VASOPRESSIN ANALOGUES WITH EFFECT ON CENTRAL NERVOUS SYSTEM: SYNTHESIS AND BIOLOGICAL PROPERTIES*

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Synthesis of four vasopressin analogues which do not contain the glycinamide residue in position 9 and have a basic non-coded amino acid in position 8 is described. All the analogues exhibit very low endocrine activities and are effective in the passive avoidance test.

Various biologically active peptides influence the central nervous system. This activity has been found also for neurohypophysial hormones and some of their analogues[1,2]. The effect of vasopressin and its analogues on learning and memory has been utilized also in the clinical praxis[3-6]. Since these compounds pass through the blood-brain barrier only with difficulty[7], they must be applied in doses substantially higher than usual in utilization of their endocrine activities. Therefore, it is desirable that such analogues have the two following properties: their endocrine activities should be as low as possible and their half-life as long as possible, *i.e.* they should be suitably modified to prevent their metabolic inactivation. In this communication we describe vasopressin analogues which, under retention of effects on central nervous system, exhibit endocrine activities by four orders of magnitude lower than those of the parent hormone. A part of these results has been already published[8].

A common feature of all the four synthesized analogues is the absence of the glycinamide moiety in position 9 and replacement of the arginine** residue in position 8 by a basic non-coded amino acid. The substitution of cysteine for β-mercaptopropionic acid in position 1 and introduction of the carba-bridge instead of the disulfide bond represent modifications, generally enhancing the enzymatic resistance of vasopressin analogues. Our synthesis employed the fragment condensation (using the

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** The chiral amino acids in this work are of the L-configuration, except arginine which has the D-configuration. The nomenclature and symbols for the amino acids, peptides and protecting groups obey the published recommendations[9]. Mpr denotes a β-mercaptopropionic acid moiety.

azide or carbodiimide method) of the amino-terminal hexapeptide (or pentapeptide) with the carboxy-terminal dipeptide ester. In the synthesis of the analogues *Ia* and *Ib* the cysteine sulfhydryl group was protected with 2,4,6-trimethylbenzyl group[10], removable under mild conditions by treatment with trifluoromethanesulfonic acid. The protected hexapeptide hydrazide *IIb* was obtained by hydrazinolysis of the corresponding ester *IIa*, prepared by azide condensation of the tripeptides. The tripeptide *IIIa* was synthesized from the protected amino-terminal dipeptide and methyl phenylalaninate, either in the presence of N,N'-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole or using the azide method; it was then converted into the hydrazide *IIIb*. The tripeptide *IV* was prepared by stepwise synthesis using 2,4,5-trichlorophenyl esters or 1-(β-naphthalenesulfonyloxy)benzotriazole[11] in the last step and after removal of the benzyloxycarbonyl group it was acylated with the tripeptide azide prepared from compound *IIIb*. The thus-obtained hexapeptide was subjected to azide condensation with the dipeptide esters arising by removal of *o*-nitrobenzenesulfenyl group from compounds *V* and *VI*. After gel filtration on Sephadex LH-20 the protect-

X-Tyr-Phe-Gln-Asn-Cys-Pro-Y

Ia, X = Cys, Y = D-Arg
Ib, X = Cys, Y = Orn
Ic, X = $S(CH_2)_2CO$, X = D-Arg
Id, X = $(CH_2)_3CO$, Y = D-Arg

Z-Cys(Bzl(2,4,6-Me_3))-Tyr-Phe-Gln-Asn-Cys(Bzl(2,4,6-Me_3))-X

IIa, X = OMe *IIb*, X = N_2H_3

Z-Cys(Bzl(2,4,6-Me_3))-Tyr-Phe-X

IIIa, X = OMe *IIIb*, X = N_2H_3

Z-Gln-Asn-Cys(Bzl(2,4,6-Me_3))OMe

IV

Nps-Pro-D-Arg(Tos)OBzl Nps-Pro-Orn(Z)OBzl

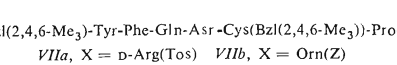
V *VI*

Z-Cys(Bzl(2,4,6-Me_3)-Tyr-Phe-Gln-Asn-Cys(Bzl(2,4,6-Me_3))-Pro-X-OBzl

VIIa, X = D-Arg(Tos) *VIIb*, X = Orn(Z)

Mpr(Bzl)-Tyr-Phe-Gln-Asn-Cys(Bzl)-Pro-D-Arg(Tos)OBzl

VIII



IX

ed octapeptides *VIIa* and *VIIb* were deblocked with trifluoromethanesulfonic acid in the presence of trifluoroacetic acid and thioanisole. The disulfhydryl compounds were oxidized with air oxygen and the analogues *Ia* and *Ib* were purified by gel filtration on Sephadex G-15 (ref.[12]).

Also the analogue *Ic* was prepared by fragment condensation. S-Benzyl-β-mercaptopropionyl-tyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-benzylcysteine hydrazide[13] was converted into the azide and this was used for acylation of the dipeptide *V* from which the amino-protecting group had been removed. The obtained peptide *VIII* was purified by gel filtration, reduced with sodium in liquid ammonia and oxidized with potassium ferricyanide to give, after gel filtration, the analogue *Ic*.

Lactam of tyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-(γ-carboxypropyl)cysteine[14] was condensed with the dipeptide *V* (after removal of the *o*-nitrobenzenesulfenyl group) in the presence of N,N'-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole to give the peptide *IX* which was again purified by gel filtration. Both the protecting groups were cleaved off with trifluoromethanesulfonic acid in the presence of trifluoroacetic acid and thioanisole and the obtained analogue *Id* was purified by free-flow electrophoresis.

The endocrine biological activities of the synthesized analogues are given in Table I, together with the data for [8-arginine]vasopressin as standard. The uterotonic, galactogogic and pressor activities are by up to three orders of magnitude lower than those of the parent hormone. The relatively high antidiuretic activity of the analogue *Ic* (as compared with the dosis necessary for provoking an effect on central nervous system) presents a disadvantage. The analogue *Ia* (in which the β-mercaptopropionic acid in position 1 was replaced by cysteine) is by an order of magnitude less active

TABLE I

Endocrine biological activities (I.U./mg) determined in rats

Compound	Uterotonic	Galactogogic	Pressor	Antidiuretic	
				A[a]	A[b]
Ia	0·07	0·12	<0·2	4·5	0·4
Ib	0·09	0·02	<0·2	0·3	0·1
Ic	0·08	0·01	<0·2	14·5	5·0
Id	0·09	—	inhibition	0·8	0·2
AVP[c,d]	17	69[e]	465	465	

[a] Anesthetized rat; [b] conscious rat; the activity expressed in % of effect of [8-D-arginine]deamino-vasopressin at the threshold level; [c] [8-arginine]vasopressin; [d] values taken from ref.[29]; [e] rabbit.

and even lower activity was found for the analogues *Ib* and *Id*. A comparison of the analogues *Ic* and *Id* is particularly interesting: it shows that the antidiuretic activity drops strongly on going from the disulfide prototype *Ic* to the carba-analogue *Id*. A reverse situation has been found with the corresponding analogues containing an unchanged glycinamide moiety in position 9; the carba-analogue being more active than the disulfide compound[14,15].

The prepared analogues *Ia–Id* enhanced the avoidance response[16] when administered immediately after the shock-trial. They were also active when the administration preceded the test-trial by 0·5, 3 or 20 h. [8-Lysine, 9-desglycinamide]vasopressin increased avoidance latencies when administered shortly after the shock trial or shortly before the test-trial. No effect was found when [8-lysine, 9-desglycinamide]-vasopressin was administered 20 h before the retention test. Details of these findings will be published separately. Analogue *Ic* can attenuate the avoidance response in the situation of passive avoidance when it is administered in connection with the forced extinction procedure[17]. The same analogue was also found to improve spatial working memory in rats[18] in experiments with 12- or 24-arm maze (food accessibility serving as motivation). The compound reduced significantly the number of errors in doses of 3 μg/kg rat.

EXPERIMENTAL

Analytical samples were dried over phosphorus pentoxide at room temperature and 150 Pa. Melting points were determined on a Kofler block and are uncorrected. Thin-layer chromatography on silica gel was carried out on Silufol plates (Kavalier) 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), 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 6) (S4), n-heptane–tert-butyl alcohol–pyridine (5 : 1 : 1) (S5), 1-butanol–acetic acid–ethyl acetate–water (1 : 1 : 1 : 1) (S7), benzene–methanol (8 : 2) (S9), 1-butanol–water–acetic acid (50 : 40 : 15) (S13), ethyl acetate–pyridine–acetic acid–water (5 : 5 : 1 : 3) (S23). Electrophoresis was performed on a Whatman 3MM paper in a moist chamber at 20 V/cm for 1 h in 1M acetic acid (pH 2·4) or in a pyridine–acetate buffer (pH 5·7). The compounds were detected with ninhydrin or by chlorination method. The solvents were evaporated on a rotatory evaporator at bath temperature 30°C, dimethylformamide at the same temperature at 150 Pa. Samples for amino acid analyses were hydrolyzed with 6M-HCl at 105°C and 150 Pa for 20 or 40 h. Amino acid analyses were carried out on a two-column instrument (type 6020, Developmental Workshops of Czechoslovak Academy of Sciences). Preparative free-flow electrophoresis was done on a previously described instrument[19,20].

Benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteinyl-tyrosine Methyl Ester

A solution of benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteine (3·9 g) and 1-hydroxybenzotriazole (1·36 g) in dimethylformamide (10 ml) was added to tyrosine methyl ester hydrochloride (2·32 g) and N-ethylpiperidine (1·37 ml) in dimethylformamide (10 ml). The mixture was cooled to −5°C and N,N′-dicyclohexylcarbodimide (2·07 g) was added. After stirring for 1 h at −5°C and for 12 h at room temperature, the separated N,N′-dicyclohexylurea was filtered off, the filtrate

taken down and the residue dissolved in ethyl acetate. The solution was washed successively with sodium hydrogen carbonate solution, water, 0·5M-HCl and again water, dried and taken down. Crystallization from ethyl acetate afforded 5·6 g (99%) of the dipeptide, m.p. 182—183°C, $[\alpha]_D$ −19·1° (*c* 0·5, dimethylformamide), R_F 0·88 (S1), 0·74 (S2), 0·85 (S3), 0·75 (S4), 0·40 (S5). For $C_{31}H_{36}N_2O_6S$ (564·7) calculated: 65·94% C, 6·43% H, 4·96% N, 5·68% S; found: 65·65% C, 6·49% H, 4·84% N, 5·72% S.

Benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteinyl-tyrosine

The protected dipeptide methyl ester (2·26 g) was suspended in methanol (12 ml) and 4M-NaOH (4 ml) was added. The mixture was stirred for 1 h at room temperature, 1M-HCl (17 ml) was added and the product was precipitated with water (200 ml). Crystallization gave 2·1 g (95%) of the protected dipeptide, m.p. 201—202°C, $[\alpha]_D$ −9·8° (*c* 0·8, methanol), $[\alpha]_D$ −15·6° (*c* 0·5, dimethylformamide). R_F 0·87 (S1), 0·57 (S2), 0·85 (S3), 0·64 (S4), 0·05 (S5). For $C_{30}H_{33}N_2O_6S$ (549·7) calculated: 65·55% C, 6·05% H, 5·10% N, 5·83% S; found: 65·38% C, 6·24% H, 5·36% N, 6·01% S.

Benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteinyl-tyrosine Hydrazide

The dipeptide methyl ester (4·5 g) was dissolved in dimethylformamide (13 ml) and mixed with 80% hydrazine hydrate (4 ml). After standing for 20 h at room temperature, the product was precipitated with water (200 ml), filtered, washed with water and crystallized from dimethylformamide and water; yield 4·2 g (93%), m.p. 256—258°C, $[\alpha]_D$ −29·5° (*c* 0·2, dimethylformamide), R_F 0·77 (S1), 0·60 (S2). For $C_{30}H_{36}N_4O_5S.0{\cdot}5\,H_2O$ (573·7) calculated: 62·81% C, 6·50% H, 9·77% N, 5·59% S; found: 63·09% C, 6·27% H, 9·79% N, 5·61% S.

Benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteinyl-tyrosyl-phenylalanine Methyl Ester (*IIIa*)

a) Dicyclohexylcarbodiimide (0·62 g) was added at −10°C to a stirred solution of the protected dipeptide acid (1·65 g) and 1-hydroxybenzotriazole (0·41 g) in dimethylformamide (8 ml) and the stirring was continued at −5°C for 1 h. A solution of phenylalanine methyl ester hydrochloride (0·65 g) and triethylamine (0·42 ml) in dimethylformamide (2 ml) was added and the mixture was kept at room temperature for 2 h. N,N′-Dicyclohexylurea was filtered off, dimethylformamide was evaporated and the residue was triturated successively with water, 0·5M-HCl, water, saturated solution of sodium hydrogen carbonate, water and ether. Crystallization from aqueous methanol afforded the methyl ester *IIIa* (2·1 g; 100%), m.p. 192—194°C, $[\alpha]_D$ −31·1° (*c* 0·5, dimethylformamide), R_F 0·86 (S1), 0·79 (S2), 0·85 (S3), 0·84 (S4), 0·32 (S5). For $C_{40}H_{45}N_3O_7S$ (711·9) calculated: 67·49% C, 6·37% H, 5·90% N, 4·50% S; found: 67·42% C, 6·39% H, 6·20% N, 4·56% S.

b) A 3·36M solution of HCl in dioxane (1·8 ml) was added to a solution of the dipeptide hydrazide (1·69 g) in dimethylformamide (10 ml). After cooling to −20°C, a solution of butyl nitrite (0·31 g) in dimethylformamide (1 ml) was added and the mixture was stirred at −20° for 20 min. After cooling to −40°C and neutralization with N-ethylpiperidine (0·85 ml), a solution of phenylalanine methyl ester hydrochloride (0·65 g) and N-ethylpiperidine (0·42 ml) in dimethylformamide (2 ml) was added. The mixture was set aside at 0°C for 48 h, dimethylformamide was evaporated and the residue was worked up as described in the preceding experiment. The obtained product (1·79 g; 84%) was crystallized from aqueous methanol; m.p. 192—194°C, $[\alpha]_D$ −31·2° (*c* 0·5, dimethylformamide).

Benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteinyl-tyrosyl-phenylalanine Hydrazide (*IIIb*)

A mixture of 80% hydrazine hydrate (2 ml), the tripeptide *IIIa* (2·13 g) and dimethylformamide (6 ml) was set aside for 15 h at room temperature. The product was precipitated with water (100 ml) and crystallized from dimethylformamide–water; yield 2·10 g (99%), m.p. 228–229°C, $[\alpha]_D$ −31·2° (*c* 0·5, dimethylformamide); R_F 0·85 (S1), 0·72 (S2), 0·83 (S3), 0·82 (S4), 0·13 (S5). For $C_{39}H_{45}N_5O_6S$ (710·9) calculated: 65·80% C, 6·37% H, 9·84% N, 4·50% S; found: 65·82% C, 6·42% H, 9·80% N, 4·67% S.

S-(2,4,6-Trimethylbenzyl)cysteine Methyl Ester Hydrochloride

Thionyl chloride (8 ml) was added dropwise to methanol (50 ml) at a temperature lower than −5°C. To the thus-obtained reagent, S-(2,4,6-trimethylbenzyl)cysteine[10] (7·5 g) was added portionwise the temperature being kept below −5°C. The mixture was stirred at −5°C for 30 min and then at 43°C for 2 h. Methanol was distilled off and the residue was codistilled several times with methanol. The crystalline material was suspended in water (15 ml) and mixed with a solution of sodium hydrogen carbonate (7·5 g) in water (75 ml). The formed suspension was filtered, the filtrate extracted with ethyl acetate, the organic extract washed with saturated solution of sodium hydrogen carbonate and water, dried and taken down. The obtained S-(2,4,6-trimethylbenzyl) cysteine methyl ester was converted into the hydrochloride with 2M-HCl in ether (3 ml). Crystallization from methanol and ether afforded 7 g (78%) of the product melting at 166 to 168°C, $[\alpha]_D$ +23·9° (*c* 0·5, dimethylformamide), R_F 0·58 (S1), 0·57 (S2), 0·54 (S3), 0·65 (S4), 0·78 (S7); $E_{2,4}^{His}$ 0·73, $E_{5,7}^{His}$ 0·80. For $C_{14}H_{21}NO_2S.HCl$ (303·9) calculated: 55·34% C, 7·30% H, 4·61% N, 10·55% S; found: 55·61% C, 7·41% H, 4·67% N, 10·30% S.

Benzyloxycarbonylasparaginyl-S-(2,4,6-trimethylbenzyl)cysteine Methyl Ester

Benzyloxycarbonylasparagine *p*-nitrophenyl ester (1·16 g) was added to a solution of S-(2,4,6-trimethylbenzyl)cysteine methyl ester (0·91 g) in dimethylformamide (6 ml). After addition of triethylamine (0·42 ml), the mixture was stirred for 12 h. The formed gel was mixed with water, the solid filtered, washed with water, saturated solution of sodium hydrogen carbonate, water, 10% HCl and acetone. Crystallization from aqueous acetic acid gave 1·3 g (84%) of the dipeptide, m.p. 178–180°C, $[\alpha]_D$ −10·7° (*c* 0·5, dimethylformamide), R_F 0·72 (S1), 0·60 (S2), 0·72 (S3), 0·71 (S4), 0·31 (S5). For $C_{26}H_{33}N_3O_6S$ (515·6) calculated: 60·56% C, 6·45% H, 8·15% N, 6·35% S; found: 60·73% C, 6·42% H, 8·11% N, 6·22% S.

Benzyloxycarbonylglutaminyl-asparaginyl-S-(2,4,6-trimethylbenzyl)cysteine Methyl Ester (*IV*)

a) The dipeptide methyl ester (3·1 g) was dissolved in acetic acid (18 ml) and 4M-HBr in acetic acid (6 ml) was added. After standing for 5 min at room temperature, the hydrobromide was precipitated with ether and dissolved in dimethylformamide (15 ml). The solution was adjusted to pH 10 with N-ethylpiperidine and a solution of benzyloxycarbonylglutamine *p*-nitrophenyl ester (2·41 g) in dimethylformamide (5 ml) was added. The mixture was set aside at room temperature for 48 h, dimethylformamide was evaporated *in vacuo*, and the residue was triturated successively with water, saturated solution of potassium hydrogen carbonate, water, 0·5M-HCl, water and ether. Crystallization from dimethylformamide and water afforded 3·0 g (78%) of the tripeptide *IV*, m.p. 248–250°C; $[\alpha]_D$ −12° (*c* 0·5, dimethylformamide), R_F 0·61 (S1), 0·47 (S2), 0·62 (S3), 0·69 (S4), 0·81 (S7). For $C_{31}H_{41}N_5O_8S$ (643·8) calculated: 57·84% C, 6·42% H, 10·88% N, 4·98% S; found: 57·92% C, 6·15% H, 11·07% N, 4·90% S.

b) Triethylamine (0·7 ml) was added at 0°C to a stirred solution of benzyloxycarbonylglutamine (1·4 g) in dimethylformamide (10 ml). To the thus-obtained salt 1-(β-naphthalenesulfonyloxy)benzotriazole[11] (1·7 g) was added at 0°C and the mixture was stirred at this temperature for 1 h and at room temperature for another 1 h. To this solution of the active ester a solution of the protected dipeptide hydrobromide (2·58 g; prepared as described under *a*)) in dimethylformamide (10 ml) was added and the mixture was adjusted to pH 10 with triethylamine. The mixture was stirred at room temperature for 12 h, taken down and the crude product was purified as described under *a*), affording 2·8 g (87%) of the protected tripeptide *IV*, m.p. 248–250°C, $[\alpha]_D$ −12·2° (*c* 0·5, dimethylformamide), identical in all respects with the product obtained by the procedure *a*).

Benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteinyl-tyrosyl-phenylalanyl-glutaminyl--asparaginyl-S-(2,4,6-trimethylbenzyl)cysteine Methyl Ester (*IIa*)

A solution of the tripeptide *IV* (1·93 g) in acetic acid (12 ml) was mixed with 4M-HBr in acetic acid (6 ml) and after standing for 30 min at room temperature the hydrobromide was precipitated with ether and dried.

A suspension of the protected tripeptide hydrazide *IIIb* (2·14 g) in dimethylformamide (15 ml) was mixed with 4·6M-HCl in dioxane (1·3 ml). After dissolution, butyl nitrite (0·31 g) in dimethylformamide (1 ml) was added at −20°C. The mixture was stirred at −20°C for 20 min, cooled to −40°C and neutralized with N-ethylpiperidine. A solution of hydrobromide of the tripeptide *IV* (prepared above) in dimethylformamide (15 ml) was added to the thus-prepared azide and the mixture was adjusted to pH 10 with N-ethylpiperidine. After standing at 0°C for 70 h, dimethylformamide was evapored and the residue triturated successively with a saturated solution of sodium hydrogen carbonate, water, 0·5M-HCl, water, methanol and ether, affording 3·2 g (90%) of the hexapeptide *IIa*, m.p. 248–250°C; $[\alpha]_D$ −31·5° (*c* 0·5, dimethylformamide), $E^{Gly}_{2,4}$ 0·80, $E^{His}_{5,7}$ 0·28 (after removal of the benzyloxycarbonyl group). Amino acid analysis: Tyr 0·94, Phe 1·00, Glu 1·02, Asp 1·06, Cys 1·92. For $C_{62}H_{76}N_8O_{12}S_2$ (1 189) calculated: 62·61% C, 6·44% H, 9·42% N, 5·39% S; found: 62·65% C, 6·48% H, 9·72% N, 5·41% S.

Benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteinyl-tyrosyl-phenylalanyl-glutaminyl--asparaginyl-S-(2,4,6-trimethylbenzyl)cysteine Hydrazide (*IIb*)

A mixture of the hexapeptide *IIa* (2·4 g), 80% hydrazine hydrate (2 ml) and dimethylformamide (20 ml) was set aside at room temperature for 2 days, the mixture was diluted with water (200 ml) and the precipitate washed with water to neutrality. Crystallization from dimethylformamide and water afforded 20 g (84%) of product, melting at 246–258°C; $[\alpha]_D$ −34·9° (*c* 0·5, dimethylformamide). For $C_{61}H_{76}N_{10}O_{11}S_2$ (1 189) calculated: 61·60% C, 6·44% H, 11·78% N, 5·39% S; found: 61·38% C, 6·64% H, 11·58% N, 5·39% S.

o-Nitrobenzenesulfenylprolyl-N^G-*p*-toluenesulfonyl-D-arginine Benzyl Ester (*V*)

A solution of *o*-nitrobenzenesulfenylproline 2,4,5-trichlorophenyl ester (2·5 g) and N^G-toluenesulfonyl-D-arginine benzyl ester hydrobromide (2·5 g) in dimethylformamide (5 ml) was stirred at room temperature for 40 h and taken down *in vacuo*. The residue was dissolved in ethyl acetate and the solution was extracted successively with saturated sodium hydrogen carbonate, water, a $KHSO_4/K_2SO_4$ solution (pH 2) and water. After drying over magnesium sulfate and evaporation of the solvent, the residue was crystallized from ethyl acetate–light petroleum, yielding 3·0 g (90%) of the product, m.p. 90–92°C, $[\alpha]_D$ −40·4° (*c* 0·4, methanol); R_F 0·88 (S1), 0·75 (S2).

0·75 (S3), 0·83 (S4), 0·60 (S9). For $C_{31}H_{36}N_6O_7S_2$ (668·8) calculated: 55·67% C, 5·43% H, 12·57% N, 9·59% S; found: 55·92% C, 5·38% H, 12·68% N, 9·38% S.

o-Nitrobenzenesulfenylprolyl-N$^{\delta}$-benzyloxycarbonylornithine Benzyl Ester (*VI*)

Triethylamine (1·39 ml) was added under cooling to a solution of N$^{\delta}$-benzyloxycarbonylornithine benzyl ester hydrochloride (3·92 g) in dimethylformamide (10 ml) and a solution of *o*-nitrobenzenesulfenylproline 2,4,5-trichlorophenyl ester (4·48 g) in dimethylformamide (15 ml) was added to the formed suspension. After stirring for 24 h at room temperature, the mixture was taken down, the residue dissolved in ethyl acetate and the solution washed with saturated sodium hydrogen carbonate solution, water, a solution of $KHSO_4$ and K_2SO_4 (pH 2) and again with water. After drying and evaporation of the solvent, the residue was crystallized from ethyl acetate–light petroleum to give 5·58 g (92%) od the product *VI*, m.p. 115–116°C, $[\alpha]_D$ −67·4° (*c* 1, methanol); R_F 0·80 (S1), 0·74 (S2), 0·75 (S3), 0·77 (S4), 0·24 (S5). For $C_{31}H_{34}N_4O_7S$ (606·7) calculated: 61·37% C, 5·65% H, 9·23% N, 5·28% S; found: 61·55% C, 5·71% H, 9·22% N, 5·33% S.

Benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteinyl-tyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-(2,4,6-trimethylbenzyl)cysteinyl-prolyl-N^{G}-*p*-toluenesulbonyl-D-arginine Benzyl Ester (*VIIa*)

To a stirred solution of N$^{\delta}$-benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteinyl-tyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-(2,4,6-trimethylbenzyl)cysteine hydrazide (*IIb*; 238 mg) in dimethyl formamide (2 ml) was added 3M-HCl in dioxane (130 µl). The solution was cooled to −20°C and butyl nitrite (21 mg) in dimethylformamide (0·5 ml) was added. The mixture was stirred for 20 min, cooled to −10°C, neutralized with N-ethylpiperidine to pH 7 (moist pH-paper) and treated with a solution, prepared in the following way:

A solution of the compound *V* (200 mg) in dimethylformamide (1 ml) was mixed with 2M-HCl in ether (0·5 ml). After standing at room temperature for 5 min, the mixture was diluted with ether, the precipitated hydrochloride collected on a filter, washed with ether and dissolved in dimethylformamide (2 ml). The solution was adjusted to pH 10 with N-ethylpiperidine and added to the above-described azide solution. The mixture was set aside for 60 h at 0°C, taken down and the residue triturated successively with 0·5M-HCl, water, saturated solution of sodium hydrogen carbonate and water. Purification by crystallization from dimethylformamide–water and gel filtration on a Sephadex LH-20 column in dimethylformamide afforded 310 mg (93%) of the compound *VIIa*, m.p. 230–232°C, $[\alpha]_D$ −28·6° (*c* 0·5, dimethylformamide). R_F 0·94 (S1), 0·75 (S2), 0·90 (S3), 0·86 (S4). For $C_{86}H_{105}N_{13}O_{14}S_3.H_2O$ (1 691) calculated: 61·08% C, 6·26% H, 10·77% N, 5·69% S; found: 60·89% C, 6·23% H, 10·75% N, 5·42% S.

[8-D-Arginine, 9-desglycinamide]vasopressin (*Ia*)

Thioanisole (100 µl) was added to a solution of the protected octapeptide *VIIa* (100 mg) in trifluoroacetic acid (1·25 ml). The solution was cooled to 0°C and trifluoromethanesulfonic acid (1·0 ml), cooled to the same temperature, was added. After standing for 0·5 h at 0°C, the mixture was diluted with ether (100 ml) and the separated product was filtered, washed with ether and dissolved in water (300 ml). The solution was adjusted to pH 6·85 with 0·1M-NaOH and oxidized with air oxygen for 1 h at room temperature. Acetic acid was then added (to pH 4) and the solution was filtered through a column of Amberlite IR-4B (acetate form; 12 × 2 cm). The eluates were freeze-dried, the residue was applied on a column of Sephadex G-15 (100 × 1·5 cm)

and eluted with 50% acetic acid. The peptidic material was freeze-dried and the residue was again applied on the same column. Elution with 0·2M acetic acid and freeze-drying afforded 14 mg (23%) of the product, $[\alpha]_D$ −17·7° (*c* 0·27, 1M acetic acid); $E^{His}_{2.4}$ 0·56, $E^{His}_{5.7}$ 0·32; R_F 0·34 (S4), 0·42 (S13), 0·91 (S23). Amino acid analysis: Phe 1·02, Tyr 0·91, Asp 1·02, Glu 1·04, Pro 1·02, Arg 1·00; Cys (O_3H) 1·92 (the value for cysteic acid was obtained from a separate sample after oxidation with peroxyformic acid). For $C_{44}H_{61}N_{13}O_{12}S_2.2\,CH_3CO_2H.2\,H_2O$ (1 124) calculated: 49·14% C, 6·19% H, 16·20% N; found: 49·02% C, 5·98% H, 15·94% N.

Benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)-cysteinyl-tyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-(2,4,6-trimethylbenzyl)cysteinyl-prolyl-N^{δ}-benzyloxycarbonylornithine Benzyl Ester (*VIIb*)

A solution of the azide was prepared from the hydrazide *IIb* (238 mg) as described for the preparation of the compound *VIIa*.

The benzyl ester *VI* (182 mg) was dissolved in dimethylformamide (1 ml) and 3M-HCl in ether (0·5 ml) was added. After 5 min the mixture was diluted with ether, the precipitate was decanted and washed with ether. The obtained hydrochloride was dissolved in dimethylformamide (2 ml), the solution adjusted to pH 10 with N-ethylpiperidine and added to the above-mentioned solution of the azide. The mixture was set aside for 60 h at 0°C, taken down and the residue triturated successively with 0·5M-HCl, water, saturated solution of sodium hydrogen carbonate and water. The product was purified by crystallization from dimethylformamide–water and gel filtration in dimethylformamide, yielding 300 mg (93%) of *VIIb*, m.p. 238—240°C, $[\alpha]_D$ −27·6° (*c* 0·15, dimethylformamide); R_F 0·86 (S1), 0·67 (S2), 0·82 (S3), 0·91 (S4). For $C_{86}H_{103}N_{11}O_{16}.S_2.2\,H_2O$ (1 617) calculated: 63·88% C, 6·67% H, 9·53% N, 3·97% S; found: 64·00% C, 6·38% H, 9·57% N, 3·95% S.

[8-Ornithine, 9-desglycinamide]vasopressin (*Ib*)

Thioanisole (100 µl) was added to a solution of the protected octapeptide *VIIb* (100 mg) in trifluoroacetic acid (1·25 ml). The solution was cooled to 0°C and trifluoromethanesulfonic acid (1 ml), precooled to 0°C, was added. After standing at 0°C for 30 min, the mixture was diluted with ether and the separated precipitate was collected on filter and dissolved in water (300 ml). The solution was adjusted to pH 6·8 with 0·1M-NaOH and oxidized with air oxygen for 1 h. Acetic acid was added to pH 3·9 and the solution was filtered through a column of Amberlite IR-4B (acetate form). The effluents were freeze-dried and the residue (89 mg) dissolved in 50% acetic acid and purified by gel filtration on a Sephadex G-15 column in 50% acetic acid to give 15·3 mg (24%) of *Ib*, $[\alpha]_D$ −15·1° (*c* 0·2, 1M acetic acid); $E^{G1}_{2.4}$ 0·98, $E^{His}_{5.7}$ 0·34; R_F 0·37 (S4), 0·43 (S13), 0·89 (S23). Amino acid analysis: Phe 1·01, Tyr 0·92, Glu 1·00, Asp 1·00, Pro 1·03, Orn 0·99. Cystine was determined as cysteic acid in a separate sample after oxidation with peroxyformic acid: 1·92. For $C_{43}H_{59}N_{11}O_{12}S_2.2\,CH_3CO_2H.2\,H_2O$ (1 142) calculated: 49·42% C, 6·26% H, 13·48% N; found: 49·18% C, 5·95% H, 13·30% N.

S-Benzyl-β-mercaptopropionyl-tyrosyl-phenylalanyl-glutaminyl-asparaginyl--S-benzylcysteinyl-prolyl-N^{G}-*p*-toluenesulfonyl-D-arginine Benzyl Ester (*VIII*)

A solution of S-benzyl-β-mercaptopropionyl-tyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-benzylcysteine hydrazide[13] (239 mg) in dimethylformamide (3 ml) was mixed with 2M-HCl in dioxane (0·25 ml), the mixture was cooled to −20°C and butylnitrite (25·8 mg) in dimethylformamide (0·8 ml) was added. The mixture was kept at −20°C for 20 min, cooled to −40°C and neutralized

with N-ethylpiperidine. To the thus-obtained azide a solution of benzyl ester hydrochloride (prepared from 250 mg *V* and 2M-HCl in ether) in dimethylformamide (2 ml) was added. The mixture was warmed to 0°C and set aside at this temperature for 3 days. After evaporation of dimethylformamide, the residue was triturated with 1% HCl, the solid was collected on filter, washed with water, saturated solution of sodium hydrogen carbonate and again with water. The crude product was purified by gel filtration on Sephadex LH-20 in dimethylformamide. Reprecipitation from dimethylformamide and water afforded 280 mg (78%) of compound *VII*, m.p. 174–175°C, $[\alpha]_D^{22}$ −29·9° (*c* 0·2, dimethylformamide); R_F 0·77 (S1), 0·70 (S2), 0·82 (S3), 0·83 (S4), 0·80 (S21), 0·91 (S22). For $C_{72}H_{86}N_{12}O_{14}S_3.0{\cdot}5\ H_2O$(1 449) calculated: 59·69% C, 6·05% H, 11·60% N, 6·64% S; found: 59·49% C, 6·15% H, 11·52% N, 6·58% S. Amino acid analysis: Cys(Bzl) 1·03, Tyr 0·97, Phe 1·04, Glu 1·00, Asp 0·98, Pro 1·03, Arg 0·95.

Cyclic Disulfide of β-Mercaptopropionyl-tyrosyl-phenylalanyl-glutaminyl-asparaginyl--cysteinyl-prolyl-D-arginine (*Ic*)

The protected octapeptide *VIII* (100 mg) was dissolved under stirring in liquid ammonia (300 ml) and reduced with sodium until the blue colouration persisted for 15 s. The mixture was decomposed with ammonium chloride and ammonia was evaporated under diminished pressure (water pump). The dry residue was dissolved with cooling in 0·01M-HCl (200 ml) and the solution was adjusted to pH 6·75. The solution was washed with ether, made up to 300 ml and oxidized with 0·01M potassium ferricyanide during 1 h. The mixture was filtered through a column of Amberlite IR-4B (Cl^- form; 2·5 × 27 cm; 90 g of moist resin). The effluents were freeze-dried, the residue was dissolved in 50% acetic acid (4 ml) and filtered through a column of Sephadex G-15 (1·5 × 100 cm, 50% acetic acid). The fractions, containing peptidic material, were filtered through a column (1·3 × 100 cm) of Bio-Gel P-4 in 1M acetic acid and eluates, containing the pure analogue, were freeze-dried. The product (10 mg) had $[\alpha]_D^{20}$ −56·7° (*c* 0·1, 1M acetic acid); R_F 0·32 (S1), 0·54 (S4), 0·71 (S7), 0·71 (S23). $E_{2.4}^{Gly}$ 0·79, $E_{5.7}^{Gly}$ 1·0. For $C_{44}H_{60}N_{12}O_{12}S_2.C_2H_4O_2.2\ H_2O$ (1 109) calculated: 49·81% C, 6·18% H, 15·15% N; found: 49·85% C, 5·97% H, 15·09% N. Amino acid analysis: Tyr 0·96, Phe 1·06, Glu 1·03, Asp 0·98, Pro 1·02, Arg 0·94.

Lactam of Tyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-(γ-carboxypropyl)cysteinyl--prolyl-N^G-*p*-toluenesulfonyl-D-arginine Benzyl Ester (*IX*)

A solution of the protected benzyl ester *V* (177 mg) in dimethylformamide (1 ml) was mixed with 2·6M-HCl in ether (1 ml). After standing for 4 min at room temperature, the hydrochloride was precipitated with ether and dried ($E_{2.6}^{Gly}$ 0·96, $E_{5.7}^{His}$ 0·70). The hydrochloride was dissolved in dimethylformamide (1 ml), the solution was adjusted approximately to pH 10 with N-ethylpiperidine and a solution of 1-deamino-1-carba-pressinoic acid[14] (100 mg) and 1-hydroxybenzotriazole (123 mg) in dimethylformamide (1·5 ml) was added. After cooling to −30°C, N,N'--dicyclohexylcarbodiimide (31 mg) in dimethylformamide (0·5 ml) was added and the mixture was stirred at −5° for 4 h and at room temperature for 20 h. N,N'-Dicyclohexylurea was filtered off and the filtrate was taken down. The residue was triturated with hydrochloric acid (pH 2), washed on the filter with water, saturated solution of sodium hydrogen carbonate, water and ether. The crude product (160 mg) was purified by gel filtration on a column (200 × 1 cm) of Sephadex LH-20 in dimethyl formamide. Effluents, containing the pure compound, were taken down and the residue crystallized from dimethylformamide and water, affording 100 mg (60%) of the product, m.p. 152–154°C, $[\alpha]_D$ −34·0° (*c* 0·46, dimethylformamide); R_F 0·50 (S1), 0·54 (S3), 0·66 (S4). Amino acid analysis: Pro 0·98, Arg 0·98, Cys($C_3H_6CO_2H$) 0·94, Glu 1·05, Asp 1·04, Tyr 0·92, Phe 1·08. For $C_{59}H_{74}N_{12}O_{14}S_2.H_2O$ (1 257) calculated: 56·36% C, 6·09% H, 13·37% N, 5·10% S; found: 56·52% C, 6·13% H, 13·27% N, 5·09% S.

Lactam of Tyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-(γ-carboxypropyl)cysteinyl-prolyl--D-arginine (*Id*)

Trifluoromethanesulfonic acid (200 μl) and thioanisole (20 μl) were added at 0°C to a solution of the protected lactam *IX* (30 mg) in trifluoroacetic acid (300 μl). After standing at 0°C for 30 min, the crude free lactam was precipitated with ether, filtered, washed with ether, dissolved in water and the solution was filtered through a column of Amberlite IR-4B in acetate form. The effluents were freeze-dried and the residue was purified by free-flow electrophoresis (2 500 V, 135 mA), affording 6 mg of the product, $[\alpha]_D -47°$ (*c* 0·1, 1M acetic acid); R_F 0·34 (S1), 0·57 (S4), 0·73 (S23), $E^{Gly}_{2,4}$ 0·80. Amino acid analysis: Arg 1·01, Pro 1·04, Glu 1·01, Asp 1·02, Phe 0·98, Tyr 0·94, $Cys(C_3H_6CO_2H)$ 0·98. For $C_{45}H_{62}N_{12}O_{12}S.CH_3COOH.3\,H_2O$ (1 109) calculated: 50·89% C, 6·54% H, 15·15% N; found: 50·68% C, 6·45% H, 14·92% N.

Pharmacological Methods

The *in vitro* uterotonic assay was carried out on isolated rat uterine strip[21,22]. Galactogogic activity was determined on ethanol-anesthetized rats[23,24] (4—15 days after delivery), pressor activity on despinalized rats[25]. Antidiuretic activity was determined on ethanol-anesthetized rats[26,27] and on conscious Wistar rats[28].

In the test of passive avoidance behaviour[16] the analogues were administered *s.c.* in the dose of 5 μg/kg either immediately after the electrical footshock or 0·5, 3 or 20 h before the retention test.

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