SYNTHESIS AND PROPERTIES OF CARBA-6-ANALOGUES OF OXYTOCIN CONTAINING A DEAMINOPENICILLAMINE RESIDUE IN POSITION 1*

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A study was made of the influence of the so-called carba substitution of the disulphide bridge on the properties of inhibitors obtained by the introduction of deaminopenicillamine into position 1 of the oxytocin molecule. Two analogues -- [dPen^1]carba-6-oxytocin (*Ia*) and [dPen^1, Tyr(Me)^2]carba-6-oxytocin (*Ib*) were prepared and their biological activities were assayed. Compound *Ia* is a strong agonist in the uterotonic assay *in vivo* (280 I.U./mg) and a weak antagonist in the pressor assay. Compound *Ib* inhibited the uterotonic activity of oxytocin *in vitro* ($pA_2 = 8.43$) and *in vivo* ($pA_2 = 7.13$) as well as the pressor action of lysine vasopressin ($pA_2 = 7.43$).

Considerable attention is being paid to studies of the inhibitors of neurohypophysial hormones (for review, see[1,2]). The introduction of alkyl groups at the β atom of cysteine** in position 1 of the peptide chain was found to be one of the most effective modifications for producing inhibitory analogues of oxytocin and vasopressin[3]. When an additional modification was performed in position 2, highly potent antagonists of the uterotonic action of oxytocin were obtained[4]. One of the important features of the analogues containing penicillamine in position 1 is their greater rigidity, which was established by measuring the relaxation time in ^{13}C NMR spectra (for review, see[5]). The rigidity may lead to slower metabolic degradation of the analogue. Moreover, it may hamper the establishment of the conformation necessary for evoking a biological response while binding to the receptor would remain unaltered or even increased due to the presence of lipophilic substituents in the analogue molecule.

The carba substitution of the disulphide bridge in most cases led to an increase in the activity of analogues[6,7], with some exceptions[8]. The increase in activity could be due to higher affinity to the receptor concerned, or to greater flexibility of the molecule, enabling it to assume the biologically active conformation more easily.

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** The abbreviations and nomenclature follow the recommendations published in Biochemical Nomenclature and Related Documents, International Union of Biochemistry 1978.

We decided to investigate the effect of the two above-mentioned simultaneous modifications in an oxytocin analogue. We prepared compounds *Ia* and *Ib*.

In order to synthetize a carba analogue modified in the region of the disulphide bridge, we required a suitable amino acid derivative that would enable the cyclization. We decided to prepare a deaminopenicillamine analogue because the presence or absence of the α-amino group did not have any great influence on the activity of the resultant analogues[9,10]. In a trial experiment, we performed the addition of cysteine to 2-methyl-2-butenoic acid and obtained dicarboxylic acid *II* the structure of which was confirmed by ^{1}H and ^{13}C NMR and mass spectrometry. The addition of homocysteine was more difficult, resulting in a mixture of products *IIIa* and *IV*, the structure of which was determined by ^{1}H NMR spectroscopy. When alkylation was performed at pH 8, the products formed were completely racemic and so the alkylation was carried out at pH 7 for a longer time. We were unable to produce the derivative *IIIc* necessary for the preparation of the α-peptide by esterification using a methanol solution of hydrogen chloride as in the case of S-(β-carboxyethyl)homocysteine[11]. This may be attributed to the very strong steric hindrance of the ω-carboxyl. It was necessary to perform total esterification resulting in compound *IIIb*, followed by the selective hydrolysis of the α-ester group, producing derivative *IIIc*. The amino group was protected by tert-butyloxycarbonyl group (*IIIf*) and tetrapeptide *IIIg* was then obtained by condensation with carboxy-terminal tripeptide. In an alternative procedure, the derivative *IIIe* with both carboxyl groups free was condensed by the carbodiimide method with the terminal tripeptide, and the preferentially (70%) formed tetrapeptide *IIIh* was purified by silica gel chromatography, or the free form *IIIi* by ion-exchange chromatography. The peptide chain was synthetized stepwise using active esters and *o*-nitrobenzenesulphenyl protecting groups. The removal of the protecting group has to be performed under conditions that do not favour the formation of the chloride which could split off the tert-butyl substituent of sulphur[12]. We used thiosemicarbazide hydrochloride and/or a solution of hydrogen chloride in ether containing a surplus of ethanethiol. We performed the hydrolysis of methyl ester at the stage of heptapeptide *VIIb*. The octapeptide, obtained by acylation with a derivative of tyrosine, was cyclized by the method of Krojidlo[13], *i.e.* by removing the protecting group, converting the octapeptide hydrochloride to an N-hydroxybenzotriazolyl ester and transferring it to an alkaline medium. By constrast with similar cyclizations of other carba analogs of oxytocin, we had to increase the reaction temperature in the present experiments. This was probably necessitated by the steric hindrance of the ω-carboxyl of the homocysteine derivative. The resultant analogue *Ia* was purified by reverse-phase liquid chromatography.

Analogue *Ib* was obtained in the same way, using N-tert-butyloxycarbonyl-O-methyltyrosine as active ester for acylation in the penultimate step.

The biological activities of the compounds prepared are given in Table I, together

TABLE I

Activities of some oxytocin analogues (I.U./mg)

Compound	Uterus *in vitro*	Uterus *in situ*	Antidiuretic activity	Pressor activity	Galactogogic activity
Oxytocin	450	450	3	3	450
[Deaminopenicillamine]oxytocin	pA_2 6·94[a–d] 7·14	—	—	pA_2 6·27[e]	
Deamino-6-carba-oxytocin	929[f]	2792[f]	117·9[f]	1·5[f]	456[g]
[2-O-Methyltyrosine]deamino-6-carba-oxytocin	3·1[h]	75[h]	—		18[h]
[Deaminopenicillamine]-6-carba-oxytocin (*Ia*)	16·2[i]	279·8[i]	4·81[i]	pA_2 6·82[i]	6·9[i]
[Deaminopenicillamine, 2-O-methyltyrosine]-oxytocin	pA_2 7·76[j]	pA_2 6·86[j]	0·02[k]	pA_2 7·59[j]	pA_2 6·94[k]
[Deaminopenicillamine, 2-O-methyltyrosine]-6-carba-oxytocin (*Ib*)	pA_2 8·43[i]	pA_2 7·13[i]	0·525[i]	pA_2 7·43[i]	inactive in doses reaching $2 . 10^{-2}$ mg

[a] Schulz and coworkers[3]; [b] Chan and coworkers[19]; [c] Manning and coworkers[18]; [d] Vavrek and coworkers[29]; [e] Nestor and coworkers[20]; [f] Barth and coworkers[6]; [g] Barth and coworkers[7]; [h] Barth and coworkers[21]; [i] this paper; [j] Lowbridge and coworkers[4]; [k] Sawyer and coworkers[30].

with the activities of some other compounds used for comparison. It is surprising that analogue *Ia* was an agonist in most tests; its activity was similar to that of oxytocin (uterus *in vivo*) or even higher (antidiuretic assay). A weak inhibitory effect was observed only in the pressor assay.

$$\begin{array}{lll} (CH_3)_2\text{—}C\text{————}S\text{—}CH_2\text{————} & CH_2 & \\ \quad | & | & \\ \quad CH_2\text{—CO-Tyr(R)-Ile-Gln-Asn-NH—} & CH & \text{—CO-Pro-Leu-Gly-}NH_2 \end{array}$$

Ia, R = H *Ib*, R = CH_3

$HCys(C(CH_3)_2CH_2COOH)OH$

II

R^1-Hcy($C(CH_3)_2CH_2COOR^2$)OR^3

IIIa, $R^1 = R^2 = R^3 = H$,
IIIb, $R^1 = H$, $R^2 = R^3 = CH_3$,
IIIc, $R^1 = H$, $R^2 = CH_3$, $R^3 = H$,
IIId, $R^1 = H$, $R^2 = H$, $R^3 = CH_3$,
IIIe, R^1 = Boc, $R^2 = R^2 = H$,
IIIf, R^1 = Boc, $R^2 = CH_3$, $R^3 = H$,
IIIg, R^1 = Boc, $R^2 = CH_3$, R^3 = Pro-Leu-Gly-NH_2,
IIIh, R^1 = Boc, $R^2 = H$, R^3 = Pro-Leu-Gly-NH_2,
IIIi, $R^1 = R^2 = H$, R^3 = Pro-Leu-Gly-NH_2

HHcy($CH(COOH)CH(CH_3)_2$)OH

IV

Nps-Asn-Hcy($C(CH_3)_2CH_2COOCH_3$)-Pro-Leu-Gly-NH_2

V

Nps-Gln-Asn-Hcy($C(CH_3)_2CH_2COOCH_3$)-Pro-Leu-Gly-NH_2

VI

R^1-Ile-Gln-Asn-Hcy($C(CH_3)_2CH_2COOR^2$)-Pro-Leu-Gly-NH_2

VIIa, R^1 = Nps, $R^2 = CH_3$, *VIIc*, R^1 = Boc-Tyr(Bu^t), $R^2 = H$,
VIIb, $R^1 = H$, $R^2 = H$, *VIId*, R^1 = Boc-Tyr(Me), $R^2 = H$

The substitution of the tyrosine hydroxyl usually increased the inhibitory properties of the resultant analogue. However, this modification alone did not produce an antagonist. The influence of this substitution has been studied in detail (for review, see[14]). The introduction of O-methyltyrosine into the carba-6 analogue containing deaminopenicillamine in position 1 resulted in a very potent inhibitor of uterotonic activity *in vitro* and *in vivo*. Its potency was similar to that of the most effective uterotonic inhibitor[15], namely [β,β-Et_2-Mpa[1], Tyr(Me)[2],Orn[8]] oxytocin with the pA_2 value equal to 7·35. The transformation of a strong agonist into an antagonist by alkylating the tyrosine residue has not been reported as yet; analogues containing an S—S group and a dialkyl group in position 1 had greatly reduced activities or were antagonists. A similar situation exists in the case of deamino-6-carba-oxytocin, where a change of configuration of the alkylated phenylalanine in position 2 resulted in very potent inhibitors[16].

The agonistic activity of *Ia* suggests that a more rigid molecular structure is really important for obtaining inhibitors. The S—S grouping is considerably more rigid than the CH_2—S group and the conformational constraints brought about by introducing alkyl groups to position 1 can be compensated more easily in the carba-analogue than in a compound containing a disulphide bridge.

Unexpectedly, the galactogogic activity of *Ia* was very low, whereas its uterotonic activity was high. The contrary is more common – almost any modification decreased the uterotonic activity more than the galactogogic effect (the analogues with the most specific galactogogic effect were produced in this way[17]). The fact that the compound *Ia* has a higher antidiuretic activity than oxytocin is not so surprising when we take into account that it is a carba-6-analogue and that this type of analogue is very potent antidiuretically.

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 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. Amino acid analyses were performed on a two-column apparatus type 6020 (Development Workshops, Czechoslovak Academy of Sciences). The NMR spectra were measured in C^2HCl_3 or ^2H_2O on a Varian XL-200 instrument in the FT mode, the ^1H NMR spectra at 200 MHz and the ^{13}C NMR spectra at 50·3 MHz. Chemical shifts are referenced to tetramethylsilane. The multiplicity of signals in the ^3C NMR spectra was determined by a ^1H-off-resonance decoupling experiment. Mass spectra were taken on an AEI-MS-902 spectrometer (70 eV, direct inlet). 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).

S-[2-(2-Methyl-1-carboxypropyl)]cysteine (*II*)

Cysteine hydrochloride (2 h) was dissolved in water (40 ml) and the pH value of the solution was adjusted to 8 by adding 1 mol . l^{-1} NaOH. A solution of 2-methyl-2-butenoic acid (3 g) in methanol (40 ml), with pH adjusted to 8 by the addition of 1 mol . l^{-} NaOH, was added to the first solution and the mixture was refluxed for 4 h. The mixture was stirred in the presence of air for 4 days and the precipitate was removed (electrophoresis revealed cystine). Acetone (400 ml) was added and the precipitate was collected, dissolved in a mixture of methanol and water (1 : 1) and applied to a column of Dowex 50 (70 ml). The column was washed with the mixture of methanol and water and the product was eluted with a 15% solution of pyridine. The yield was 1·8 g (64%) of product, m.p. 154—156°C. R_F 0·38 (S1), 0·02 (S2), 0·33 (S3), 0·29 (S4); $E_{5.7}^{\text{picr}}$ 0·65, $E_{2.4}^{\text{Gly}}$ 0·46. For $C_8H_{15}NO_4S$ (221·3) calculated: 43·42% C, 6·83% H 6·33% N; found: 43·18% C, 6·90% H, 6·14% N. $[\alpha]_D$ −11·9° (*c* 0·13; dimethylformamide); −8·8° (*c* 0·1; 1 mol l^{-1} HCl). ^{1}H NMR spectrum (^2H_2O): 1·44 s, 6 H, (CH_3), 2·62 s, 2 H (CH_2—COOH), 3·13 m, 2 H (CH_2—S), 3·94 m, 1 H (CH—COOH). ^{13}C NMR spectrum (^2H_2O), reference hexadeuterio-acetone, $\delta_{(\text{methyl})}$ = 29·8): 28·48 q and 28·55 q (CH_3), 28·99 t (CH_2—CH), 44·28 s (quaternary C), 46·99 t (CH_2—CO), 54·41 d (CH—CH_2), 172·3 s (CO—CH), 175·2 s (CO—CH_2).

The acetone solution left after the precipitation of the product was evaporated, the residue was dissolved in a mixture of 1 mol l^{-1} HCl and ethyl acetate, the aqueous layer was washed with ethyl acetate and after evaporation yielded 400 mg of the hydrochloride of compound *II* 12%), m.p. ~317°C.

S-[2-(2-Methyl-1-carboxypropyl)]homocysteine (*IIIa*)

Homocystine (10 g) was dissolved in liquid ammonia (~500 ml) and reduced with sodium until the blue colour was stable for 8 min. The solution was decolourized by adding acetic acid and lyophilized. The residue was dissolved in water (160 ml), bubbled with helium, a solution of 2-methyl-2-butenoic acid (20 g) in methanol (160 ml, bubbled with helium) was added and the pH of the mixture was adjusted to 7 by adding 1 mol l^{-1} NaOH. The mixture was refluxed under nitrogen atmosphere for 20 h and applied to a column of Dowex 50 (300 ml). The column was washed with a mixture of methanol and water (1 : 1) and the product was eluted with 15% pyridine. The eluate was evaporated, dissolved in 1 mol l^{-1} acetic acid (150 ml) and applied in 50 ml portions to a column of Dowex 1 (100 × 4 cm). After elution with 1 mol l^{-1} acetic acid and evaporation, fractions were obtained containing a neutral compound (0·82 g; homocystine), a compound with $E_{5.7}^{\text{picr}}$ 0·32 (4·1 g, 21%, compound *IIIa*) and a compound with $E_{5.7}^{\text{picr}}$ 0·55 (1·1 g, 5·6%, compound *IV*). Compound *IIIa*: m.p. 217—220°C (1 mol l^{-1} acetic acid), $[\alpha]_D$ +4·1° *c* 0·3; water), +20·8° (*c* 0·3; 1 mol l^{-1} HCl): R_F 0·40 (S1), 0·02 (S2), 0·36 (S3), 0·36 (S4); $E_{2.4}^{\text{Gly}}$ 0·48. For $C_9H_{17}NO_4S$ (235·3) calculated: 45·94% C, 7·28% H, 5·95% N; found: 45·93% C, 7·27% H, 5·94% N. ^{1}H NMR spectrum ($^2H_3CSOC^2H_3$—^2H_2O): 1·33 s, 6 H (CH_3), 2·48 s, 2 H (CH_2—COOH), 3·49 t, J = 6 Hz, 1 H (CH—COOH), 1·86 m, 4 H (CH_2—CH_2). Mass spectrum: M^+ 235.

Compound *IV*: m.p. 210°C. $[\alpha]_D$ −4·4° (*c* 0·18; dimethylformamide); +11.4° (*c* 0·12; 1 mol . . l^{-1} HCl). R_F 0·42 (S1), 0·02 (S2), 0·38 (S3), 0·32 (S4); $E_{2.4}^{\text{Gly}}$ 0·48. For $C_9H_{17}NO_4S$ (235·3) found: 45·44% C, 7·13% H, 5·80% N. ^{1}H NMR spectrum ($^2H_3CSOC^2H_3$): 0·89 and 0·96 d and d, J = 6·5 and 6·5 Hz, 6 H (CH_3), 2·48 s, 2 H (CH_2—COOH), 3·40 t, J = 5 Hz, 1 H (NH_2—CH—COOH), 2·93 d, J = 8·6 Hz, 1 H (S—CH—COOH), 1·90 bm, 3 H (CH—CH_2——CH_2 and CH_3—CH), 2·44 t, J = 4 Hz, 2 H (S—CH_2). Mass spectrum: M^+ 235. Neither of the compounds was affected by the action of a mixture of HBr and acetone, whereas both were oxidized in the presence of sodium periodate.

Cleavage of Compound *IIIa*

Compound *IIIa* (10 mg) was dissolved in methanol and incubated with the following reagents: *a*) *o*-nitrobenzenesulphenyl chloride, *b*) a mixture of *o*-nitrobenzenesulphenyl chloride and thiosemicarbazide hydrochloride, *c*) thiosemicarbazide hydrochloride, *d*) ethanethiol, *e*) a mixture of *o*-nitrobenzenesulphenyl chloride and ethanethiol. After 5 and 15 min, electrophoresis of the reaction mixtures was carried out. A neutral compound was formed only in case *a*).

S-[2-(2-Methyl-1-methoxycarbonylpropyl)]homocysteine (*IIIc*)

Compound *IIIa* (4·2 g) was dissolved in methanol (300 ml) and the solution was saturated with gaseous hydrogen chloride at 0°C. The mixture was left at room temperature overnight. Electrophoresis proved the formation of diester *IIIb*. The mixture was repeatedly evaporated with methanol, dissolved in methanol (50 ml) and water (20 ml), cooled to 3°C and the pH value was adjusted to 11·7 by 1 mol l^{-1} NaOH. After 10 min, HCl was added until pH 5 was reached in the mixture and methanol was then evaporated in vacuum. The residue was applied to a column of Dowex 50 (120 ml) which was then washed with water and the product was eluted with 15% pyridine. The fractions obtained were evaporated in vacuum and dissolved in 1 mol l^{-1} acetic acid. The resultant solution was applied to a column of Dowex 1 (100 × 4 cm) and 1 mol l^{-1} acetic acid was used for elution. The eluted fractions were freeze-dried and the residue was re-precipitated from methanol and ether. The yield was 2·77 g (62%), m.p. 197—198°C, $[\alpha]_D$ +34·8° (*c* 0·17; dimethylformamide); +19·8° (*c* 0·1; 1 mol l^{-1} HCl). R_F 0·40 (S1), 0·16 (S2), 0·36 (S3), 0·48 (S4); $E_{5.7}^{His}$ 0·00, $E_{2.4}^{Gly}$ 0·43. For $C_{10}H_{19}NO_4S$ (249·3) calculated: 48·17% C, 7·68% H, 5·62% N; found: 47·70% C, 7·61% H, 5·56% N.

Tert-butyloxycarbonyl-S-[2-(2-methyl-1-carboxypropyl)]homocysteine (*IIIe*)

Compound *IIIa* (2·72 g) was dissolved in a mixture of dioxane (20 ml), water (10 ml) and 1 mol . . l^{-1} NaOH (10 ml). Di-tert-butyl dicarbonate (2·8 g) was then added and pH 8 was maintained. After stirring for 40 min at room temperature, dioxane was evaporated in vacuum, the mixture was washed with ethyl acetate, the aqueous layer was acidified to pH 2 with a solution of $KHSO_4$ and the product was extracted by ethyl acetate. The organic layer was washed with a solution of sodium sulphate, dried with sodium sulphate and evaporated. The oil obtained (2·9 g) was dissolved in ethyl acetate (55 ml), dicyclohexylamine (3·39 ml) and light petroleum (300 ml) were added, the crystals obtained were collected by filtration and dried in vacuum. The yield was 3·42 g (42%) of product, m.p. 155—157°C (reaction at 145°C); $[\alpha]_D$ +3·9° (*c* 0·38; dimethylformamide). For $C_{38}H_{71}N_3O_6$ (698·1) calculated: 65·38% C, 10·25% H, 6·02% N; found: 65·02% C, 10·45% H, 5·87% N. The dicyclohexylammonium salt was suspended in ethyl acetate, washed with 0·2 mol l^{-1} H_2SO_4 and with water, dried and evaporated, producing 1·46 g (37%) of substance, m.p. 140—143°C. After crystallization from ethanol and water (1 : 2), the product obtained had a m.p. of 144—145°C. R_F 0·41 (S1), 0·18 (S2), 0·90 (S3), 0·69 (S4). For $C_{14}H_{25}$. .NO_6S (335·4) calculated: 50·14% C, 7·51% H, 4·18% N; found: 50·36% C, 7·44% H, 3·93% N.

Tert-butyloxycarbonyl-S-[2-(2-methyl-1-methoxycarbonylpropyl)]homocysteine (*IIIf*)

Compound *IIIc* (1·45 g) was dissolved in a mixture of dioxane (12 ml) and water (6 ml) and 1 mol l^{-1} NaOH was added to obtain pH 8. After adding di-tert-butyl dicarbonate (2 g) in dioxane (4 ml), the mixture was stirred for 1 h while maintaining pH 8. Dioxane was removed by evaporation in vacuum, the mixture was washed with ethyl acetate, acidified with citric acid and the product was extracted by ethyl acetate. The extract was washed with water, dried with sodium sulphate and evaporated. The yield was 2 g (100%) of oil. R_F 0·81 (S1), 0·33 (S2), 0·72 (S3), 0·66 (S4). A part of the product was transformed into the dicyclohexylammonium salt and then recrystallized from ethanol: m.p. 147—151°C, $[\alpha]_D$ −14·3° (*c* 0·11; dimethylformamide). For $C_{27}H_{50}N_2O_6S$ (530·8) calculated: 61·10% C, 9·49% H, 5·28% N; found: 61·42% C, 9·61% H, 5·04% N.

Tert-Butyloxycarbonyl-S-[2-(2-methyl-1-methoxycarbonylpropyl)]homocysteinyl-prolyl-leucyl-glycine Amide (*IIIg*)

Compound *IIIf* (1·95 g), prolyl-leucyl-glycine amide (2·1 g) and N-hydroxybenzotriazole (0·78 g) were dissolved in dichloromethane (30 ml), cooled to −10°C, dicyclohexylcarbodiimide (1·32 g) was then added, the mixture was stirred for 1 h at −10°C and overnight at room temperature. The mixture was passed through a filter, the solvent evaporated, the residue dissolved in ethyl acetate and the solution was washed with a solution of $KHSO_4$, water, dried with sodium sulphate and concentrated to a small volume. The product was precipitated with light petroleum, filtered off, washed with light petroleum and dried in vacuum. The yield was 3·11 g (91%) of product with m.p. 84—85°C, $[\alpha]_D$ −34° (*c* 0·2; dimethylformamide). R_F 0·66 (S1), 0·58 (S2), 0·63 (S3), 0·78 (S4); $E^{His}_{5.7}$ 0·61, $E^{Gly}_{2.4}$ 0·93. For $C_{28}H_{49}N_5O_8S$ (615·8) calculated: 54·61% C, 8·02% H, 11·37% N; found: 55·01% C, 8·17% H, 11·32% N. k' = 3·50 (methanol–water 3 : 1). Amino acid analysis: Pro 0·97, Gly 1·04, Leu 1·01, Hcy($C_5H_9O_2$) 0·87, Hcy 0·12.

S-[2-(2-Methyl-1-carboxypropyl)]homocysteinyl-prolyl-leucyl-glycine Amide (*IIIi*)

a) Compound *IIIe* (250 mg), prolyl-leucyl-glycine amide (220 mg) and N-hydroxybenzotriazole (102 mg) were dissolved in dimethylformamide, the solution was cooled to −20°C, dicyclohexylcarbodiimide (155 mg) was then added, the mixture was stirred for 2 h at −20°C and overnight at room temperature. The solution was filtered and evaporated, the residue was dried in vacuum and a portion (100 mg) was subjected to chromatography on a column (25 × × 0·8 cm) of Separon SI-VSK in a mixture of chloroform and methanol (19 : 1). Thus, 54 mg of compound *IIIh* was obtained; after reaction with diazomethane, its mobility during chromatography and electrophoresis was the same as that of compound *IIIg*. The remainder of the product (230 mg) was dissolved in trifluoroacetic acid (3 ml) and after 10 min at room temperature, the mixture was repeatedly evaporated with toluene, the residue was dissolved in 0·2 mol l^{-1} pyridine and applied on a column of Dowex 50 (100 × 1·5 cm) in pyridine cycle. The column was eluted with 0·2 mol l^{-1} pyridine, the fractions obtained were evaporated and 108 mg (35%) of product, m.p. 129—132°C were obtained. $[\alpha]_D$ −47·3° (*c* 0·85; dimethylformamide), R_F 0·44 (S1), 0·09 (S2), 0·38 (S3), 0·56 (S4). k' = 1·46 (methanol–0·1% trifluoroacetic acid 63 : 37); $E^{His}_{5.7}$ 0·13, $E^{Gly}_{2.4}$ 0·81. For $C_{22}H_{39}N_5O_6S.H_2O$ (519·7) calculated: 50·85% C, 7·95% H, 13·48% N; found: 51·32% C, 7·71% H, 13·09% N.

b) Compound *IIIg* (30 mg) was dissolved in trifluoroacetic acid (0·2 ml) and, after 10 min, the solution was evaporated. The residue was dissolved in water (0·6 ml) and 1 mol l^{-1} NaOH was added until pH 12 was achieved in the mixture. After 1 h at room temperature, the solution was acidified with 1 mol l^{-1} HCl and applied on a column of Dowex 50 (5 ml). After washing with water and elution with 15% pyridine and evaporation, the yield was 22 mg of substance *IIIi* which had the same properties as the product obtained by procedure *a*).

o-Nitrobenzenesulphenylasparaginyl-S-(2-(2-methyl-1-methoxycarbonylpropyl))-homocysteinyl-prolyl-leucyl-glycine Amide (*V*)

Compound *IIIg* (3·1 g) was dissolved in trifluoroacetic acid (30 ml), left for 30 min at room temperature, the solution was then repeatedly evaporated with toluene, the residue was triturated with ether, filtered and dried in vacuum. The product was dissolved in dimethylformamide (40 ml) and, after adjusting the pH value to 8 by adding N-ethylpiperidine (moist pH paper), *o*-nitrobenzenesulphenylasparagine 2,4,5-trichlorophenyl ester (3·15 g) was added to the solution. The mixture was stirred for 120 h and then evaporated, the residue was triturated with ether, the product filtered, washed with ether and water, and dried in vacuum. After reprecipitation from dimethylformamide and water, the yield was 2·5 g (63%) of product, m.p. 183—186°C. $[\alpha]_D$ −55·8° (*c* 0·2; dimethylformamide). R_F 0·40 (S1), 0·36 (S2), 0·34 (S4); $E_{5.7}^{His}$ 0·37, $E_{2.4}^{Gly}$ 0·78, k' = 3·23 (methanol–water 7 : 3). For $C_{33}H_{50}N_8O_{10}S_2.H_2O$ (800·9) calculated: 49·49% C, 6·54% H, 13·99%; found: 49·66% C, 6·19% H, 14·24% N. Amino acid analysis: Asp 1·02, Pro 1·02, Gly 0·98, Leu 1·00, Hcy 0·86.

o-Nitrobenzenesulphenylglutaminyl-asparaginyl-S-(2-(2-methyl-1-methoxycarbonylpropyl))-homocysteinyl-prolyl-leucyl-glycine Amide (*VI*)

a) Compound *V* (330 mg) was dissolved in methanol (14 ml). After adding 2·05 mol l^{-1} HCl in ether (0·6 ml) the mixture was concentrated in vacuum and the product precipitated with ether, dried and dissolved in dimethylformamide (4 ml). The pH value was adjusted to 8 by adding N-ethylpiperidine (moist pH paper) and *o*-nitrobenzenesulphenylglutamine 2,4,5-trichlorophenyl ester (0·35 g) was added. The solution was stirred for 60 h at room temperature while maintaining the above-mentioned pH. The product was then precipitated with ether and reprecipitated from dimethylformamide and water. The precipitate was dissolved in methanol and water 1 : 1 (12 ml), applied in three portions to a column of Partisil ODS (50 × 0·9 cm) and eluted with a mixture of methanol and water (58 : 42), k' = 9·1. The resultant fractions were evaporated and the yield was 170 mg of product, m.p. 147—150°C, $[\alpha]_D$ −33·9° (*c* 0·18; dimethylformamide). R_F 0·32 (S1), 0·14 (S2), 0·33 (S3), 0·67 (S4). $E_{5.7}^{His}$ 0·38, $E_{2.4}^{Gly}$ 0·77 (after removing the Nps group), hydrolysis of a sample with 1 mol l^{-1} NaOH resulted in a compound with $E_{5.7}^{His}$ 0·15. For $C_{38}H_{58}N_{10}$.$O_{12}S_2.H_2O$ (929·1) calculated: 49·13% C, 6·51% H, 15·08% N; found: 49·38% C, 6·84% H, 14·86% N. ^1H NMR spectrum ($C^2H_3SOC^2H_3$): 1·32 s, 6 H $(CH_3)_2C$=, 3·56 s, $-COOCH_3$. Amino acid analysis: Asp 0·95, Glu 0·95, Pro 1·03, Gly 1·00, Leu 1·07, Hcy 0·87, Hcy($C_5H_9O_2$) 0·02.

b) Compound *V* (1·96 g) was dissolved in a mixture of dimethylformamide (10 ml), ethanethiol (1 ml) and 3·4 mol l^{-1} HCl in ether (1·6 ml). After 5 min at room temperature, the product was precipitated with ether, filtered, washed with ether and dried in vacuum. Next, the procedure used in case *a*) was followed. The product obtained by precipitation with ether was reprecipitated from dimethylformamide using a mixture of moist ethyl acetate and ether (1 : 1). The yield was 2·1 g of product, m.p. 140—145°C which was identical with the product of procedure *a*).

o-Nitrobenzenesulphenylisoleucyl-glutaminyl-asparaginyl--S-(2-(2-methyl-1-methoxycarbonylpropyl))homocysteinyl-prolyl-leucyl-glycine Amide (*VIIa*)

a) Compound *VI* (0·91 g) was dissolved in a mixture of dimethylformamide (20 ml), ethanethiol (2 ml) and 3·4 mol l^{-1} HCl in ether (0·7 ml), and left for 5 min at room temperature. The product was precipitated with ether, filtered and dried in vacuum. Then, it was dissolved in dimethylformamide (6 ml), the pH value of the solution was adjusted to 8 by adding N-ethylpiperidine, *o*-nitrobenzenesulphenylisoleucine N-hydroxysuccinimide ester (0·9 g) was added and the mixture was stirred for 120 h at room temperature. The product was precipitated with ether, filtered and washed with ether and water. The yield was 0·62 g (61%) of compound *VIIa*, m.p. 192—195°C. The sample for analysis was reprecipitated from dimethylformamide and ether; the product then had a m.p. of 195—196°C. $[\alpha]_D$ −57·1° (*c* 0·19; dimethylformamide). R_F 0·41

(S1), 0·21 (S2), 0·38 (S3), 0·77 (S4); $E^{His}_{5.7}$ 0·35, $E^{Gly}_{2.4}$ 0·61. For $C_{44}H_{69}N_{22}O_{13}S_2.H_2O$ (1 042) calculated: 50·71% C, 6·87% H, 14·78% N; found: 50·34% C, 6·55% H, 14·91% N. Amino acid analysis: Asp 1·01, Glu 0·94, Pro 1·03, Gly 1·04, Ile 0·97, Leu 1·05, Hcy 0·91, Hcy($C_5H_9O_2$) 0·03.

b) Compound *VI* (150 mg) was dissolved in methanol (7 ml), thiosemicarbazide hydrochloride was added and the mixture was left for 15 min at room temperature. The product was precipitated with ether, dried, dissolved in water and the solution was applied to a column (25 × 0·4 cm) of Separon SI-C-18. The column was washed with water and the product was eluted with a mixture of methanol and water (1 : 1). The fractions obtained were evaporated and the reaction with the active ester was carried out as in procedure *a*). The yield was 73 mg (43%) of product identical with that obtained by procedure *a*).

Isoleucyl-glutaminyl-asparaginyl-S-(2-(2-methyl-1-carboxypropyl))homocysteinyl-prolyl-leucyl--glycine Amide (*VIIb*)

Compound *VIIa* (0·57 g) was dissolved in a mixture of dimethylformamide (6 ml), ethanethiol (1 ml) and 2·25 mol l^{-1} HCl in ether (0·6 ml) and the solution was left for 5 min at room temperature. The product was precipitated with ether, filtered and dried in vacuum. The substance was dissolved in a mixture of methanol (4 ml) and water (12 ml), 1 mol l^{-1} NaOH was added to attain pH 12·5 and the mixture was stirred for 1 h. The mixture was acidified with acetic acid to pH 6 and applied to a column of Dowex 50 (25 ml). The column was washed with water and the product was eluted with 20% pyridine. The fractions obtained were freeze-dried and the yield was 350 mg (73%) of product. The sample for analysis was reprecipitated from methanol and ether; m.p. 135°C. $[\alpha]_D$ −63·3° (*c* 0·1; dimethylformamide). R_F 0·09 (S1), 0·01 (S2), 0·05 (S3), 0·63 (S4); $E^{His}_{5.7}$ 0·13, $E^{Gly}_{2.4}$ 0·73, $k' = 8·54$ (methanol–0·05% trifluoroacetic acid 36 : 64). For $C_{37}H_{64}N_{10}O_{11}.2\,H_2O$ (891·3) calculated: 49·73% C, 7·67% H, 15·68% N; found: 49·92% C, 7·63% H, 15·60% N. Amino acid analysis: Asp 1·01, Glu 0·94, Pro 1·04, Gly 0·98, Ile 1·00, Leu 1·02, Hcy($C_5H_9O_2$) 0·26, Hcy 0·82.

Tert-butyloxycarbonyl-O-tert-butyl-tyrosyl-isoleucyl-glutaminyl-asparaginyl--S-(2-(2-methyl-1-carboxypropyl))homocysteinyl-prolyl-leucyl-glycine Amide (*VIIc*)

Free heptapeptide *VIIb* (200 mg) was dissolved in dimethylformamide (3 ml), N-tert-butyloxycarbonyl-O-tert-butyltyrosine N-hydroxysuccinimide ester (0·7 g) was added and N-ethylpiperidine was used for obtaining pH 8. The mixture was stirred for 44 h at room temperature, the product was precipitated with ether, filtered, washed with ether and dried in vacuum. The yield was 236 mg (86%) of product, m.p. 205—207°C. The sample for analysis was reprecipitated from dimethylformamide and ether, m.p. 209—210°C, $[\alpha]_D$ −26·2° (*c* 0·18; dimethylformamide). R_F 0·39 (S1), 0·12 (S2), 0·56 (S3), 0·73 (S4); $E^{His}_{5.7}$ 0·11, $E^{Gly}_{2.4}$ 0·64 (after removing the Boc group). For $C_{35}H_{89}N_{11}O_{15}S.H_2O$ (1 176) calculated: 55·31% C, 7·68% H, 12·90% N; found: 55·62% C, 7·52% H, 12·58% N. Amino acid analysis: Asp 1·05, Glu 0·97, Pro 1·03, Gly 1·01, Ile 0·94, Leu 1·03, Tyr 0·99, Hcy($C_5H_9O_2$) 0·82.

[1-Penicillamine]deamino-6-carba-oxytocin (*Ia*)

Octapeptide *VIIc* (100 mg) was dissolved in trifluoroacetic acid (3 ml), the solution was left for 1 h at room temperature, evaporated, the residue was dissolved in methanol, 2 mol l^{-1} HCl in ether (0·1 ml) was then added, the mixture was evaporated and dried in vacuum. The residue was dissolved in dimethylformamide (2 ml), N-hydroxybenzotriazole was added (120 mg), the mixture was cooled to 0°C and dicyclohexylcarbodiimide (132 mg) was added. The mixture was stirred for 30 min at 0°C and for 2 h at room temperature, passed through a filter into a solution of N-ethylpiperidine (300 μl) in methanol (100 ml) heated to 50°C. The mixture was heated for 2 h and after concentration in vacuum, the product was precipitated with ether and dried. The product obtained (78 mg) was dissolved in a mixture of methanol (3 ml) and water (7 ml) and applied to a column of Separon SI-C-18 (25 × 1·2 cm). Elution was carried out with a gradient of methanol in 0·05% trifluoroacetic acid (30–70% in the course of 80 min at a flow rate of 7 ml/min; the product was eluted after 40 min). The fractions obtained were concentrated in vacuum and freeze-dried. The yield was 13·1 mg (16%) of product, R_F 0·20 (S1), 0·18 (S2), 0·23 (S3), 0·66 (S4); k' = 3·29 (methanol–0·05% trifluoroacetic acid 1 : 1). Oxidation with periodate resulted in single sulphoxide with k' = 2·27. $[\alpha]_{300}$ −448° ± 12° (*c* 0·4; 0·01 mol l^{-1} phosphate pH 7·5). For $C_{46}H_{71}N_{11}O_{12}S.2\,H_2O$ (1 038) calculated: 53·22% C, 7·28% H, 14·83% N; found: 53·54% C, 6·91% H, 14·56% N. Amino acid analysis: Asp 0·98, Glu 1·02, Pro 1·04, Gly 1·00, Ile 1·00, Leu 1·04, Tyr 0·98, Hcy($C_5H_9O_2$) 0·56.

[1-Penicillamine, 2-O-methyltyrosine]deamino-6-carba-oxytocin (*Ib*)

To a solution of free heptapeptide *VIIb* (113 mg) in dimethylformamide (1·5 ml), N-tert-butyloxycarbonyl-O-methyltyrosine 2,4,5-trichlorophenyl ester (0·5 g) was added and N-ethylpiperidine was added until pH 8 was reached. The mixture was stirred for 100 h at room temperature, then diluted with ether, the precipitated product was collected by filtration, dried, dissolved in dimethylformamide and precipitated with water. The mixture was kept for 60 h at 4°C, the product was then collected by filtration and dried in vacuum. The yield was 76 mg (51%) of compound *VIId*, m.p. 216–217°C. R_F 0·49 (S1), 0·40 (S2), 0·51 (S3), 0·77 (S4). $E_{5.7}^{His}$ 0·10, $E_{2.4}^{Gly}$ 0·58 (after removing Boc group).

The octapeptide obtained (70 mg) was cyclized by the same procedure as in the case of compound *Ia*. The crude product (57 mg) was purified on the same column; elution was carried out with 48% methanol in 0·05% aqueous trifluoroacetic acid. The fractions required were freeze-dried; the yield was 6·8 mg (12%) of compound *Ib* with k' = 8·44 (methanol–0·05%aqueous trifluoroacetic acid (1 : 1). Oxidation with periodate resulted in a mixture of sulphoxides with k' = 4·13 and 4·70. R_F 0·22 (S1), 0·18 (S2), 0·23 (S3), 0·66 (S4). $[\alpha]_{300}$ −225° ± 25° (*c* 0·02; 0·01 mol l^{-1} phosphate, pH 7·5). For $C_{47}H_{73}N_{11}O_{12}.2\,H_2O$ (1 052) calculated: 53·65% C, 7·38% H, 14·64% N; found: 53·47% C, 7·61% H, 14·31% N. Amino acid analysis: Asp 0·94, Glu 0·92, Pro 1·06, Gly 1·04, Ile 1·03, Leu 1·04, Tyr 0·62, Tyr(Me) 0·35, Hcy($C_5H_9O_2$) 0·58.

Biological Assays

The uterotonic assay *in vitro* was carried out on isolated rat uterine strips[22,23]. The uterotonic activity *in vivo* was determined in experiments on oestrogenised rats in ethanol anaesthesia[24]. The galactogogic activity was determined on ethanol-anaesthetised rats (4–15 days after delivery[7,25]). The pressor activity was assayed on pithed male rats[26] and the antidiuretic activity on ethanol-anaesthetised male rats[27,28].

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