ANALOGUES OF NEUROHYPOPHYSIAL HORMONES, CONTAINING TERT-LEUCINE: SYNTHESIS AND PROPERTIES*

Michal LEBL, Jan POSPÍŠEK, Jan HLAVÁČEK, Tomislav BARTH, Petr MALOŇ, Linda SERVÍTOVÁ, Karel HAUZER and Karel JOŠT

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6

Received June 16th, 1981

Analogues of oxytocin, deamino-oxytocin and deamino-vasopressin, containing tert-leucine in position 8, were prepared by fragment condensation in solution. Stepwise synthesis in solution afforded analogues of 1-carba- and 6-carba-deamino-oxytocin with tert-leucine in position 2. [8-Tert-leucine]oxytocin proved to be the most interesting of this series; it exhibits a relatively high and specific galactogogic activity and is cleaved with chymotrypsin by an order of magnitude more slowly than oxytocin.

It is well known that even mild structural changes in the molecules of neurohypophysial hormones oxytocin (*Ia*) and vasopressin (*Ib*) can influence their spatial arrangement, interaction with receptors or enzymatic resistance and change thus their biological properties. One of the most frequent modifications of the hormone structure is substitution in the side-chains of the amino acid moieties.** Non-coded amino acids are often used for this purpose, introducing thus some specific structural features into the analogue molecules.

Some time ago we described several derivatives and simple peptides of tert-leucine, *inter alia* also a carboxy-terminal oxytocin tripeptide in which leucine was replaced by the sterically more demanding tert-leucine[2]. The reactivity of functional groups in tert-leucine (including hydrolysis of its esters) is substantially lower than the reactivity of other α-amino acids, containing aliphatic side chains[2]. It was also observed that the tert-butyl side chain of tert-leucine affects significantly the conformation of peptides, containing this amino acid[3]. For this reason, we introduced tert-leucine into the position 8 which is critical for cleavage of neurohypophysial hormones and at the same time offers enough freedom for structural modifications to which receptors of both hormones are relatively tolerant. Replacement of leucine

* Part CLXXVII in the series Amino Acids and Peptides; Part CLXXVI: This Journal *47*, 338 (1982).

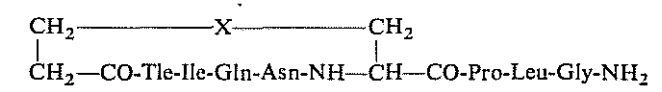
** The amino acids used in this paper are of the L-series. The nomenclature and symbols obey the published recommendations[1]. Tle denotes tert-leucine, Mpr β-mercaptopropionic acid and Hcy homocysteine moieties.

in position 8 of the oxytocin molecule by a more bulky amino acid (analogues *Ic* and *Id*) could show how the steric demands of an amino acid in this position affect the biological activity and also whether steric features are more important than lipophilicity. In the vasopressin molecule (analogue *Ie*), the same change, introducing an increased hydrophobicity (under simultaneous elimination of the basic group), should substantially reduce the vasopressin activities but since conformation of the tripeptide chain in vasopressin should differ from that in the oxytocin molecule, its effect on oxytocic activities could be different from the case of oxytocin.

$$\text{X-Tyr-Y-Gln-Asn-Cys-Pro-Z-Gly-NH}_2$$

Ia, X = Cys, Y = Ile, Z = Leu
Ib, X = Cys, Y = Phe, Z = Arg
Ic, X = Cys, Y = Ile, Z = Tle
Id, X = Mpr, Y = Ile, Z = Tle
Ie, X = Mpr, Y = Phe, Z = Tle

We prepared also two oxytocin analogues in which tert-leucine was introduced into the position 2 (instead of tyrosine). For this position the effect of substitution with an aliphatic amino acid is also known: introduction of leucine or valine reduces the biological activities substantially more than introduction of isoleucine[4–6]. The introduction of tert-leucine should show whether the bulky substituent would resemble leucine or isoleucine in its effect on biological activities. Since the effect of substitution with aliphatic amino acids is known also in the 1-carba[7] and 6-carba[8] analogues which, moreover, retain higher biological activities as compared with the parent disulfide compounds, we prepared also [2-tert-leucine]deamino-1-carba-oxytocin (*IIa*) and [2-tert-leucine]deamino-6-carba-oxytocin (*IIb*).



$$\begin{array}{l} \text{CH}_2\text{———X———CH}_2 \\ \quad\vert\qquad\qquad\qquad\vert \\ \text{CH}_2\text{—CO-Tle-Ile-Gln-Asn-NH—CH—CO-Pro-Leu-Gly-NH}_2 \end{array}$$

IIa, X = CH_2—S *IIb*, X = S—CH_2

In the synthesis of analogues *Ic*–*Ie* we used fragment condensation of the protected hexapeptides *IIIa*, *IIIc* and *IIId* with prolyl-tert-leucyl-glycinamide (*IV*). The hexapeptide *IIIb* was prepared by azide condensation[9] of S-benzylmercaptopropionyl--tyrosyl-isoleucine hydrazide[10] with the tripeptide *Va*, obtained by removal of the protecting group from the peptide *Vb*. The protected intermediate *IIIe* for the preparation of [8-tert-leucine]oxytocin was prepared using the pentafluorophenol-dicyclohexylcarbodiimide complex[11,12] in the presence of 1-hydroxybenzotriazole; this method proved to be the method of choice also in other cases[13,14]. The protecting groups were removed from the compound *IIIe* with sodium in liquid ammonia,

the disulfide bond was closed by air oxidation and the product was purified by countercurrent distribution and gel filtration. The corresponding deamino derivative *IIIf* was prepared by azide condensation but the product was formed in only a 40% yield and its separation from side products by gel filtration on Sephadex LH-20 in dimethylformamide was very difficult. We purified therefore the protected peptide for analytical purposes only and the free peptide *Id* was prepared directly from the crude protected peptide. After removal of the protecting groups with sodium in liquid ammonia and oxidative cyclization with potassium ferricyanide, the reaction mixture was desalted on a series of three Sep-pak C^{18} cartridges. The peptide mixture was eluted with methanol and purified by high performance liquid chromatography (HPLC) on a reversed phase. The fractions were characterized by amino acid analysis and uterotonic activity. On the other hand, the azide condensation in the preparation of the vasopressin analogue gave high yield of very pure product. The protected peptide *IIIg* was reduced and oxidized in the same manner as the compound *IIIf* and the analogue *Ie* was purified by gel filtration only. In the synthesis of the carba-analogues *IIa* and *IIb* the partially protected octapeptides *VIb* and *VId* were prepared by acylation of the corresponding heptapeptides *VIa* (ref.[15]) and *VIc* (ref.[16]), using symmetric anhydride of *o*-nitrobenzenesulfenyl-tert-leucine. After transformation into the *p*-nitrophenyl or 2,4,5-trichlorophenyl ester and removal of the amino-protecting group, the compounds were cyclized in pyridine under conditions of high dilution. The analogues *IIa* and *IIb* were purified either by countercurrent distribution and gel filtration, followed by HPLC on a reversed phase, or by

X(Bzl)-Tyr-Y-Gln-Asn-Cys(Bzl)-Z

IIIa, X = Tos-Cys, Y = Ile, Z = OH
IIIb, X = Mpr, Y = Ile, Z = OMe
IIIc, X = Mpr, Y = Ile, Z = N_2H_3
IIId, X = Mpr, Y = Phe, Z = N_2H_3
IIIe, X = Tos-Cys, Y = Ile, Z = Pro-Tle-Gly-NH_2
IIIf, X = Mpr, Y = Ile, Z = Pro-Tle-Gly-NH_2
IIIg, X = Mpr, Y = Phe, Z = Pro-Tle-Gly-NH_2

Pro-Tle-Gly-NH_2

IV

X-Gln-Asn-Cys(Bzl)-OMe

Va, X = H *Vb*, X = Nps

X-Ile-Gln-Asn-Y-Pro-Leu-Gly-NH_2

VIa, X = H, Y = Cys($C_3H_6CO_2H$) *VIb*, X = Nps-Tle, Y = Cys($C_3H_6CO_2H$)
VIc, X = H, Y = Hcy($C_2H_4CO_2H$) *VId*, X = Nps-Tle, Y = Hcy($C_2H_4CO_2H$)

HPLC only. The second method was quite satisfactory for obtaining pure analogues; it was faster and gave no sulfoxides, found as side products when the compounds were purified by the first method.

Biological activities of the prepared analogues, together with those of some reference peptides, are given in Table I. The most interesting compound of the prepared tert-leucine analogues appears to be [8-tert-leucine]oxytocin (*Ic*) which exhibits a marked dissociation of galactogogic and uterotonic activities. In the case of its deamino derivative *Id*, however, this dissociation is suppressed and the compound exhibits higher uterotonic than galactogogic activity. Anyway, it is obvious that the presence of tert-leucine in position 8 does not destroy the uterotonic activity; the same behaviour was observed also with the vasopressin analogue *Ie*. However, the uterotonic activity of the analogues *Ic*–*Ie* is lower than that of the parent compounds; it cannot be excluded that this is due to a lower flexibility of the tripeptide side chain caused by the steric bulk of the tert-leucine moiety. Drop in the uterotonic activity is observed also with the carba-analogues. As compared with the 2-isoleucine analogues, compound *IIa* is by two orders of magnitude and compound *IIb* by one order of magnitude less active.* The difference in galactogogic activity of the tert-leucine and isoleucine analogues is not so pronounced; this indicates lower steric demands of the galactogogic than of the uterotonic receptor. All the analogues, described in this paper, except compound *Id*, exhibit higher galactogogic than uterotonic activity. To a certain extent this difference can be caused by a different sensitivity to enzymatic cleavage of bonds containing either coded amino acids or tert-leucine (uterotonic activity was determined *in vitro*, galactogogic activity *in vivo*).

We studied therefore the rate of oxytocin cleavage with chymotrypsin and compared it with that of the analogue *Ic*. The cleavage rate was determined both by following the uterotonic activity of the sample on incubation with chymotrypsin, and by HPLC, the latter method being substantially more accurate. Cleavage of the glycinamide moiety in *Ic* was about ten times slower than in oxytocin (at the time, corresponding to 53% cleavage of oxytocin, only 6% of the analogue *Ic* reacted). These results agree with those obtained by the uterotonic tests. Similar resistance of analogue *Ic* toward enzymatic cleavage was found also with post-proline-cleaving enzyme[17] by which this compound was cleaved again by an order of magnitude more slowly than oxytocin.

We studied also kinetics of oxidation of both the carba-analogues. It has already been shown previously[18] that the 6-sulfur atom is less accessible to the attack by the oxidation reagent than the 1-sulfur atom. Whether this shielding is caused by interaction of the aromatic system in position 2 with the 6-sulfur atom or only by steric reasons, could be decided by studying the pair *IIa* and *IIb* in which the

* In our previous work[8] the uterotonic activity of [2-isoleucine]deamino-6-carba-oxytocin was erroneously given to be 3 I.U./mg. The actual value is 66 I.U./mg.

interaction between the aromatic system and sulfur atom is excluded but, on the other hand, the steric demands of the side chain in the amino acid in position 2 are large. Since both analogues have very similar retention times, it was not possible to carry out a competitive oxidation of their mixture. Similarly to oxytocin carba-analogues[19], the 6-carba-analogue *IIb* has shorter retention time and the arising stereoisomeric sulfoxides cannot be separated, whereas oxidation of the 1-carba-analogue *IIa* gives separable sulfoxides (in the ratio 2·4 : 1). Half-time of oxidation of compounds *IIa* and *IIb* was 11·5 and 7·3 min, respectively. This behaviour shows thus again the non-equivalence of the bridge sulfur atoms and the lesser accessibility of the atom in position 6; however, in comparison with tyrosine-containing compounds[18] this difference is somewhat smaller. The shielding effect is thus mainly of steric origin but some contribution of the sulfur–aromate interaction cannot be excluded[20].

The shape of CD spectra of the analogue *Ic* (in phosphate buffer pH 7·5, in 0·01M-HCl and in hexafluoroacetone trihydrate) does not differ significantly from that of oxytocin (*Ia*) (see Table I and Fig. 4 in ref.[21] and Table III in ref.[22]), small differences being only in intensities of the dichroic bands. The positive complex band at 224–228 nm due to the tyrosine $^1A_{1g} \rightarrow {}^1B_{1u}$ transition and the amide $n-\pi^*$ transition is by about 30% less intense for *Ic* than for oxytocin in all solvents employed. Similarly, the intensity of the negative short wavelength complex band at 195–197 nm (contributed by the $^1A_{1g} \rightarrow {}^1E_{1u}$ transition of tyrosine aromatic chromophore and the amide $\pi-\pi^*$ transition) is invariably higher by about 30%. The long

TABLE I

Biological activities of the prepared analogues (I.U./mg)

Compound	Uterotonic (*in vitro*)	Galactogogic (*in situ*)	Pressor	Antidiuretic
Oxytocin[a] (*Ia*)	450	450	5	5
Deamino-oxytocin[a] (*Ib*)	795	266[b]	1·4	19
Deamino-lysine-vasopressin[a] (*Ic*)	12	33[c]	131	313
[Ile^2]deamino-1-carba-oxytocin[b,d]	347	327	—	2
[Ile^2]deamino-6-carba-oxytocin[e]	66	90	—	—
Ic	45	508	2·7	0·7
Id	184	120	1·4	—
Ie	2·9	17·1	3·2	6
IIa	3·3	73·6	<0·2	—
IIb	7·3	44·6	—	—

[a] Ref.[30]; [b] ref.[27]; [c] determined on rabbit; [d] ref.[7]; [e] ref.[8].

wavelength bands of the disulfide chromophore and the band due to the $^1A_{1g} \rightarrow {}^1B_{2u}$ transition of the aromatic chromophore are within the experimental error identical with those of oxytocin. On the basis of CD spectra we can thus conclude that conformation of the analogue *Ic* in solution is approximately the same as that of oxytocin and that minor conformational changes, if any, probably do not involve the cyclic part of the molecule. The observed intensity differences can be interpreted as the result of a greater fixation of the terminal tripeptide and possibly also of an interaction with the tyrosine side chain.

EXPERIMENTAL

Analytical samples were dried over phosphorus pentoxide *in vacuo* (150 Pa) at room temperature. Melting points were determined on a Kofler block and are uncorrected. Thin-layer chromatography was performed on Silufol plates (Kavalier, Czechoslovakia) in the following systems: 2-butanol–98% formic acid–water (75 : 13·5 : 11·5) (S1), 2-butanol–25% aqueous ammonia–water (85 : 7·5 : 7·5) (S2), 1-butanol–acetic acid–water (4 : 1 : 1) (S3), and 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 6) (S4). Electrophoresis was carried out on a 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 with ninhydrin or chlorination method. Solvents were evaporated on a rotatory evaporator (bath temperature 30°C) in the vacuum of a water pump; dimethylformamide was evaporated at the same temperature at 150 Pa. Peptides, contăining tert-leucine, were hydrolyzed with a mixture of propionic acid and 12M-HCl (ref.[23]) in the presence of phenol at 160°C and 150 Pa for 6 h. Amino acid analyses were performed on a two-column apparatus type 6020 (Development Workshops, Czechoslovak Academy of Sciences). Countercurrent distribution was carried out in an all-glass Steady State Distribution Machine (Quickfit & Quartz, Stone, Staffordshire, England) with possibility of transfer of both phases; solvent system 2-butanol–0·05% aqueous acetic acid. The peptide material was detected by the Folin–Ciocalteau reagent. In the gel filtrations, peptides were detected by absorption at 280 nm. Optical rotations were measured on a Perkin–Elmer 141 MCA instrument. High performance liquid chromatography was carried out on an SP-8700 instrument (Spectra-Physics, Santa Clara, USA), equipped with an SP-8400 detector and SP-4100 integrator of the same provenience. Analytical chromatography was done on a 25 × 0·4 cm Separon SI-C-18 column (Laboratorní přístroje, Prague), preparative runs on a 50 × 0·9 cm Partisil-ODS-2 column (Whatman, New Jersey, USA).

N^{α}-*p*-Toluenesulfonyl-S-benzylcysteinyl-tyrosyl-isoleucyl-glutaminyl-asparaginyl--S-benzylcysteinyl-prolyl-tert-leucyl-glycine Amide (*IIIe*)

Prolyl-tert-leucyl-glycinamide[2] (0·125 g) was added to a solution of the hexapeptide *IIIa* (ref.[13]; 0·24 g), 1-hydroxybenzotriazole (0·95 g) and dicyclohexylcarbodiimide–pentafluorophenol complex[11,12] (0·33 g)in dimethylformamide (5 ml). After stirring for 3 days at room temperature, the separated dicyclohexylurea was filtered off and dimethylformamide evaporated. The residue was successively triturated with 5% $NaHCO_3$ solution, water, 1M-HCl and again water and crystallized from aqueous dimethylformamide, affording 0·26 g (90%) of the product, m.p. 208—210°C, $[\alpha]_D$ —13·6° (*c* 0·25, dimethylformamide); R_F 0·51 (S1), 0·37 (S2), 0·77 (S4). Amino acid analysis: Asp 1·06, Glu 1·00, Pro 1·06, Gly 1·06, Tle 1·04, Ile 0·96, Tyr 0·99, Cys(Bzl) 1·75. For $C_{62}H_{96}N_{12}O_{13}S_3$ (1 313) calculated: 56·69% C, 7·37% H, 12·79% N, 7·32% S; found: 56·48% C, 7·38% H, 12·53% N, 7·26% S.

[8-Tert-leucine]oxytocin (*Ic*)

The protected nonapeptide *IIIe* (0·15 g) was reduced with sodium in liquid ammonia till the blue colour persisted. After 20 s the excess sodium was destroyed with NH_4Cl and ammonia was evaporated. The residue was dissolved in dilute HCl (pH of the solution 3·5), the solution was extracted with ether, adjusted to pH 6·9 with 0·1M-NaOH (total volume of the solution 700 ml) and the compound was oxidized with air for 2 h. After adjusting to pH 3·5 with acetic acid, the solution was concentrated and freeze-dried. The product was purified by countercurrent distribution (220 transfers of the upper and 50 of the lower phases, $K = 0{\cdot}34$) and gel filtration (Bio-Gel P-4, 1M acetic acid); yield 25 mg, R_F 0·19 (S1), 0·08 (S2), 0·10 (S3), 0·32 (S4); $E_{2,4}^{Gly}$ 0·67, $E_{5,7}^{His}$ 0·32. $[\alpha]_D$ −19·4° (*c* 0·3, 1M acetic acid), $k' = 4{\cdot}4$ (methanol–0·01M phosphate buffer pH 4·0, 1 : 1). For $C_{43}H_{66}N_{12}O_{12}S_2.C_2H_4O_2.5\,H_2O$ (1 157) calculated: 46·70% C, 6·97% H, 14·52% N; found: 46·70% C, 6·76% H, 14·80% N.

Methyl Ester of *o*-Nitrobenzenesulfenylglutaminyl-asparaginyl-S-benzylcysteine (*Vb*)

2,4,5-Trichlorophenyl ester of *o*-nitrobenzenesulfenylglutamine (5·2 g) was added at room temperature to a stirred solution of methyl asparaginyl-S-benzylcysteinate hydrobromide (4·15 g) and N-ethylpiperidine in dimethylformamide (25 ml). After two days, dimethylformamide was evaporated, the residue washed successively with light petroleum, ether, 0·5M sodium hydrogen carbonate solution, water, and dried. Trituration with ethyl acetate afforded 3·85 g (62%) of the product, m.p. 192—194°C; R_F 0·62 (S1), 0·50 (S2), 0·70 (S3), 0·72 (S4). The analytical sample was crystallized from dimethylformamide, m.p. 199—200°C. $[\alpha]_D$ −23·0° (*c* 0·2, dimethylformamide). For $C_{26}H_{32}N_6O_9S_2$ (636·7) calculated: 49·05% C, 5·06% H, 13·20% N; found: 49·15% C, 5·10% H, 13·20% N.

Methyl Ester of S-Benzyl-β-mercaptopropionyl-tyrosyl-isoleucyl-glutaminyl-asparaginyl--S-benzylcysteine (*IIIb*)

n-Butyl nitrite (160 μl) was added to a solution of S-benzyl-β-mercaptopropionyl-tyrosyl-isoleucine hydrazide[10] (0·42 g) in a mixture of dimethylformamide (16 ml) and 7M-HCl in tetrahydrofuran (1·75 ml), cooled to −30°C. After stirring for 4 min at this temperature, the mixture was treated with dimethylformamide solution (10 ml) of hydrochloride of the tripeptide *Va*, obtained from the protected tripeptide *Vb* (1·24 g) by treatment with HCl (2 ml, 2·26M in ether) in dimethylformamide (10 ml) and precipitation with ether. The pH was adjusted to 8·5 with N-ethylpiperidine and after standing for 110 h at 0°C the solution was diluted with 1M-HCl (100 ml), the precipitate filtered, washed with 1M-HCl, water and dried, affording 0·64 g (85%) of the product, m.p. 236—240°C. The analytical sample was precipitated from dimethylformamide and water; m.p. 238—241°C, $[\alpha]_D$ −18·5° (*c* 0·2, dimethylformamide). For $C_{45}H_{59}N_7O_{10}S_2$ (922·1) calculated: 58·61% C, 6·45% H, 10·63% N; found: 58·39% C, 6·78% H, 10·40% N. Amino acid analysis: Asp 1·04, Glu 1·02, Ile 0·96, Tyr 0·98, Cys(Bzl) 0·48.

S-Benzyl-β-mercaptopropionyl-tyrosyl-isoleucyl-glutaminyl-asparaginyl-S-benzylcysteine Hydrazide (*IIIc*)

The methyl ester *IIIb* (0·6 g) was dissolved in dimethylformamide (20 ml) and mixed with hydrazine hydrate (1 ml). The mixture was stirred at room temperature for 80 h, diluted with water (80 ml) and cooled to 0°C. The precipitate was filtered, washed with water and ethanol and dried, affording 0·57 g (95%) of the product, m.p. 256—258°C. The analytical sample was precipitated

from dimethylformamide and water; m.p. 258—261°C. $[\alpha]_D$ —27·4° (*c* 0·2, dimethylformamide). For $C_{44}H_{59}N_9O_9S_2.2\,H_2O$ (958·1) calculated: 55·16% C, 6·63% H, 13·16% N; found: 54·91% C, 6·27% H, 13·19% N.

S-Benzyl-β-mercaptopropionyl-tyrosyl-isoleucyl-glutaminyl-asparaginyl-
-S-benzylcysteinyl-prolyl-tert-leucyl-glycine Amide (*IIIf*)

A solution of the hexapeptide hydrazide *IIIc* (200 mg) in dimethylformamide (10 ml) and 4·4M-HCl in tetrahydrofuran (0·45 ml) was cooled to —30°C and mixed with n-butyl nitrite (50 µl). After 4 min, a solution of hydrochloride of the tripeptide *IV* (0·25 g) and N-ethylpiperidine (0·5 ml) in dimethylformamide (2 ml) was added and the resulting mixture was set aside for 70 h at 0°C (pH 8·5). The product was precipitated with 1M-HCl, filtered and washed with 1M-HCl and water. Drying on air afforded 230 mg of compound, whose amino acid analysis gave only 40% of the theoretical values for Pro, Tle and Gly. The product was dissolved in dimethylformamide and applied on a column of Sephadex LH-20 (2·5 × 100 cm). The chromatography, however, showed only slight separation. A part of the product (40 mg) was chromatographed twice on a column (1 × 200 cm), filled with the same material, affording 5·3 mg of compound of m.p. 229—234°C; $[\alpha]_D$ —15·0° (*c* 0·4, dimethylformamide). R_F 0·46 (S1), 0·37 (S2), 0·54 (S3), 0·64 (S4). For $C_{57}H_{79}.N_{11}O_{13}S_2.4\,H_2O$ (1 263) calculated: 54·22% C, 6·93% H, 12·20% N; found: 53·75% C, 6·48% H, 12·53% N. Amino acid analysis: Asp 1·01, Glu 1·04, Pro 0·86, Gly 0·89, Ile 1·03, Tle 0·86, Tyr 0·90, Cys(Bzl) 0·36.

[8-Tert-leucine]deamino-oxytocin (*Id*)

The protected crude peptide *IIIf* (180 mg) was dissolved in liquid ammonia (40 ml) and reduced and oxidized in the same manner as described for the preparation of compound *Ie*. After cyclization, the solution was filtered through a series of three Sep-pak C^{18} columns which were then washed with water and the peptide material was eluted with methanol. The thus-obtained solution was diluted with water (5 ml) and concentrated to a small volume (7 ml). A part (2 ml) was applied on a column of Partisil ODS-2 (50 × 0·9 cm) and chromatographed in a mixture of methanol and water (2 : 3). Of the seven fractions obtained, only one contained a compound of the desired amino acid analysis. This fraction was also the only one, which exhibited uterotonic activity. Freeze-drying afforded 4·3 mg of compound, R_F 0·16 (S1), 0·13 (S2), 0·36 (S3), 0·62 (S4), pure according to HPLC ($k' = 3·3$, methanol–water 1 : 1). $[\alpha]_D$ —61·1° (*c* 0·14, 3M acetic acid). For $C_{43}H_{65}N_{11}O_{12}S_2.2\,H_2O$ (1 028) calculated: 50·23% C, 6·77% H, 14·98% N; found: 50·62% C, 6·57% H, 14·86% N. Amino acid analysis: Asp 0·99, Glu 1·06, Pro 0·96, Gly 0·95, Tle 0·96, Ile 1·06, Tyr 0·83, Cys 0·16.

S-Benzyl-β-mercaptopropionyl-tyrosyl-phenylalanyl-glutaminyl-asparaginyl-
-S-benzylcysteinyl-prolyl-tert-leucyl-glycine Amide (*IIIg*)

A mixture of a solution of the hexapeptide hydrazide *IIId* (ref.[24]; 0·5 g) in dimethylformamide (10 ml) and 6·6M-HCl in tetrahydrofuran (0·75 ml) was cooled to —30°C and n-butyl nitrite (100 µl) was added. After stirring for 4 min at this temperature, a solution of hydrochloride of the tripeptide *IV* (0·5 g) and N-ethylpiperidine (0·85 ml) in dimethylformamide (4 ml) was added. After 5 h, a further amount of the base (0·1 ml) was added and the mixture was set aside for 96 h at 0°C, taken down and triturated with 1M-HCl. The product was filtered and washed with 1M-HCl and water, affording 0·60 g (96%) of the product, m.p. 193—199°C. (Filtration of the washings through Dowex 50 afforded the unreacted free tripeptide *IV*.) Part of the product

(107 mg) was dissolved in dimethylformamide and purified by gel filtration through a column of Sephadex LH-20 (100 × 2·5 cm). The pertinent fractions were taken down to give 76 mg of compound, m.p. 197–202°C. $[\alpha]_D$ −35·2° (*c* 0·2; dimethylformamide), R_F 0·48 (S1), 0·39 (S2), 0·54 (S3), 0·67 (S4). For $C_{60}H_{77}N_{11}O_{12}S_2$ (1 208) calculated: 59·63% C, 6·42% H, 12·75% N; found: 59·75% C, 6·32% H, 12·67% N. Amino acid analysis: Asp 1·03, Glu 1·03, Pro 1·04, Gly 0·99, Tle 0·95, Tyr 0·90, Phe 0·98, Cys(Bzl) 0·30.

[8-Tert-leucine]deamino-vasopressin (*Ie*)

The protected nonapeptide *IIIg* (40 mg) was dissolved in liquid ammonia (20 ml) and reduced with a sodium rod until the blue coloration of the mixture persisted for 1 min. The solution was decolorized with acetic acid and freeze-dried. The residue was dissolved in 0·1M-HCl (6 ml) and the solution washed with ethyl acetate. After short evacuation, the solution was adjusted to pH 7·0 with 0·1M-NaOH, the total volume of the mixture being 80 ml. A solution of $K_3Fe(CN)_6$ (22 mg) in water (5 ml) was added dropwise, together with 0·1M-NaOH which was added so as to maintain the pH 7·0. After stirring for 1 h at room temperature, the mixture was adjusted to pH 4·0 with acetic acid and the solution was applied on a column of Amberlite CG-50-I (6 ml). After washing the column with 0·25% acetic acid, the peptide was eluted with 50% acetic acid. Freeze-drying afforded 14 mg of the compound which was further purified by gel filtration through Bio-Gel P-4 (110 × 1 cm) in 1M acetic acid. Freeze-drying of the pertinent fractions gave 9·2 mg of compound *Ie*; R_F 0·18 (S1), 0·15 (S2), 0·37 (S3), 0·62 (S4), pure according to HPLC: $k' = 2·3$ (methanol–water 55 : 45). $[\alpha]_D$ −38·0° (*c* 0·08, acetic acid). For $C_{46}H_{63}N_{11}O_{12}S_2$ (1 026) calculated: 53·84% C, 6·19% H, 15·01% N; found: 53·68% C, 6·23% H, 14·95% N. Amino acid analysis: Asp 1·04, Glu 1·04, Pro 1·00, Tle 0·96, Gly 0·96, Tyr 1·02, Phe 0·96, Cys 0·44.

Symmetric Anhydride of *o*-Nitrobenzenesulfenyl-tert-leucine

Dicyclohexylcarbodiimide (0·65 g) was added to a stirred solution of *o*-nitrobenzenesulfenyl-tert-leucine (1·8 g) in dichloromethane (72 ml) which had been cooled to −20°C. After 20 min the mixture was warmed to 0°C and kept at this temperature for 4 days. The separated dicyclohexylurea was filtered off and the solution taken down. Crystallization from benzene–light petroleum afforded 1·13 g (65%) of the product, m.p. 89–90°C; $[\alpha]_D$ −7·9° (*c* 0·5, dimethylformamide). For $C_{24}H_{30}N_4O_7S_2$ (550·6) calculated: 52·35% C, 5·49% H, 10·18% N; found: 52·32% C, 5·30% H, 9·77% N.

o-Nitrobenzenesulfenyl-tert-leucyl-isoleucyl-glutaminyl-asparaginyl-S-(γ-carboxypropyl)cysteinyl-prolyl-leucyl-glycine Amide (*VIb*)

The symmetric anhydride from the preceding preparation (0·38 g) was added to a solution of the heptapeptide *VIa* (0·5 g) in dimethylformamide (25 ml), the mixture was stirred for 1 h and set aside for 4 days at room temperature. Dimethylformamide was evaporated and the residue triturated with ether. The crystalline portion was collected on filter, washed with water, 0·5M-H_2SO_4 and again with water, affording 0·63 g (94%) of product, melting at 227–228°C; $[\alpha]_D$ −43·8° (*c* 0·5, dimethylformamide). Amino acid analysis: Asp 1·01, Glu 0·97, Pro 1·03, Gly 1·01, Ile 1·01, Leu 1·01, Tle 1·00, Cys(C_3H_6COOH) 0·73. For $C_{47}H_{74}N_{12}O_{14}S_2.H_2O$ (1 113) calculated: 50·72% C, 6·70% H, 15·29% N; found: 50·92% C, 6·88% H, 15·30% N.

o-Nitrobenzenesulfenyl-tert-leucyl-isoleucyl-glutaminyl-asparaginyl-
-S-(β-carboxyethyl)homocysteinyl-prolyl-leucyl-glycine Amide (*VId*)

A mixture of heptapeptide *VIc* (0·3 g), anhydride of *o*-nitrobenzenesulfenyl-tert-leucine (0·3 g) and dimethylformamide (5 ml) was stirred for 110 h at room temperature. The product was precipitated with ether, filtered and washed with ether and water; m.p. 215—225°C; yield 0·31 g (77%). R_F 0·48 (S1), 0·07 (S2), 0·41 (S3), 0·63 (S4); $E_{5.7}^{His}$ 0·25, $E_{2.4}^{Gly}$ 0·57. $[\alpha]_D$ −41·6° (*c* 0·22, dimethylformamide). For $C_{47}H_{74}N_{12}O_{14}S_2.2\,H_2O$ (1 131) calculated: 49·91% C, 6·95% H, 14·86% N; found: 50·07% C, 6·71% H, 14·67% N. Amino acid analysis: Asp 1·06, Glu 0·99, Pro 0·97, Gly 1·03, Ile 1·00, Leu 0·94, Tle 0·83, Hcy(C_2H_4COOH) 0·12.

[2-Tert-leucine]deamino-6-carba-oxytocin (*IIb*)

Bis(*p*-nitrophenyl) sulfite (0·7 g) was added at room temperature to a solution of the protected octapeptide *VId* (200 mg) in dimethylformamide (7 ml) and pyridine (7 ml) with stirring and introduction of nitrogen into the mixture. After 6 h another portion of the sulfite (0·7 g) was added and after 12 h again (0·35 g). After further 5 h pyridine was evaporated and the product was precipitated with ether, filtered and dried *in vacuo*. Its solution in dimethylformamide (6 ml) was mixed with 2·26M-HCl in ether (0·52 ml) and after standing for 10 min at room temperature the mixture was diluted with ether. The precipitate was isolated by centrifugation, washed with ether and dissolved in dimethylformamide (8 ml). This solution was added dropwise during 2 h at 60°C into a mixture of pyridine (250 ml) and N-ethylpiperidine (60 μl) with simultaneous bubbling of nitrogen. The mixture was kept at 60°C for 4 h more, concentrated to a small volume and the product was precipitated with ether, filtered and dried *in vacuo* (210 mg). A part (100 mg) was dissolved in 3M acetic acid and purified by gel filtration on a column of Bio-Gel P-4 (100 × × 1 cm). The obtained lyophilisate (78 mg), however, consisted still of at least 5 components (HPLC). A part (30 mg) was dissolved in aqueous methanol (1 : 1; 2 ml) and applied on a column of Partisil ODS (50 × 0·9 cm). Concentration and freeze-drying of the pertinent fractions afforded the product (8 mg) which was pure according to HPLC ($k' = 4{\cdot}04$; methanol–0·01M phosphate buffer pH 4·4, 55 : 45) as well as TLC: 0·22 (S1), 0·41 (S2), 0·37 (S3), 0·66 (S4). $[\alpha]_D$ −84·0° (*c* 0·25; 3M acetic acid). For $C_{41}H_{69}N_{11}O_{11}S.2\,H_2O$ (960·1) calculated: 51·29% C, 7·66% H, 16·04% N; found: 51·39% C, 7·34% H, 15·69% N. Amino acid analysis: Asp 0·96, Glu 1·03, Pro 1·04, Gly 0·95, Ile 1·00, Leu 1·02, Tle 0·98, Hcy(C_2H_4COOH) 0·13.

[2-Tert-leucine]deamino-1-carba-oxytocin (*IIa*)

Bis(2,4,5-trichlorophenyl)sulfite (1 g) was added to a stirred solution of the protected octapeptide *VIb* (321 mg) in a mixture of dimethylformamide (10 ml) and pyridine (10 ml) into which nitrogen was introduced. After 8 h another portion of the sulfite (1 g), together with pyridine (10 ml) was added once more. After 8 h, the mixture was taken down, the residue triturated with ether, filtered and washed with ether and water. The material was dried, dissolved in dimethylformamide (7 ml) and mixed with 3M-HCl in ether (0·2 ml). After 8 min the peptide hydrochloride was precipitated with ether, filtered and washed with ether. It was dissolved in dimethylformamide (10 ml) again and this solution was added dropwise at 60°C during 3 h to a mixture of pyridine (400 ml) and N-ethylpiperidine (82 μl) with stirring and bubbling of nitrogen into the mixture. After standing for 12 h at room temperature the solution was taken down and the residue triturated with ether, affording 232 mg of the crude product. A part (100 mg) was purified by countercurrent distribution; after 95 transfers of the upper phase the peak of $K = 2{\cdot}2$ was concentrated and freeze-dried, affording 12·7 mg of the product which, according to chromatography in S1

and S3, contained a small amount of another compound. Another part of the crude product was purified on a column of Partisil-ODS in the same manner as described for the compound *IIb*. The obtained product was pure according to thin-layer, as well as high performance liquid, chromatography: R_F 0·22 (S1), 0·41 (S2), 0·37 (S3), 0·66 (S4), $k' = 4{\cdot}14$ (methanol–0·01M phosphate buffer pH 4·4; 55 : 45). $[\alpha]_D$ −128·3° (*c* 0·02, 3M acetic acid). Amino acid analysis: Asp 1·02, Glu 0·98, Pro 0·95, Gly 1·04, Ile + aIle 1·02, Leu 1·02, Tle 1·02, Cys(C_3H_6COOH) 0·83(the hydrolysis was carried out in the absence of phenol; the values for Pro and Cys(C_3H_6COOH) were obtained from the sample, hydrolyzed for 20 h with 6M-HCl). For $C_{41}H_{69}N_{11}O_{11}S.3\ H_2O$ (978·1) calculated: 50·35% C, 7·74% H, 15·75% N; found: 50·21% C, 7·16% H, 15·89% N.

Cleavage of the Analogue *Ic* and Oxytocin with Chymotrypsin

The incubation mixture (100 µl) contained $5{\cdot}0 \cdot 10^{-1}$ mg/ml of the cleaved compound, 20 mM sodium-phosphate buffer pH 7·8, and $6{\cdot}0 \cdot 10^{-1}$ mg/ml of chymotrypsin. The samples were incubated at 37°C and the reaction was stopped by heating in a steam bath for 3 min. Incubation mixture of the same composition, boiled in the zero incubation time, was taken as standard. The amount of the non-cleaved compound was determined both by biological test (rat uterus *in vitro*[25]) and HPLC (in this case 10 µl of the incubation solution were injected without boiling). The results are listed in Table II.

Oxidation of Compounds *IIa* and *IIb*

The oxidation was carried out in aqueous methanol (1 : 1) at 0°C (concentration 2·5 mg/ml), with a ten-fold molar excess of periodate, ensuring thus monomolecular character of the reaction which was followed by HPLC.

TABLE II

Cleavage of compounds *Ia* and *Ic* with chymotrypsin (amount of non-cleaved compound in %)

Incubation time min	*Ia*		*Ic*	
	Bioassay[a]	HPLC	Bioassay[a]	HPLC
0	100	100	100	100
20	57	53	96	97
40	45	47	92	94
80	29	36	85	92

[a] Mean of 4 determinations.

Pharmacological Methods

The uterotonic assay[25] was carried out on uterine strips of adult Wistar strain rats. For the determination of the milk-ejecting activity[26,27], lactating female rats (5—14 days after delivery) of the same strain were used. Pressor activity[28] was determined on despinalized Wistar strain rats. Antidiuretic activity was estimated on anesthetized rats[29].

Spectroscopic Measurements

The spectra of circular dichroism were recorded on a Roussel–Jouan Dichrographe CD 185 model II in quartz cells with an optical pathlength of 0·02—1·00 cm. The solutions were prepared by weighing the freeze-dried substance.

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Translated by M. Tichý.