XV-A) toward

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5) K. Hofmann, *Ann. Rev. Biochem.*, **31**, 213 (1962).

6) J. Meienhofer and V. du Vigneaud, *J. Am. Chem. Soc.*, **83**, 142 (1961). Main content of this literature was the synthesis of [Sar^9]-vasopressin, and there was a brief description that [Sar^9]-oxytocin possessed the extreme low activity.

7) B. M. Ferrier and V. du Vigneaud, *J. Med. Chem.*, **9**, 55 (1966).

8) We followed the rules naming synthetic modifications of natural peptides in *Biochemistry*, **6**, 362 (1967); *e. g.*, the semitrivial name is [9-leucine]-oxytocin and the abbreviated form is [Leu^9]-oxytocin. Oxytocinoyl-glycinamide may be alternately named as 9a-endo-glycine-oxytocin.

9) R. A. Boissonnas, St. Guttman, P.-A. Jaquenoud and J.-P. Waller, *Helv. Chim. Acta*, **38**, 1491 (1955).

10) V. du Vigneaud, C. Ressler, J. M. Swan, C. R. Roberts, P. G. Katsoyannis and S. Gordon, *J. Am. Chem. Soc.*, **75**, 4879 (1953).

11) S. Sakakibara and Y. Shimonishi, This Bulletin, **38**, 1412 (1965).

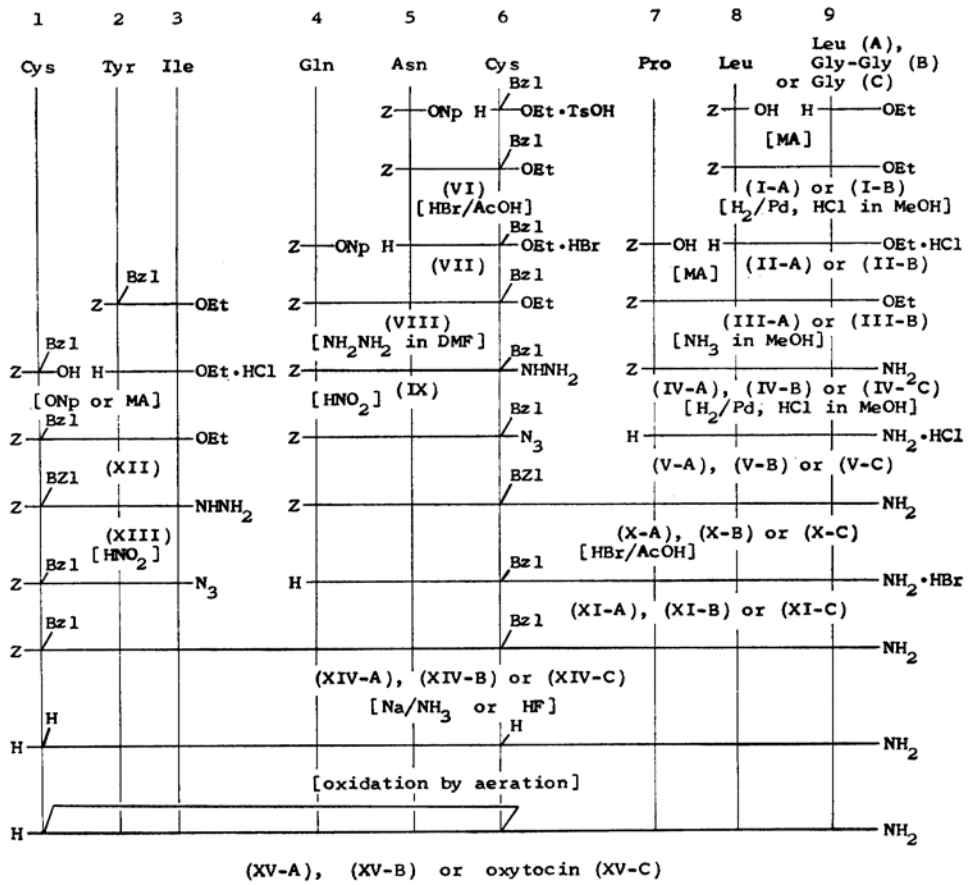

Fig. 1. Schematic diagram of syntheses of oxytocin and its analogs.
Z, benzyloxycarbonyl; Bzl, benzyl; ONp, *p*-nitrophenyl ester; [MA], mixed anhydride method; DMF, dimethylformamide.

TABLE 1. TOTAL ACTIVITIES (UNITS) OF PRODUCTS DERIVED FROM THE PROTECTED PEPTIDES (0.06 mmol)

Compound	Oxytocic (rat)		Depressor (fowl)	
	Na/NH_3	HF	Na/NH_3	HF
	(%)			
[Leu^9]-oxytocin (XV-A)	1050(8)	225	850	190
Oxytocinoyl-Gly-NH_2 (XV-B)	63(0.5)	15	55	12
Oxytocin (XV-C)	12800(100)	2650	13100	2700

oxytocin should be considered as that of very approximation since the oxidized solution which was subjected to the biological assay might contain several by-products. It would be noteworthy that glycyl-oxytocin[12,13] carrying glycine residue to the amino group of *N*-terminal hemicystine residue showed 0.07% oxytocic potency compared with oxytocin.[13] The [9-leucine]-oxytocin preparation, however, showed appreciable biological activities; thus the replacement of the glycin residue with bulky leucine did not cause a remarkable decrease in the activities. These effects are of considerable significance to the question of the relation of structure to the biological activity of oxytocin molecule. The nature of the effects may become apparent when the role of glycine at position 9 toward the activity will be elucidated in future.

12) V. du Vigneaud, P. S. Fitt, M. Bodanszky and M. O'Connell, *Proc. Soc. Exp. Biol. (N. Y.)*, **104**, 653 (1960).
13) K. Jost, J. Rudinger and F. Sorm, *Collection Czech. Chem. Commun.*, **28**, 2021 (1963).

Experimental

All melting points are uncorrected.

Z-Leu-Leu-OEt (I-A). To a chilled solution of benzyloxycarbonyl-L-leucine (4.7 g; 20 mmol) and

triethylamine (2.8 ml) in tetrahydrofuran (40 ml), isobutyl chloroformate (2.6 ml) was added. After 15 min, a mixture of L-leucine ethyl ester hydrochloride (4.7 g; 20 mmol), triethylamine (2.8 ml) and chloroform (40 ml) was added to the solution. The reaction mixture was allowed to stand overnight and then evaporated *in vacuo*. The residual oil was dissolved in ethyl acetate, and the solution was washed successively with 4% sodium bicarbonate, 3% hydrochloric acid and water, dried over sodium sulfate and then evaporated *in vacuo*. The residual oil was solidified by the addition of petroleum ether. It was recrystallized from ethyl acetate-ether-petroleum ether; yield, 6.8 g (84%); mp 89—90°C; $[\alpha]_D^{25}$ −31.6° (*c* 1, AcOH).

Found: C, 64.92; H, 8.39; N, 7.09%. Calcd for $C_{22}H_{34}O_5N_2$: C, 65.00; H, 8.43; N, 6.89%.

H-Leu-Leu-OEt·HCl (II-A). The compound I-A (11.0 g; 27 mmol) was subjected to hydrogenolysis in the presence of palladium black and 0.76 N methanolic hydrogen chloride (37 ml). The filtrate from the catalyst was evaporated *in vacuo* leaving an oil; yield, 8.0 g (96%).

H-Leu-Gly-Gly-OEt·HCl (II-B). Z-Leu-Gly-Gly-OEt (I-B)[14] (6.7 g; 16.5 mmol) was treated as described above; yield of a hygroscopic powder, 4.2 g (92%).

Z-Pro-Leu-Leu-OEt (III-A). The mixed anhydride prepared from benzyloxycarbonyl-L-proline (3.8 g; 15 mmol) was coupled with II-A (4.7 g; 15 mmol), following the procedure used in the preparation of I-A. The crude product was recrystallized from ethanol-ether; yield, 5.6 g (73%); mp 138—139°C; $[\alpha]_D^{25}$ −88.6° (*c* 1, AcOH).

Found: C, 64.14; H, 7.96; N, 8.38%. Calcd for $C_{27}H_{41}O_6N_3$: C, 64.39; H, 8.21; N, 8.34%.

Z-Pro-Leu-Gly-Gly-OEt (III-B). The mixed anhydride prepared from benzyloxycarbonyl-L-proline (3.8 g) was coupled with II-B (4.7 g; 15.3 mmol) as has been described for the preparation of I-A. The product was recrystallized from ethyl acetate - ether - petroleum ether; yield, 4.9 g (65%); mp 133—135°C; $[\alpha]_D^{25}$ −64.8° (*c* 1, AcOH).

Found: C, 59.35; H, 7.13; N, 11.19%. Calcd for $C_{25}H_{36}O_7N_4$: C, 59.51; H, 7.19; N, 11.10%.

Z-Pro-Leu-Leu-NH_2 (IV-A). The compound III-A (6.3 g; 12.5 mmol) was dissolved in methanol (60 ml) saturated with ammonia. After it had been allowed to stand for 3 days, the solution was evaporated *in vacuo*. The remaining crystals were collected with the aid of ether. Recrystallization from ethanol-ether gave 3.9 g (66%); mp 190°C; $[\alpha]_D^{25}$ −88.5° (*c* 1, AcOH).

Found: C, 63.13; H, 7.94; N, 11.69%. Calcd for $C_{25}H_{38}O_5N_4$: C, 63.27; H, 8.07; N, 11.81%.

Z-Pro-Leu-Gly-Gly-NH_2 (IV-B). The compound III-B (5.2 g; 10.7 mmol) was converted into the amide as described above. Recrystallization from ethanol-ether gave 4.2 g (84%); mp 192—193°C; $[\alpha]_D^{25}$ −75.0° (*c* 2, AcOH).

Found: C, 57.99; H, 6.93; N, 14.75%. Calcd for $C_{23}H_{33}O_6N_5$: C, 58.09; H, 7.00; N, 14.73%.

H-Pro-Leu-Leu-NH_2·HCl (V-A). The compound IV-A (4.8 g; 10 mmol) was treated as has been described for the preparation of II-A. The product was obtained as a hygroscopic powder; yield, 3.8 g (99%).

H-Pro-Leu-Gly-Gly-NH_2·HCl (V-B). The compound IV-B (2.4 g; 5 mmol) was treated as has been described for the preparation of II-A. The product was obtained as a hygroscopic powder; yield, 1.8 g (95%).

H-Pro-Leu-Gly-NH_2·HCl (V-C). Z-Pro-Leu-Gly-NH_2 (IV-C)[15] (6.7 g; 16 mmol) was treated as described above; yield of a hygroscopic powder (V-C), 5.4 g (99%). Boissonnas *et al.* reported the synthesis of the oily hydrobromide from IV-C with hydrogen bromide-acetic acid.[9]

Z-Asn-Cys(Bzl)-OEt (VI). To a solution of *S*-benzyl-L-cysteine ethyl ester *p*-toluenesulfonate[16] (8.2 g; 20 mmol) in a mixture of dimethylformamide (20 ml) and triethylamine (4.0 ml), benzyloxycarbonyl-L-asparagine *p*-nitrophenyl ester[17] (7.7 g; 20 mmol) was added. The solution was allowed to stand at room temperature and a mass of crystals was deposited within about 1 hr. After 2 hr this mass was triturated with water (300 ml), collected by filtration, and washed successively with 4% sodium bicarbonate, 3% hydrochloric acid and water. It was recrystallized from ethanol; yield, 6.2 g (64%); mp 191°C; $[\alpha]_D^{29}$ −30.2° (*c* 2, DMF).

Found: C, 59.11; H, 6.04; N, 8.73%. Calcd for $C_{24}H_{29}O_6N_3S$: C, 59.13; H, 6.00; N, 8.62%.

H-Asn-Cys(Bzl)-OEt·HBr (VII). The compound VI (6.0 g; 12.3 mmol) was dissolved in acetic acid (40 ml) and 6.9 N hydrogen bromide - acetic acid (30 ml). After it had been allowed to stand at room temperature for 1 hr, the solution was evaporated *in vacuo*. The resulting residue was washed with ether and dried *in vacuo;* yield, 5.0 g (94%).

Z-Gln-Asn-Cys(Bzl)-OEt (VIII). To a solution of VII (5.0 g; 11.5 mmol) and triethylamine (2.5 ml) in dimethylformamide (25 ml), benzyloxycarbonyl-L-glutamine *p*-nitrophenyl ester[17] (4.6 g; 11.5 mmol) was added. After it had been allowed to stand at 30°C for 2 hr, the precipitate which formed was triturated with water (300 ml) and treated as has been described for the preparation of VI. It was recrystallized from dimethylformamide-ether; yield, 6.2 g (82%); mp 234—235°C; $[\alpha]_D^{25}$ −21.5° (*c* 2, DMF).

Found: C, 56.80; H, 6.13; N, 11.31%. Calcd for $C_{29}H_{37}O_8N_5S$: C, 56.57; H, 6.06; N, 11.38%.

Z-Gln-Asn-Cys(Bzl)-$NHNH_2$ (IX). To a solution of VIII (3.0 g; 4.8 mmol) in dimethylformamide (30 ml), hydrazine hydrate (2.5 ml) was added. After it had been allowed to stand at room temperature overnight, the mixture which contained crystals was allowed to cool for several hours. The product was then collected by filtration, and washed with water and acetone; yield, 2.7 g (95%); mp 246—248°C. Boissonnas *et al.* reported the synthesis of IX from Z-Gln-Asn-Cys(Bzl)-OMe with hydrazine in methanol by heating for 3 hr; mp 248°C.[9]

Z-Gln-Asn-Cys(Bzl)-Pro-Leu-Leu-NH_2 (X-A). To a chilled solution of IX (0.60 g; 1 mmol) in glacial acetic acid (25 ml) and 2 N hydrochloric acid (2 ml),

14) M. Bergmann, L. Zervas and J. S. Fruton, *J. Biol. Chem.*, **111**, 225 (1935).

15) M. Zaoral and J. Rudinger, *Collection Czech. Chem. Commun.*, **20**, 1183 (1955).

16) T. Kato, S. Makisumi, M. Ohno and N. Izumiya, *Nippon Kagaku Zasshi (J. Chem. Soc. Japan, Pure Chem. Sect.)*, **83**, 1151 (1962).

17) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

1 N sodium nitrite (1.1 ml) was added at 0°C. After 6 min, cold water (80 ml) was added to the solution. The azide which precipitated as a white mass was collected by filtration, washed with 4% sodium bicarbonate and water, and dried *in vacuo* at 0°C. The azide was added to a solution of V-A (0.40 g; 1 mmol) and triethylamine (0.14 ml) in dimethylformamide (10 ml). The mixture was stirred for 1 day at 0°C and further 1 day at room temperature, and then evaporated *in vacuo*. The precipitate which formed upon the addition of water was collected, and washed with 4% sodium bicarbonate, 3% hydrochloric acid and water. It was recrystallized from dimethylformamide - ethyl acetate - ether; yield, 0.59 g (66%); mp 220—221°C; $[\alpha]_D^{17}$ −56.9° (*c* 1, DMF).

Found: C, 58.06; H, 6.98; N, 13.76%. Calcd for $C_{44}H_{63}O_{10}N_9S$: C, 58.07; H, 6.98; N, 13.85%.

Z-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-Gly-NH_2 (X-B). The azide derived from IX (2.0 g; 3.3 mmol) was condensed with V-B (1.4 g; 3.3 mmol) as was described above. The product was recrystallized from dimethylformamide - ethyl acetate - ether; yield, 1.7 g (56%); mp 188—191°C; $[\alpha]_D^{17}$ −46.1° (*c* 1, DMF).

Found: C, 54.38; H, 6.41; N, 15.08%. Calcd for $C_{42}H_{58}O_{11}N_{10}S \cdot H_2O$: C, 54.29; H, 6.51; N, 15.08%.

Z-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (X-C). The azide from IX (2.46 g; 4.1 mmol) was condensed with V-C (1.32 g; 4.1 mmol) The product was recrystallized from dimethylformamide - ethyl acetate; yield, 1.68 g (48%); mp 206—208°C; $[\alpha]_D^{17}$ −53.5° (*c* 1, DMF).

Found: C, 55.59; H, 6.47; N, 14.52%. Calcd for $C_{40}H_{55}O_{10}N_9S \cdot \frac{1}{2}H_2O$: C, 55.67; H, 6.53; N, 14.61%.

Rudinger *et al.* reported the synthesis of the similar hemihydrate (mp 209—210°C),[18] and Bodanszky *et al.* reported the synthesis of the compound which has no water of crystallization (mp 233—234°C).[19]

H-Gln-Asn-Cys(Bzl)-Pro-Leu-Leu-NH_2·HBr (XI-A). The compound X-A (1.83 g; 2 mmol) was treated as has been described for the preparation of VII. The product was obtained as a hygroscopic powder; yield, 1.61 g (94%).

H - Gln - Asn-Cys(Bzl)-Pro-Leu-Gly-Gly-NH_2·HBr (XI-B). The compound X-B (0.93 g; 1 mmol) was treated as has been described for the preparation of VII. The product was obtained as a hygroscopic powder; yield, 0.85 g (96%).

H-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2·HBr (XI-C). The compound X-C (1.71 g; 2 mmol) was converted to XI-C as a hygroscopic powder; yield, 1.52 g (95%).

Z-Cys(Bzl)-Tyr-Ile-OEt (XII). *(a) ONp Method.* To a solution of H-Tyr-Ile-OEt·HCl[20] (1.9 g; 5 mmol) and triethylamine (0.7 ml) in dimethylformamide (10 ml), benzyloxycarbonyl-S-benzyl-L-cysteine *p*-nitrophenyl ester[17] (2.3 g; 5 mmol) was added at 0°C. The solution was allowed to stand overnight at room temperature. After the addition of water, the precipitate was collected by filtration and treated as has been described for the preparation of VI. It was recrystallized from ethyl acetate - ether - petroleum ether; yield, 2.5 g (80%); mp 143°C. Iselin *et al.* prepared this compound XII by the coupling of benzyloxycarbonyl-*S*-benzylcystein cyanomethyl ester and H-Try-Ile-OEt; mp 141—143°C.[21]

(b) MA Method. The product XII was also prepared from benzyloxycarbonyl-*S*-benzyl-L-cysteine and H-Tyr-Ile-OEt·HCl by the mixed anhydride method using isobutyl chloroformate; yield, 50%; mp 143°C.

Z-Cys(Bzl)-Tyr-Ile-$NHNH_2$ (XIII). The compound XII (5.2 g; 8 mmol) was converted into the hydrazide as has been described for the preparation of IX; yield, 4.0 g (80%); mp 236—238°C. Boissonnas *et al.* prepared the compound XIII from Z-Cys(Bzl)-Tyr-Ile-OMe with hydrazine in methanol; mp 238°C.[9]

Z-Cys(Bzl) - Tyr - Ile - Gln-Asn-Cys(Bzl)-Pro - Leu-Leu-NH_2 (XIV-A). The compound XIII (0.50 g; 0.8 mmol) in glacial acetic acid (40 ml) was converted to the corresponding azide with 1N hydrochloric acid (2.5 ml) and 1N sodium nitrite (0.9 ml). The azide was added to a solution of XI-A (0.69 g; 0.8 mmol) and triethylamine (0.11 ml) in dimethylformamide (8 ml). The mixture was treated in the same manner as has been described for the preparation of X-A. The crude product was recrystallized from dimethylformamide-ether; yield, 0.47 g (44%); mp 213—217°C; $[\alpha]_D^{25}$ −31.2° (*c* 1, DMF).

Found: C, 60.02; H, 6.55; N, 11.98%. Calcd for $C_{69}H_{94}O_{14}N_{12}S_2$: C, 60.07; H, 6.87; N, 12.18%.

Z-Cys(Bzl)-Tyr-Ile-Gln - Asn - Cys(Bzl) - Pro - Leu-Gly-Gly-HN_2 (XIV-B). The azide derived from XIII (0.64 g; 1 mmol) was condensed with XI-B (0.89 g; 1 mmol) as was described above. It was recrystallized from dimethylformamide-methanol-ether; yield, 0.78 g (57%); mp 225—227°C; $[\alpha]_D^{21}$ −42.0° (*c* 1, DMF).

Found: C, 58.02; H, 6.63; N, 12.85%. Calcd for $C_{67}H_{89}O_{15}N_{13}S_2$: C, 58.29; H, 6.50; N, 13.19%.

Z - Cys(Bzl) - Tyr - Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (XIV-C). The azide from XIII (1.02 g; 1.6 mmol) was condensed with XI-C (1.26 g; 1.6 mmol) as described above; yield, 0.77 g (37%) (found: C, 58.78; H, 6.41; N, 12.55%); mp 235—237°C; $[\alpha]_D^{21}$ −45.5° (*c* 1, DMF). Lit., mp 241°C, $[\alpha]_D$ −51.5° (AcOH);[9] mp 243—245°C, $[\alpha]_D$ −43° (DMF).[22]

Removal of the Protecting Groups from XIV. *(a) Na/NH_3 Method.* Each 0.06 mmol of the protected peptide (83 mg of XIV-A, 83 mg of XIV-B or 80 mg of XIV-C) was dissolved in liquid ammonia (*ca.* 50 ml), and sodium (*ca.* 40 mg) was added until a blue color persisted for 3 min. Ammonium chloride (100 mg) was added to the solution and the ammonia was allowed to evaporate spontaneously. The residue was dissolved in water (100 ml) and the pH was adjusted to 6.5 with acetic acid, and carbon dioxide free-air was bubbled through for about 4 hr until the nitroprusside reaction for sulfhydryl groups was negative. The solution was further acidified to pH 3.5 with acetic acid, and an aliquot of the solution was subjected to the tests of the rat uterine-contracting and avian depressor activities (Table 1).[23]

18) J. Rudinger, J. Honzl and M. Zaoral, *Collection Czech. Chem. Commun.*, **21**, 202 (1956).
19) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
20) H. Aoyagi, K. Arakawa and N. Izumiya, This Bulletin, **41**, 433 (1968).
21) B. Iselin, M. Feurer and R. Schwyzer, *Helv. Chim. Acta*, **38**, 1508 (1955).
22) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 2504 (1959).
23) We wish to express our thanks to Dr. S. Matsushima of Teikoku Hormone MFG. Co., Ltd. for the biological assays.

(b) HF Method.[24] Each 0.06 mmol of XIV was treated with anhydrous hydrogen fluoride (HF) as has been described in the literature.[11] An aliquot of the oxidized solution (100 m*l*) was subjected to the biological assays as was described above (Table 1). Sakakibara and Shimonishi obtained oxytocin solution with 133 units (rat-uterine test) per mg of XIV-C by HF treatment, whereas we obtained oxytocin solution with 21 units per mg of XIV-C as calculated from Table 1. We cannot give a reasonable explanation for this low yield at present.

24) One (H. A.) of us carried out the experiments with HF at Sakakibara's Laboratory of Osaka University. We wish to express our thanks to Dr. S. Sakakibara and Dr. Y. Kishida of Osaka University for their courtesy.