OXYTOCIN ANALOGUES WITH NON-CODED AMINO ACID RESIDUES IN POSITION 8: [8-NEOPENTYLGLYCINE]OXYTOCIN AND [8-CYCLOLEUCINE]OXYTOCIN*,**

Jan HLAVÁČEK[a,c], Jan POSPÍŠEK[a], Jiřina SLANINOVÁ[a],
Walter Y. CHAN[b] and Victor J. HRUBY[c]

[a] *Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6, Czechoslovakia*
[b] *Department of Pharmacology, Cornell University Medical College, New York, NY 10021, U.S.A. and*
[c] *Department of Chemistry, University of Arizona, Tucson, AZ 85721 U.S.A.*

Received February 2nd, 1987

[8-Neopentylglycine]oxytocin (*II*) and [8-cycloleucine]oxytocin (*III*) were prepared by a combination of solid-phase synthesis and fragment condensation. Both analogues exhibited decreased uterotonic potency *in vitro*, each being about 15—30% that of oxytocin. Analogue *II* also displayed similarly decreased uterotonic potency *in vivo* and galactogogic potency. On the other hand, analogue *III* exhibited almost the same potency as oxytocin in the uterotonic assay *in vivo* and in the galactogogic assay.

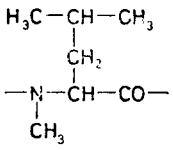
Generally, in the biological activities of oxytocin (*I*), the carboxyterminal tripeptide sequence appears to be important for obtaining high potency in oxytocin analogues[2,3]. Therefore, structural modifications of the side-chains moieties in the C-terminal tripeptide oxytocin might lead to analogues with variable biological properties at different oxytocin target tissues. Such variations in biological activity could result from conformational changes influencing interactions of the analogues with the receptor[2,4], from steric demands, or from changed susceptibility of the molecules to enzymic cleavage[5]. In this regard, non-coded amino acids often can be profitably used since they allow one to introduce some specific structural features into the analogue molecule which are normally not available.

Structural modifications in position 8 of oxytocin do not usually affect the biological properties as dramatically as those in other parts of the molecule[2,3,5]. However, most of them do decrease the oxytocin-like activities, generally having greater effect on uterotonic than galactogogic activity[5]. This dissociation of biological

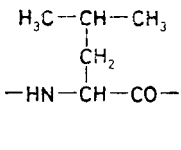
* Part CCII in the series Amino Acids and Peptides;
Part CCI: Collect. Czech. Chem. Commun. *52*, 2309 (1987).

** The nomenclature and symbols follow published recommendations of the IUPAC–IUB[1]. Additional abbreviations include: Neo, neopentylglycine; Cle, cycloleucine.

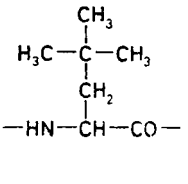
activities was especially noticeable for [8-tert-leucine]oxytocin[6,7]. This analogue with a shortened lipophilic bulky side-chain in position 8, exhibited a full galactogogic potency, but only 10% of the uterotonic potency of oxytocin.



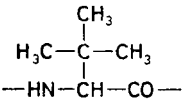
N-methylleucine



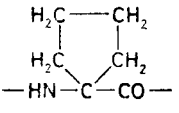
leucine



neopentylglycine



tert-leucine



cycloleucine

We now describe the synthesis and biological activities of two oxytocin analogues, [8-neopentylglycine]oxytocin (*II*) and [8-cycloleucine]oxytocin (*III*) in which the leucine side-chain was replaced either with a bulkier side-chain of the same length or with a cyclic shortened side-chain (of the same length as in [8-tert-leucine]oxytocin). Some aspects of this research have been published as a preliminary communication[8,9].

The analogue *II* was prepared by fragment condensation 6 + 3 of the protected hexapeptide acid *IVa* with prolyl-neopentylglycyl-glycine amide[10]. The compound *IVa* was synthesized on a Merrifield resin using the corresponding tert-butyloxycarbonylamino acids and dicyclohexylcarbodiimide with 1-hydroxybenzotriazole as coupling reagents, in a mixture of dichloromethane and dimethylformamide. The side chain group of both Cys and Tyr residues were protected with benzyl group. Prior to each coupling, the Boc-peptide resin was deprotected with 50% trifluoroacetic acid in dichloromethane containing 10% anisole and neutralized with 10% triethylamine in dichloromethane. The coupling reaction was monitored by the ninhydrin test[11]. The protected hexapeptide was removed from the resin under mild alkaline conditions[12] and coupled to prolyl-neopentylglycyl-glycine amide using the "complex" dicyclohexylcarbodiimide–pentafluorophenol in the presence of 1-hydroxybenzotriazole in dimethylformamide[13]. The protected nonapeptide amide *IVb* was treated with 35% hydrogen bromide in acetic acid to remove the Boc protecting group, and then was reduced with sodium in liquid ammonia. The disulfide bridge was closed by oxidation with $K_3Fe(CN)_6$. The analogue *II* was purified by means of partition chromatography and gel filtration.

H-Cys-Tyr-Ile-Gln-Asn-Cys-R

I, R = Pro-Leu-Gly-NH_2; *II*, R = Pro-Neo-Gly-NH_2; *III*, R = Pro-Cle-Gly-NH_2

R^1-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-R^2

IVa, R^1 = Boc, R^2 = OH; *IVb*, R^1 = Boc, R^2 = Pro-Neo-Gly-NH_2
IVc, R^1 = H, R^2 = Pro-Cle-Gly-NH_2

In the synthesis of analogue *III*, solid phase procedures were used exclusively. Starting from Boc-glycine-benzyl-ester resin, the peptide chain was extended using the dipeptide Boc-Pro-Cle-OH to avoid a possible cyclization to the 2,5-dioxopiperazine derivative due to the adjacent Cle and Gly residues in the peptide chain. Boc--derivatives of glutamine and asparagine were coupled through their *o*-nitrophenyl esters[14] in dichloromethane. The N^{α}-Boc protecting groups were removed by 50% trifluoroacetic acid in dichloromethane containing 10% anisole followed by neutralization of the peptide resin using 10% diisopropylethylamine in dichloromethane. The protected derivative of analogue *III* (*IVc*) was obtained by cleavage from the solid support using ammonia in methanol. The peptide was deprotected, oxidized and purified as in the preparation of analogue *II*.

Biological evaluation showed (Table I) that the uterotonic and galactogogic activities of *II* were about one order of magnitude lower than those of the parent hormone. The decrease was less pronounced in the uterotonic test *in vivo*. Interestingly, for [8-neopentylglycine]oxytocin (*II*) the introduction of the bulky tert-butyl moiety into the side-chain in position 8 led to a similar decrease in biological activities as in the analogue with N-methylleucine[16]. Both analogues also exhibit proportionally decreased pressor activity. This is in contrast with the analogue modified in position 8

TABLE I

Biological activities (I.U./mg) of some oxytocin analogues modified in position 8 with non-coded amino acid residues

Compound	Uterotonic *in vitro*	Uterotonic *in vivo*	Galactogogic *in vivo*	Pressor
Oxytocin[a](OT) (*I*)	450	450	450	5
[8-Neopentylglycine]OT (*II*)	61·5	87·6	52·9	0·15
[8-Cycloleucine]OT (*III*)	149	421	368	~0·5
[8-Tert-leucine]OT	45[b]	38·5	508[b]	2·7
[8-N-Methylleucine]OT	45[c]	17·2	46[c]	0·2

[a] Ref.[15]; [b] refs[6,7]; [c] ref.[16].

with tert-leucine in which the same structural feature, but with a shorter side-chain (less a CH_2 group), leads to an analogue with an appreciable dissociation of uterotonic and galactogogic potencies. It would appear that a small structural modification in position 8 – introduction of an additional methyl group either into the backbone or the side-chain – leads to a similar effect on the conformation of the carboxy-terminal tripeptide chain, and a similar decrease in the biological potencies of the corresponding analogues. The shortened bulky side-chain of a tert-leucine residue can, on the other hand, apparently stabilize the conformation of peptide which is fully compatible with galactogogic receptor. In contrast with the analogue *II*, the introduction of a cyclic hydrocarbon side-chain of cycloleucine to give analogue *III* affected the oxytocin-like activities to a lesser extent. The cycloleucine side-chain is highly conformationally restricted (relative to a leucine side-chain) due to its cyclic nature. Moreover, this residue has additional conformational constrains due to the quarternary α-carbon atom[17] it possesses. Interestingly, this analogue maintained high uterotonic activity (Table I) *in vitro* (about 30% that of oxytocin) and almost the same uterotonic activity as oxytocin *in vivo*. While the conformational structure requirements of the galactogogic receptor for the position 8 of oxytocin are such that analogues with a tert-leucine or a cycloleucine (*III*) have comparable potency in this assay (see also refs[2,3,5] for other examples), the uterotonic receptor can more readily accommodate a cycloleucine moiety than tert-leucine moiety in this position (Table I). In the latter case, the cyclopentane ring apparently assumes a conformation of its side-chain that is more compatible with the conformational requirements for binding of the analogue to its receptor and/or for triggering of the uterotonic activity than is the case of tert-leucine.

The results obtained in this study suggest that the conformational structure requirements for the position 8 of oxytocin for interaction with the galactogogic receptor depend more on the length than on the volume of the lipophilic side-chain. A bulky but shorter side-chain (in comparison with Leu residue) is compatible with the galactogogic receptor which appears to have higher tolerance for structure modifications than the uterotonic receptor. In addition, the maintenance of the uterotonic activity *in vivo* in analogue *III* suggests that constraining the 8-position side-chain with the Cle residue, which strongly limits its conformational freedom, leads to increased interaction with the uterotonic receptor.

EXPERIMENTAL

Analytical samples were dried over phosphorus pentoxide *in vacuo* at room temperature. Melting points were determined on a Kofler block or on a Thomas–Hoover apparatus and are uncorrected. Thin-layer chromatography was performed on Silufol plates (Kavalier, Czechoslovakia) in the following solvent systems: 2-Butanol–98% formic acid–water (75 : 13·5 : 11·5) (S_1); 2-butanol–25% aqueous ammonia–water (85 : 7·5 : 7·5) (S_2); 1-butanol–acetic acid–water (4 : 1 : 1) (S_3); 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 6) (S_4); or on pre-coated TLC plates

Silica gel 60 (Merck, Darmstadt) in the following solvent systems: 1-Butanol–acetic acid–pyridine–water (15 : 3 : 10 : 12) (S_5); 1-butanol–acetic acid–water (4 : 1 : 5, upper phase only) (S_6); 1-pentanol–pyridine–water (7 : 7 : 6) (S_7). Electrophoresis was carried out on Whatman 3MM paper (moist chamber, 20 V/cm, 1 h) in 1M acetic acid (pH 2·4) and in a pyridine–acetate buffer (pH 5·7). Detection was with iodine, ninhydrin or by the chlorination method. In all cases, unless otherwise specified, single symmetrical spots were observed for purified materials. Solvents were evaporated on a rotary evaporator (bath temperature 30°C) *in vacuo*; dimethylformamide was evaporated at the same temperature *in vacuo* (150 Pa). Amino acid analyses were performed on a Beckman 120C or on a Durrum D-500 amino acid analyzer following hydrolysis in 6M-HCl for 20 h at 110°C. In the column purifications,peptides were detected by absorption at 220 or 280 nm. Optical rotations were measured on a Perkin–Elmer 141 MCA polarimeter. High performance liquid chromatography (HPLC) was carried out on a SP 8 700 instrument equipped with a SP 8 400 UV detector and a 4 100 integrator (Spectra-Physics, Santa Clara U.S.A.). An analytical column (15 × 0·4 cm) packed with Separon SIX-C-18 was used; the flow rate was 40 ml/h. N^{α}-Boc-protected amino acids were purchased from Vega Biotechnologies (Tucson, Az) or Peninsula Laboratories (San Carlos, Ca) or were prepared in the laboratory following published methods[18,19]. Just before use, all amino acid derivatives were tested for homogeneity by thin-layer chromatography in the systems S_1 through S_7 and by mixture melting point determinations with authentic samples. Progress in peptide synthesis was followed by the ninhydrin test of Kaiser *et al.*[11]. Solvents for partition chromatography were purified as previously reported[20].

N^{α}-Tert-butyloxycarbonyl-S-benzylcysteinyl-O-benzyltyrosyl-isoleucyl-glutaminyl--asparaginyl-S-benzylcysteine (*IVa*)

N^{α}-Boc-Cys(Bzl)-resin was prepared from polystyrene resin (1% cross-linked with divinylbenzene and chloromethylated to an extent of 1·2 mmol per 1 g resin) by the Gisin method[21]. The substitution level was 0·51 mmol of cysteine *per* 1 g resin. A 3·2 g (1·6 mmol) sample of this resin was used in the synthesis. The removal of the N^{α}-Boc protecting group, neutralization of the peptide salt resin, and addition of the individual N^{α}-Boc-amino acids to the growing peptide chain followed procedures previously used[22] except that the N^{α}-Boc group was removed using 50% trifluoroacetic acid in dichloromethane containing 10% of anisole, neutralization was performed with 10% triethylamine in dichloromethane, and 1-hydroxybenzotriazole (equimolar to the amino acid) dissolved in dimethylformamide was added in each coupling step. 50 ml portions of solvents or solutions were used throughout the reaction process. At the completion of the synthesis, the protected peptide resin Boc-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-resin (4·11 g, 72%) was obtained.

The protected peptide was cleaved from the resin using the method of Buis *et al.*[12]. The peptide resin (2 g) was vigorously stirred in a mixture containing dioxane (90 ml), methanol (27 ml), and 4M-NaOH (3 ml) for 3 min. Then pH was adjusted to 6 by adding acetic acid. After filtration the resin was washed gradually with methanol (2 × 70 ml), dichloromethane (2 × × 70 ml), dimethylformamide (50 ml), methanol (110 ml), and dichloromethane (50 ml). The resin was dried and the same procedure was repeated twice more. The combined washings were evaporated, the residue dissolved in methanol, the insoluble material filtered off and water was added. A precipitate of the protected hexapeptide acid *IVa* (0·5 g) was obtained with m.p. 210 to 215°C. This product was dissolved in a hot dimethylformamide (4 ml) and boiling water (150 ml) was added to this solution. After cooling in refrigerator (1 day), the product was filtered off, washed with water and dried in a desiccator, yield 0·45 g (75%), m.p. 219—222°C; TLC, R_F: 0·62 (S_1); 0·42 (S_2); 0·63 (S_3); 0·61 (S_4). Electrophoretic analysis of sample *IVa* was carried out

after removing the Boc-protecting group: $E^{Gly}_{2.4}$ 0·51; $E^{His}_{2.4}$ 0·3; $E^{His}_{5.7}$ 0·22. Amino-acid analysis: Asp 1·00; Glu 0·95; Ile 0·92; Tyr 0·87; Cys(Bzl) 1·6. For $C_{56}H_{72}N_8O_{12}S_2$ (1 113·4) calculated: 60·41% C, 6·52% H, 10·06% N; found: 60·14% C, 6·78% H, 10·27% N.

N^{α}-Tert-butyloxycarbonyl-S-benzylcysteinyl-O-benzyltyrosyl-isoleucyl-glutaminyl-S-benzylcysteinyl-prolyl-neopentylglycyl-glycine amide (*IVb*)

Prolyl-neopentylglycyl-glycine amide hydrochloride (50 mg) was dissolved in water (5 ml) and applied on the column of Dowex 50x2 (5 ml). The chloride ions were washed out with water (100 ml) and the free base eluted with 5% NH_4OH (100 ml). The eluate was evaporated at 30°C and the residue repeatedly evaporated with a mixture of ethanol–benzene. The tripeptide amide, $E^{Gly}_{2.4}$ 1·1; $E^{His}_{2.4}$ 0·72; $E^{His}_{5.7}$ 0·87, was dissolved in dimethylformamide (1 ml) and added at 0°C to the stirred mixture containing protected hexapeptide acid *IVa* (111·4 mg), 1-hydroxybenzotriazole (27 mg) and a "complex" of dicyclohexylcarbodiimide–pentafluorophenol (105 mg). This mixture was stirred for 2 h at 0°C, 40 h at 5°C, and 5 h at room temperature, and its pH maintained on value 7·5 by adding of N-ethylpiperidine (0·2 ml). The mixture was evaporated, dissolved in dimethylformamide (1 ml), cooled in the refrigerator and the dicyclohexylurea filtered off. The filtrate was applied on the top of the column packed with Sephadex LH-20 (column size 130 × 2·3 cm) in dimethylformamide. The fractions corresponding to the main peak (206—253 ml of elution volume) wre pooled and evaporated to give 126 mg (80%), m.p. 190—195°C. The product was reprecipitated from dimethylformamide–water with a yield of 105 mg (65%), m.p. 194—197°C.

Electrophoretic analysis of the sample *IVb* after claevage of the Boc-protecting group showed $E^{Gly}_{2.4}$ 0·64; $E^{His}_{2.4}$ 0·39; $E^{His}_{5.7}$ 0·29. Amino-acid analysis: Asp 1·04; Glu 1·03; Pro 1·00; Gly 1·05; Ile 1·00; Tyr 0·97; Neo 0·95; Cys(Bzl) 1·85. For $C_{70}H_{96}N_{12}O_{14}S_2$ (1 393·8) calculated: 60·33% C, 6·94% H, 12·06% N; found: 59·98% C, 6·72% H, 12·38% N.

[8-Neopentylglycine]oxytocin(*II*)

Protected nonapeptide amide *IVb* (100 mg) was treated with 35% HBr in acetic acid (2 ml) at room temperature for 20 min. Ether was added to this solution and the precipitate was filtered off, washed with ether and dried in a desiccator. The partially deprotected peptide hydrobromide was dissolved in 100 ml of anhydrous liquid ammonia and treated with sodium stick until a blue color persisted for 30 s. Acetic acid was added and the ammonia removed by evaporation under nitrogen and the last 10 ml by lyophilization. The residue was dissolved in 0·7% acetic acid (200 ml). The pH of the solution was adjusted to 8·5 with 3M-NH_4OH and the peptide was oxidized with 50 ml of 0·003M-$K_3Fe(CN)_6$ (ref.[23]). After 30 min the pH was adjusted to 4 with 10% acetic acid and the ferro and the excess of ferricyanide were removed by addition of Amberlite IR-4B (20 ml). The resin was filtered off after 30 min of stirring and washed with three 20-ml portions of 10% acetic acid. The solution was evaporated to about 100 ml *in vacuo* at 30°C and freeze-dried. The analogue *II* was purified by partition chromatography on Sephadex G-25 using the solvent system 1-butanol–3·5% acetic acid containing 1·5% of pyridine (1 : 1). The fractions corresponding to the product (R_F 0·46) were pooled and isolated as a powder which was further purified by gel filtration on Sephadex G-25 using 0·2M acetic acid as the eluant solvent. The fractions corresponding to the major peak were pooled and freeze-dried to give pure analogue *II*; 22 mg, $[\alpha]_D^{25}$ −5·3° (*c* 0·03; 1M acetic acid), TLC, R_F: 0·2 (S_1); 0·15 (S_2); 0·16 (S_3); 0·67 (S_4), $E^{Gly}_{2.4}$ 0·54; $E^{His}_{2.4}$ 0·34; $E^{His}_{5.7}$ 0·32. Amino-acid analysis: 1/2 Cys 1·82; Tyr 0·89; Ile 0·96; Glu 1·06; Asp 1·00; Pro 1·00; Neo 0·93; Gly 1·04. For $C_{44}H_{67}N_{12}O_{12}S_2.CH_3COOH.4\,H_2O$ (1 152·3)

calculated: 47·95% C, 6·91% H, 14·59% N; found: 48·12% C, 7·09% H, 14·30% N. HPLC analysis of *II* using a mobile phase 50% methanol in 0·05% aqueous trifluoroacetic acid afforded a capacity factor $k' = 4{\cdot}6$.

N$^{\alpha}$-Tert-butyloxycarbonylprolyl-cycloleucine methyl ester

1-Hydroxybenzotriazole (2·7 g) and dicyclohexylcarbodiimide (4·1 g) were added to a stirred solution of tert-butyloxycarbonylproline (4·3 g) in dimethylformamide (50 ml) at −12°C. After 30 min the cycloleucine methyl ester hydrochloride (3·6 g), prepared according to Brenner[24], and triethylamine (2·8 ml) were added and the mixture stirred overnight at a gradually elevated temperature up to room one (20 h). Dicyclohexylurea was filtered off and dimethylformamide was evaporated off *in vacuo*. The oily residue was dissolved in ether and the solution washed three times with 1M-$NaHCO_3$, 10% citric acid and water, dried over anhydrous Na_2SO_4 and the solvent evaporated off. The remaining oil was crystallized from light petroleum to yield 4·63 g of the crude product, m.p. 84—92°C. After crystallization from ethyl acetate–light petroleum the pure compound (4·4 g; 64%), m.p. 96—98°C, was obtained. TLC, R_F: C_2H_5OH 0·73; S_5 0·72; S_6 0·73; S_7 0·68. For $C_{17}H_{28}N_2O_5$ (340·4) calculated: 59·98% C, 8·29% H, 8·23% N; found: 59·82% C, 8·35% H, 8·41% N.

N$^{\alpha}$-Tert-butyloxycarbonylprolyl-cycloleucine dicyclohexylammonium salt

Aqueous NaOH (2 mol l^{-1}, 2·5 ml) was added to the solution of N$^{\alpha}$-tert-butyloxycarbonylprolyl--cycloleucine methyl ester (1·72 g) in methanol (20 ml) and the mixture stirred at room temperature for 40 h. Methanol was evaporated off, and the alkaline solution diluted with 5 ml of water, twice washed with ethyl acetate (10 ml portions) and acidified with 20% citric acid. The oily product was extracted into ethyl acetate (5 ml washings) and the combined ethyl acetate extracts were washed with water, dried over anhydrous Na_2SO_4 and the solvent evaporated off. The residue was evaporated repeatedly with anhydrous ether and the product obtained as a foam; yield 1·35 g (82%), TLC, R_F: S_5 0·61; S_6 0·63; S_7 0·62; C_2H_5OH 0·58. This product was converted to its dicyclohexylammonium salt by adding dicyclohexylamine (0·85 ml) to a solution in benzene (3 ml), ether (2 ml), and light petroleum (50 ml). The salt was crystallized overnight in the refrigerator affording 1·55 g (74%), m.p. 135—137°C. For $C_{28}H_{49}N_3O_5$ (507·7) calculated: 66·24% C, 9·73% H, 8·38% N; found: 66·01% C, 9·99% H, 8·42% N.

S-Benzylcysteinyl-O-benzyltyrosyl-isoleucyl-glutaminyl-asparaginyl-S-benzyl--cysteinyl-prolyl-cycloleucyl-glycine amide (*IVc*)

N$^{\alpha}$-Boc-Gly-resin was prepared from polystyrene resin (1% cross-linked with divinylbenzene and chloromethylated to an extent of 1·06 mmol *per* 1 g resin; Lab Systmes, San Mateo, Ca) using standard procedure[21]. The substitution level was 0·43 mmol of glycine *per* 1 g resin. A 4·65 g (2 mmol) sample of this resin was used in the synthesis. N$^{\alpha}$-Boc-protected amino acid derivatives and N$^{\alpha}$-Boc-Pro-Cle were used in the coupling reaction. The removal of the N$^{\alpha}$-Boc protecting groups, neutralization of the peptide salt resin, and addition of the individual amino--acid residues to the growing peptide chain followed published procedures[22] except that the N$^{\alpha}$-Boc group was removed using 50% trifluoroacetic acid containing 2% anisole in dichloromethane, and 1-hydroxybenzotriazole was added in each coupling step. 60 ml portions of solvent or solutions were used throughout the synthesis. Following these procedures, the partially protected peptide resin H-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro-Cle-Gly-resin (6·02 g) was obtained.

The protected peptide was cleaved from the resin on adding the resin to a solution of 80 ml freshly prepared anhydrous methanol saturated with anhydrous ammonia at −5°C. The mixture was stirred in wire shut flask at room temperature for 4 days in a desiccator. After the solvents were removed *in vacuo*, the cleaved peptide was washed from the resin with two 60-ml portions of dimethylformamide at 60°C. The combined washings were evaporated down to about 10 ml *in vacuo* and water was added (150 ml). The solid which precipitated was filtered off and washed with two 20-ml portions of water, ethanol, and ether. The precipitate was dried *in vacuo* to give 0·49 g of the compound *IVc*, m.p. 228–230°C. Amino-acid analysis: Cys(Bzl) 1·65; Tyr 0·91; Ile 0·98; Gln 1·00; Asn 1·03; Pro 1·04; Cle 0·97; Gly 1·02. For $C_{64}H_{84}N_{12}O_{12}S_2$ (1 277·6) calculated: 60·17% C, 6·63% H, 13·16% N; found: 59·91% C, 6·85% H, 13·01% N.

[8-Cycloleucine]oxytocin (*III*)

A sample of 250 mg of the protected peptide *IVc* was dissolved in 250 ml of anhydrous liquid ammonia and reduced as in the preparation of the analogue *II*. The residue was dissolved in 0·1% acetic acid (500 ml) and oxidation of the sulfhydryl groups of cysteine residues was performed as in the case of analogue *II*. The ferro and the excess ferricyanide were removed by addition of Rexyn 203 (Cl^- cycle). The analogue was purified by partition chromatography[25] on Sephadex G-25 using the solvent system 1-butanol–3·5% acetic acid containing 1·5% pyridine (1 : 1). The fractions corresponding to the product (R_F 0·22) were pooled and isolated as a powder which was further purified by gel filtration on Sephadex G-25 using 0·2M acetic acid as the eluant solvent. The fractions corresponding to the major peak were pooled and freeze-dried to give pure analogue *III*, 102 mg, $[\alpha]_D^{22}$ −8·0°; (*c* 0·4; 1M acetic cid). TLC, R_F: 0·29 (S_5); 0·76 (S_6); 0·62 (S_7), $E_{2.4}^{Gly}$ 0·62; $E_{2.4}^{His}$ 0·36; $E_{5.7}^{His}$ 0·41. HPLC in 50% methanol–0·05 trifluoroacetic acid $k' = 2·3$. Amino-acid analysis: 1/2 Cys 2·02; Tyr 0·92; Ile 1·03; Glu 1·05; Asp 1·00; Pro 0·98; Cle 0·97; Gly 1·04. For $C_{43}H_{64}N_{12}O_{12}S_2.CH_3COOH.4\,H_2O$ (1 137·3) calculated: 47·48% C, 6·74% H, 14·78% N; found: 47·71% C, 6·79% H, 14·42% N.

Biological Assay Methods

The *in vitro* uterotonic assay was carried out on isolated rat uterine strips[26–28]. For the determination of the *in vivo* activity[29] oestrogenized rats in ethanol anaesthesia were used. Galactogogic activity was determined on ethanol-anaesthesized rats (4–15 days after delivery)[30,31]. Pressor activity was measured on despinalized rats[32].

The authors are indebted to Mrs I. Hošková for the technical assistance in the biological activity tests, to Mr J. Zbrožek for the amino acid analyses and to Mrs Z. Ledvinová for optical rotation measurements. The elemental analyses were carried out in the analytical laboratory of the Institute of Organic Chemistry and Biochemistry (Dr J. Pechanec). This work was supported in part by grants from the U.S. Public Health Service AM 17420 (VJH), *the National Science Foundation* PCM-8412084 (VJH) *and by the* NIH HD-20839 (WYC).

REFERENCES

1. IUPAC–IUB: *Nomenclature and Symbolism for Amino Acids and Peptides, Recommendations 1983*, Eur. J. Biochem. *138*, 9 (1984).
2. Hruby V. J. in the book: *Biochemical Actions of Hormones* (G. Litwack, Ed.), pp. 191–241. Academic Press, New York 1986.
3. Hruby V. J., Smith C. W. in the book: *Peptides: Analysis, Synthesis, Biology* (C. W. Smith, Ed.), Vol. 8. Academic Press, New York, in press 1987.

4. Hruby V. J. in the book: *Oxytocin: Clinical and Laboratory Studies* (J. A. Amico and A. G. Robinson, Eds), pp. 405—414. Elsevier/North Holland, New York 1985.
5. *Handbook of Neurohypophyseal Hormone Analogs* (K. Jošt, M. Lebl and F. Brtník, Eds) CRC Press, Boca Raton, Florida, U.S.A., in press 1987.
6. Hlaváček J., Barth T., Pospíšek J., Frič I., Jošt K. in the book: *Peptides 1980* (K. Brunfeldt, Ed.), p. 482. Scriptor, Copenhagen 1981.
7. Lebl M., Pospíšek J., Hlaváček J., Barth T., Maloň P., Servítová L., Hauser K., Jošt K.: Collect. Czech. Chem. Commun. *47*, 689 (1982).
8. Hlaváček J., Pospíšek J., Slaninová J., Barth T., Jošt K. in the book: *Peptides 1984* (U. Ragnarsson, Ed.), p. 415. Almquist, Stockholm 1985.
9. Hlaváček J., Jošt K.: *F.E.C.S. 3rd Int. Conf. Chem. Biotechnol. Biol. Act. Natur. Prod., Sofia,* Vol. 3, p. 244. Publishing House of the Bulgarian Academy of Sciences, Sofia 1985.
10. Pospíšek J., Bláha K.: Collect. Czech. Chem. Commun. *52*, 514 (1987).
11. Kaiser E., Colescott R. L., Bossinger C. D., Cook P. I.: Anal. Biochem. *34*, 595 (1970).
12. Buis J. T. W. A. R. M., Tesser G. I., Nivard R. J. F.: Tetrahedron *32*, 2321 (1967).
13. Hlaváček J., Barth T., Bláha K., Jošt K.: Collect. Czech. Chem. Commun. *44*, 275 (1979).
14. Bodanszky M., Funk K. W., Fink M. L.: J. Org. Chem. *38*, 3565 (1973).
15. Berde B., Boissonnas R. A. in the book: *The Handbook of Experimental Pharmacology* (B. Berde, Ed.), Vol. 23, p. 802. Springer, Berlin, (1982).
16. Hlaváček J., Poduška K., Jošt K., Frič I., Barth T., Cort J. H., Bláha K., Šorm F.: Collect. Czech. Chem. Commun. *42*, 1233 (1977).
17. Marshall G. R., Bosshard H. E.: Circ. Res. Suppl. II, *30/31*, 143 (1972).
18. Schnabel E.: Ann. Chem. *702*, 188 (1967).
19. Pozdnev V. F., Podgornova N. N., Zencova N. K., Aukone G. I., Kalei U. O.: Khim. Prirod. Soed. *4*, 543 (1979).
20. Hruby V. J., Groginsky C. M.: J. Chromatogr. *63*, 427 (1971).
21. Gisin B. F.: Helv. Chim. Acta *56*, 1475 (1973).
22. Hruby V. J., Deb K. K., Yamamoto D. M.: J. Med. Chem. *22*, 7 (1979).
23. Hope D. B., Murti V. V. S., du Vigneaud V.: J. Biol. Chem. *237*, 1563 (1962).
24. Brenner M., Huber W.: Helv. Chim. Acta. *36*, 1109 (1953).
25. Yamashiro D.: Nature (London) *200*, 76 (1964).
26. Chan W. Y., Fear R., du Vigneaud V.: Endocrinology *81*, 1267 (1967).
27. Holton P.: Brit. J. Pharmacol. *3*, 328 (1948).
28. Munsick R. A.: Endocrinology *66*, 451 (1960).
29. Pliška V.: Eur. J. Pharmacol. *6*, 253 (1969).
30. Bisset G. W., Clark B. J., Haldar J., Harris M., Lewis G. P., Rocha-e-Silva M.: Brit. J. Pharmacol. Chemotherap. *31*, 537 (1967).
31. Barth T., Jošt K., Rychlík I.: Endocrinol. Exper. *9*, 35 (1974).
32. Krejčí I., Kupková B., Vávra I.: Brit. J. Pharmacol. Chemotherap. *30*, 497 (1967).