SYNTHESIS OF OXYTOCIN, ARGININE-VASOPRESSIN AND ITS DEAMINO-ANALOGUE USING 2,4,6-TRIMETHYLBENZYL GROUP FOR PROTECTION OF THE CYSTEINE SULFUR*

František Brtník, Milan Krojidlo**, Tomislav Barth and Karel Jošt

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

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Preparation of oxytocin, arginine-vasopressin and its deamino-analogue serves as an example of use of 2,4,6-trimethylbenzyl group for protection of the cysteine sulfur atom in the peptide synthesis. This modified benzyl group is sufficiently stable under conditions of solvolytic removal of common amino-protecting groups and it can be cleaved off under mild conditions with liquid hydrogen fluoride or trifluoromethanesulfonic acid.

Cysteine*** peptides have been synthesized using a great variety of protecting groups[2-4] of different structural types and different stability which can be removed under various conditions. One of the most frequently used groups is benzyl group[5,6] which can be easily introduced into the cysteine molecule; moreover, S-benzylcysteine derivatives exhibit favourable physico-chemical properties and the benzyl group is stable under wide range of conditions used in peptide synthesis. On the other hand, this group is very stable and its removal requires conditions under which the synthesized peptide chain can be damaged[3,4] (sodium in liquid ammonia or liquid hydrogen fluoride at 20°C). Some recent attempts to alter stability of the S-benzyl group by suitable substitution have been published. Introduction of methoxy group into the position 4 of the benzyl moiety[7,8] made its stability too low[9-12]. S-3,4-Methylenedioxybenzyl group[13] requires refluxing with trifluoroacetic acid for 2 hours; its stability toward hydrogen fluoride was not described. Introduction of a methyl group into the position 4 (ref.[9,10,12]) or two methyl groups into the positions 3 and 4 (ref.[14,15]) afforded derivatives suitable for the solid-phase peptide synthesis (including oxytocin[12,15] and vasopressin[12]); these protecting groups were cleaved off by action of liquid hydrogen fluoride.

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** Present address: Léčiva, Prague - Modřany.

*** The amino acids used in this work are of the L-configuration. The nomenclature and symbols obey the published recommendations[1]; the abbreviations Cys(Bzl(2,4,6-Me_3)) and Mpr designate the residue of S-(2,4,6-trimethylbenzyl)cysteine and 3-mercaptopropionic acid, respectively.

It has been found[16] that the presence of three methyl groups in the positions 2, 4 and 6 increases lability of the benzyl ester. Whereas the solvolytic cleavage of the benzyl ester must be accomplished by treatment with hydrogen bromide in acetic acid, the 2,4,6-trimethylbenzyl ester is readily cleaved by trifluoroacetic acid. Comparison of variously substituted benzyl esters showed[16] that the 2,4,6-trimethylbenzyl ester was more readily cleaved by solvolysis than *e.g.* the 4-methyl derivative. We assumed therefore that also an S-2,4,6-trimethylbenzyl protecting group bonded to the cysteine sulfur atom could be more easily removed than the corresponding 4-methyl derivative; this was actually confirmed by our preliminary experiments[17]. The S-2,4,6--trimethylbenzyl group is sufficiently stable under conditions of peptide synthesis (Table I): it is resistant toward trifluoroacetic acid as well as hydrogen bromide in acetic acid at room temperature; a 10% cleavage was observed only at 40°C.

The S-trimethylbenzyl protecting group can be quantitatively removed by liquid hydrogen fluoride[18,19] at 0°C during 30 minutes; the reaction must be performed in the presence of anisole[18,19] since otherwise even after 1 hour at 20°C the reaction mixture contains still 13% of the starting compound (Table I). This group can be removed from the cysteine sulfur atom also by action of trifluoromethanesulfonic acid. This reagent[20] was used in the synthesis of tuftsin[21], neurotensin[22,23] and

TABLE I

Stability of S-(2,4,6-Trimethylbenzyl)cysteine under Various Solvolytic Conditions

Reagent	Reaction conditions			Starting compound[a] %
	temperature °C	time min	anisole equivalents	
CF_3COOH	23	120	0	100
	23	3 600	0	96·8
	reflux	60	0	96·1
2M-HBr/CH_3COOH	23	15	0	100
	23	60	0	99·1
4M-HBr/CH_3COOH	23	15	0	97·7
	23	60	0	94·4
	40	30	0	89·2
HF (liq)	20	60	0	13·2
	0	30	2	0
CF_3SO_3H/CF_3COOH (2 : 3)[b]	0	30	5	0

[a] Determined on amino acid analyser from the ratio starting compound to product; [b] volume.

Z-Cys(Bzl(2,4,6-Me_3))-Pro-Y-Gly-NH_2

I

Nps-Asn-Cys(Bzl)2,4,6-Me_3))-Pro-Y-Gly-NH_2

II

Nps-Gln-Asn-Cys(Bzl(2,4,6-Me_3))-Pro-Y-Gly-NH_2

III

Nps-X-Gln-Asn-Cys(Bzl(2,4,6-Me_3))-Pro-Y-Gly-NH_2

IV

Nps-Tyr(Bu^t)-X-Gln-Asn-Cys(Bzl(2,4,6-Me_3))-Pro-Y-Gly-NH_2

V

Z-Cys(Bzl(2,4,6-Me_3))-Tyr(Bu^t)-X-Gln-Asn-Cys(Bzl(2,4,6-Me_3))-Pro-Y-Gly-NH_2

VI

Series *a*: X = Ile, Y = Leu; *b*: X = Phe, Y = Arg(Tos)

Mpr(Bzl(2,4,6-Me_3))-Tyr(Bu^t)-Phe-Gln-Asn-Cys(Bzl(2,4,6-Me_3))-Pro-Arg(Tos)-Gly-NH_2

VII

Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2

VIIIa

Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2

VIIIb

Mpr-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2

VIIIc

somatostatin[24]. The work with trifluoromethanesulfonic acid is simpler than with liquid hydrogen fluoride. Unfortunately, it has been found that during synthesis of biologically active arginine-containing peptides, N^G-nitroarginine was cleaved only partially and the cleavage of N^G-*p*-toluenesulfonylarginine required 60 min and elevated (40°C) temperature. However, if the reaction is performed under conditions given in Table II, the *p*-toluenesulfonyl protecting group is removed quantitatively already at 0°C after 5 to 30 min.

In order to check the applicability of S-2,4,6-trimethylbenzyl group to synthesis of biologically active peptides, we prepared oxytocin, arginine-vasopressin and its deamino-analogue by stepwise synthesis in solution, mostly using the *o*-nitrobenzenesulfenyl protecting group and active esters for formation of the peptide bond. The guanidine group of arginine was protected by *p*-toluenesulfonyl group and the tyrosine hydroxyl by tert-butyl group. Starting from the previously described tripeptides

TABLE II

Cleavage of Cyclohexylammonium Salt of N^{α}-Benzyloxycarbonyl-N^{G}-*p*-toluenesulfonylarginine (1 μM) with Trifluoromethanesulfonic Acid

$CF_3SO_3H^a$	CF_3COOH^a	Anisole[a]	Temperature °C	Time min	Formed[b] arginine %
12·5	21·6	3	23	30	100
100	173	3	0	5	99·2
100	173	3	0	30	99·6
100	173	3	0	60	100

[a] In μM; the volume ratio $CF_3SO_3H : CF_3COOH$ was 2 : 3; [b] determined on an amino acid analyser.

prolyl-leucyl-glycine amide[25,26] and prolyl-N^{G}-*p*-toluenesulfonylarginyl-glycine amide[27], we prepared tetrapeptides *Ia* and *Ib*, pentapeptides *IIa* and *IIb*, hexapeptides *IIIa* and *IIIb*, heptapeptides *IVa* and *IVb*, octapeptides *Va* and *Vb*, and finally the protected nonapeptides *VIa* and *VIb*. These were transformed into compounds with free sulfhydryl groups by treatment with liquid hydrogen fluoride which were subjected to oxidative cyclisation to give oxytocin *VIIIa* and arginine-vasopressin *VIIIb*, both fully biologically active. Moreover, arginine-vasopressin was obtained also from the protected nonapeptide *VIb* by removal of the protecting groups with trifluoromethanesulfonic acid.

Acylation of the free octapeptide (after removal of *o*-nitrobenzenesulfenyl group from the compound *Vb*) with *p*-nitrophenyl S-β-(2,4,6-trimethylbenzyl)mercaptopropionate afforded the compound *VII* from which the protecting groups were again cleaved off by action of liquid hydrogen fluoride. The obtained product was then subjected to oxidative cyclisation to give deamino-vasopressin *VIIIc*.

EXPERIMENTAL

Analytical samples were dried *in vacuo* (150 Pa) at room temperature. Melting points were determined on a Kofler block and are uncorrected. Thin-layer chromatography was performed on silica gel (Silufol, Kavalier, Czechoslovakia) in the systems: S1 2-butanol–98% formic acid–water (75 : 13·5 : 11·5), S2 2-butanol–25% aqueous ammonia–water (85 : 7·5 : 7·5), S3 1-butanol–acetic acid–water (4 : 1 : 1), S4 pyridine–1-butanol–acetic acid–water (10 : 15 : 3 : 6), S5 *n*-heptane–tert-butyl alcohol–pyridine (5 : 1 : 1), S7 1-butanol–acetic acid–ethyl acetate–water (1 : 1 : 1 : 1), S10 ethyl acetate, S13 toluene–acetone (7 : 3), S14 toluene–acetone (20 : 1). Electrophoresis was performed on a Whatman 3MM paper (moist chamber at 20 V/cm, 60 min) in 1M acetic acid (pH 2·4) or pyridine–acetic acid (pH 5·7). The reaction mixtures were taken down under diminished pressure (water pump) on a rotary evaporator at the bath temperature 30 to 40°C; dimethylformamide-containing mixtures were evaporated using an oil pump. Amino acid analyses were performed after hydrolysis of the samples (48 h in 6M-HCl, phenol, 105°C, 150 Pa).

on an automatic analyser (type 6020, Developmental Workshops, Czechoslovak Academy of Sciences). Countercurrent distribution was performed in an all-glass apparatus (Countercurrent Distribution Machine, Quickfit & Quartz, Stone, Staffordshire, England). Preparative free-flow electrophoresis was carried out under conditions described previously[28]. Gel filtration was performed on columns of Bio-Gel P-4 (Bio-Rad Laboratories, Richmond, USA). For work with liquid hydrogen fluoride we used a Toho Kasei Co (Osaka, Japan) instrument. Optical rotations were measured on a Perkin Elmer 141 MCA polarimeter. Concentrations of the solutions for pharmacological tests were determined spectrophotometrically, using N-acetyltyrosine amide as standard.

S-(2,4,6-Trimethylbenzyl)cysteine

Cystine ($[\alpha]_D$ —215·3° (*c* 1, 1M-HCl); 10 g) in liquid ammonia (500 ml) was reduced with sodium (4 g) until the blue colour of the solution persisted for 15 min. The excess sodium was destroyed by addition of ammonium chloride in small portions and, immediately after decolorization, 2,4,6-trimethylbenzyl chloride (16·8 g) was added. The suspension was stirred for 2 h, ammonia was evaporated, 1M-HCl (200 ml) was added and the mixture adjusted to pH 6. The separated solid was filtered, washed with water and ethyl acetate and crystallized from aqueous ethanol, affording 12 g (57%) of the product, melting at 235—238°C, $[\alpha]_D^{20}$ +9·4° (*c* 0·5, 1M-NaOH), R_F 0·45 (S1), 0·28 (S2), 0·45 (S3). For $C_{13}H_{19}NO_2S$ (253·4) calculated: 61·57% C, 7·56% H, 5·53% N; found: 61·43% C, 7·58% H, 5·56% N.

In order to prove its optical purity, a sample of S-(2,4,6-trimethylbenzyl)cysteine $[\alpha]_D$ +8·9° (*c* 0·5, 1M-NaOH), was reduced with hydrogen fluoride in the presence of anisole. The arising cysteine was oxidized in an aqueous solution with air (pH 7, 10 h), giving cystine (97%) of $[\alpha]_D$ —210° (*c* 1, 1M-HCl).

N-Benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteine

Benzyloxycarbonyl chloride (10 ml) was added dropwise in the course of 30 min to a stirred (Vibro-mischer) suspension of S-(2,4,6-trimethylbenzyl)cysteine (12·6 g) in 2M-NaOH (125 ml). After stirring for further 30 min, the mixture was diluted with water (500 ml), extracted with ether and made acid by addition of conc. hydrochloric acid to pH 3. The separated oil was taken up in ethyl acetate, the extract washed with water, dried and taken down. Crystallization of the residue from ethyl acetate and light petroleum afforded 13·4 g (69%) of the product, m.p. 122 to 123°C, $[\alpha]_D^{20}$ —12·3° (*c* 1, ethanol), $[\alpha]_D^{20}$ —36·9° (*c* 1, dimethylformamide). Crystallization of an analytical sample did not rise the melting point. For $C_{21}H_{25}NO_4S$ (487·5) calculated: 65·09% C, 6·50% H, 3·61% N, 8·27% S; found: 64·85% C, 6·40% H, 3·54% N, 8·37% S.

N-Tert-butyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteine

Tert-butyloxycarbonyl azide (7·8 g) was added to a suspension of S-(2,4,6-trimethylbenzyl)cysteine (6·32 g) in a mixture of dioxane (25 ml) and water (15 ml). The stirred mixture was kept for 11 h at room temperature, the pH being maintained at 10·2 with 4M-NaOH, extracted with ether, the aqueous layer acidified with citric acid and the precipitated product taken up in ethyl acetate. The organic layer was washed with water, dried over sodium sulfate, taken down and the residue was crystallized from ether–light petroleum, affording 5·8 g (66%) of the product, m.p. 101 to 103°C, $[\alpha]_D^{20}$ —11·5° (*c* 0·7, acetic acid); R_F 0·91 (S3), 0·73 (S4). The analytical sample was crystallized from diisopropyl ether without rise in the melting point. For $C_{18}H_{27}NO_4S$ (353·5) calculated: 61·16% C, 7·70% H, 3·96% N, 9·07% S; found: 61·32% C, 7·61% H, 4·13% N, 8·83% S.

N-Benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteine 2,4,5-Trichlorophenyl Ester

Dicyclohexylcarbodiimide (2·06 g) in ethyl acetate (10 ml) was added to a cold (0°C) solution of N-benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteine (3·88 g) and 2,4,5-trichlorophenol (1·97 g) in ethyl acetate (20 ml). After stirring for 1 h at 0°C and for 15 h at room temperature, the separated dicyclohexylurea was filtered and washed with ethyl acetate. The combined ethyl acetate solutions were successively washed with water, saturated sodium hydrogen carbonate solution and again water, dried and taken down. Crystallization from ethyl acetate–light petroleum gave 5·2 g (92%) of the product, m.p. 143—144°C; $[\alpha]_D^{20}$ —20·1° (*c* 0·4, ethyl acetate); R_F 0·87 (S3), 0·62 (S5), 0·77 (S10). For $C_{27}H_{26}Cl_3NO_4S$ (566·9) calculated: 57·20% C, 4·62% H, 2·47% N, 5·65% S; found: 57·28% C, 4·58% H, 2·46% N, 5·76% S.

N-Benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteine *o*-Nitrophenyl Ester

N-Benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteine (2·9 g) and *o*-nitrophenol (2·09 g) were dissolved in pyridine (7·5 ml), the solution cooled to 0°C and dicyclohexylcarbodiimide (1·55 g) in pyridine (5 ml) was added. After standing at 0°C for 30 min and at room temperature for 12 h, the separated dicyclohexylurea was filtered and the pyridine evaporated. The residue was dissolved in chloroform, the solution washed with a saturated solution of citric acid, water, 0·1M-NaOH and again water. After drying, the solvent was driven off and the product crystallized from 95% aqueous 2-propanol, yielding 3·6 g (95%) of the active ester, m.p. 147—149°C, $[\alpha]_D^{20}$ —56·6° (*c* 1, dimethylformamide); R_F 0·82 (S3), 0·89 (S5) (prior to detection with the chlorinating reagent the plates were sprayed with ethanolic ammonia and dried). For $C_{27}H_{28}N_2O_6S$ (508·6) calculated: 63·76% C, 5·55% H, 5·51% N, 6·30% S; found: 63·52% C, 5·63% H, 5·49% N, 6·39% S.

Tert-butyloxycarbonyl-*S*-(2,4,6-trimethylbenzyl)cysteine *o*-Nitrophenyl Ester

o-Nitrophenol (1·4 g) was added to a stirred solution of tert-butyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteine (1·7 g) in pyridine (5 ml), the mixture was cooled to 0°C and a solution of dicyclohexylcarbodiimide (1 g) in pyridine (5 ml) was added. The reaction mixture was kept at 0°C for 30 min and at room temperature for 12 h. The separated dicyclohexylurea was filtered off, the filtrate taken down and the residue dissolved in chloroform. After extraction with a solution of citric acid, water, 0·1M-NaOH and again water, the solution was dried over magnesium sulfate and taken down. Crystallization from 95% ethanol afforded 1·7 g (72%) of the active ester, m.p. 136—137·5°C, $[\alpha]_D^{22}$ —50·7° (*c* 1, dimethylformamide with 1% of acetic acid). For $C_{24}H_{30}N_2O_6S$ (474·6) calculated: 60·74% C, 6·37% H, 5·90% N, 6·76% S; found: 60·91% C, 6·20% H, 6·04% N, 6·70% S.

Benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteinyl-prolyl-leucyl-glycine Amide (*Ia*)

A solution of dicyclohexylcarbodiimide (1·03 g) and hydroxybenzotriazole (0·68 g) in dimethylformamide (5 ml) was added at 0°C to a solution of benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)-cysteine (1·94 g) and prolyl-leucyl-glycine amide[25,26] (1·47 g) in dimethylformamide (10 ml). The mixture was kept for 1 h at 0°C and for 20 h at room temperature. The separated dicyclohexylurea was removed by filtration, the filtrate and washings were taken to dryness and the residue washed with water, saturated sodium hydrogen carbonate solution, water, 1M-HCl and again water. Three crystallizations from aqueous methanol afforded 2·8 g (86%) of product, melting at 197—199°C; $[\alpha]_D^{21}$ —50·0° (*c* 0·5, dimethylformamide); R_F 0·75 (S1), 0·72 (S2), 0·67 (S3), 0·83 (S4). For $C_{34}H_{47}N_5O_6S$ (653·8) calculated: 62·46% C, 7·24% H, 10·71% N, 4·90% S; found: 62·16% C, 7·30% H, 11·01% N, 4·96% S.

o-Nitrobenzenesulfenylasparaginyl-S-(2,4,6-trimethylbenzyl)cysteinyl-prolyl-leucyl-glycine Amide (*IIa*)

The protected tetrapeptide *Ia* (3·27 g) was dissolved in hot acetic acid (10 ml), the solution cooled to room temperature, and 2M-HBr in acetic acid (10 ml) was added. The mixture was set aside for 30 min at room temperature. The tetrapeptide hydrobromide was precipitated by addition of ether, filtered and washed with ether ($E_{5,7}^{His}$ 0·65, $E_{2,4}^{Gly}$ 0·82). The tetrapeptide hydrobromide was dissolved in dimethylformamide (15 ml), the solution adjusted to pH 10 with N-ethylpiperidine (1·1 ml) and a solution of *o*-nitrobenzenesulfenylasparagine 2,4,5-trichlorophenyl ester (2·33 g) in dimethylformamide (5 ml) was added. After 24 h, the active ester (0·47 g) in dimethylformamide (2 ml) was again added. The mixture was set aside for further 24 h at room temperature and the dimethylformamide was evaporated. The residue was triturated with light petroleum and ether, the solid portion filtered and washed on the filter with a saturated solution of sodium hydrogen carbonate, water, a solution of $KHSO_4/K_2SO_4$ (pH 2), water and ether. Crystallization from aqueous methanol afforded 2·56 g (65%) of the product, m.p. 222—224°C, R_F 0·60 (S1), 0·42 (S2), 0·58 (S3), 0·71 (S4), 0·90 (S7). $[\alpha]_D^{20}$ —80·4° (*c* 0·5, dimethylformamide). For $C_{36}H_{50}N_8O_8S_2$ (786·9) calculated: 54·94% C, 6·40% H, 14·24% N, 8·04% S; found: 54·75% C, 6·32% H, 14·00% N, 8·04% S.

o-Nitrobenzenesulfenylglutaminyl-asparaginyl-S-(2,4,6-trimethylbenzyl)cysteinyl-prolyl-leucyl-glycine Amide (*IIIa*)

A solution of the protected pentapeptide *IIa* (2·36 g) in dimethylformamide (10 ml) was mixed with 2·5M-HCl in ether (3 ml). After 5 min the mixture was diluted with ether and the separated hydrochloride collected and dried ($E_{5,7}^{His}$ 0·45, $E_{2,4}^{Gly}$ 0·79). It was dissolved in dimethylformamide (10 ml), the solution adjusted to pH 10 with N-ethylpiperidine (0·75 ml) and a solution of *o*-nitrobenzenesulfenylglutamine 2,4,5-trichlorophenyl ester (1·44 g) in dimethylformamide (5 ml) was added. After stirring for 24 h at room temperature, another portion of the activated ester (0·29 g) in dimethylformamide (1 ml) was added. After 24 h the dimethylformamide was evaporated, the residue triturated with ether and water, filtered and washed on the filter with saturated solution of sodium hydrogen carbonate, water, solution of $KHSO_4/K_2SO_4$ (pH 2), water, ethyl acetate and ether. Crystallization from dimethylformamide–ether gave 1·8 g (66%) of the hexapeptide, m.p. 196—197°C, R_F 0·53 (S1), 0·50 (S2), 0·44 (S3), 0·67 (S4), 0·80 (S7); $[\alpha]_D^{20}$ —52·3° (*c* 0·5, dimethylformamide). Amino acid analysis: Glu 1·04, Asp 0·91, 1/2 Cys 0·93, Leu 1·05, Pro 1·00, Gly 1·04. For $C_{41}H_{58}N_{10}S_2$ (915·1) calculated: 53·81% C, 6·38% H, 15·31% N, 7·01% S; found: 53·66% C, 6·30% H, 15·11% N, 7·07% S.

o-Nitrobenzenesulfenylisoleucyl-glutaminyl-asparaginyl-S-(2,4,6-trimethylbenzyl)cysteinyl-prolyl-leucyl-glycine Amide (*IVa*)

Ethereal 2·5M-HCl (5 ml) was added to a solution of the protected hexapeptide *IIIa* (1·37 g) in dimethylformamide (7 ml) and after 4 min the hydrochloride was precipitated by addition of ether (200 ml) ($E_{5,7}^{His}$ 0·45, $E_{2,4}^{Gly}$ 0·78). The dried hydrochloride was dissolved in dimethylformamide (5 ml), the solution adjusted to pH 10 with N-ethylpiperidine (0·5 ml) and *o*-nitrobenzenesulfenylisoleucine N-hydroxysuccinimide ester (0·57 g) in dimethylformamide (2 ml) was added. After stirring at room temperature for 24 h, the active ester (0·3 g) was added and after 24 h the same amount of the ester was added again. After further 24 h dimethylformamide was evaporated, the residue triturated with light petroleum and ether, washed on the filter with water, solution of $KHSO_4/K_2SO_4$, water, methanol (10 ml) and ether, and crystallized from dimethylformamide–

-ether to give 1 g (65%) of the heptapeptide *IVa*, m.p. 238—240°C, $[\alpha]_D^{20}$ —66·0° (*c* 0·5, dimethylformamide); R_F 0·54 (S1), 0·50 (S2), 0·47 (S3), 0·66 (S4), 0·83 (S7). For $C_{47}H_{69}N_{11}O_{11}S_2$ (1028·3) calculated: 54·90% C, 6·76% H, 14·98% N, 6·23% S; found: 55·04% C, 6·64% H, 14·89% N, 5·99% S.

o-Nitrobenzenesulfenyl-O-tert-butyltyrosyl-isoleucyl-glutaminyl-asparaginyl-S-(2,4,6-trimethylbenzyl)cysteinyl-prolyl-leucyl-glycine Amide (*Va*)

Ethereal 2·5M-HCl (2 ml) was added to a solution of the protected heptapeptide *IVa* (0·83 g) in dimethylformamide (10 ml). After standing for 4 min at room temperature the mixture was diluted with ether ($E_{2,4}^{Gly}$ 0·67, $E_{5,7}^{His}$ 0·40). The separated hydrochloride was collected on filter, dissolved in dimethylformamide (8 ml), the solution adjusted to pH 10 with N-ethylpiperidine, and treated with a solution of *o*-nitrobenzenesulfenyl-O-tert-butyltyrosine N-hydroxysuccinimide ester (0·4 g) in dimethylformamide (2 ml). After standing for 24 h at room temperature, the same portion of the activated ester was added again and the mixture was set aside under the same conditions for 24 h. The solvent was evaporated, the residue triturated with light petroleum and water, washed on the filter with a solution of $KHSO_4/K_2SO_4$ (pH 2), water, methanol (15 ml), ethyl acetate and ether, affording thus 0·53 g (53%) of the octapeptide *Va*, m.p. 221—223°C; $[\alpha]_D$ +3° (*c* 0·5 dimethylformamide); R_F 0·58 (S1), 0·53 (S2), 0·63 (S3), 0·80 (S4), 0·83 (S7). For $C_{60}H_{86}N_{12}O_{13}S_2$ (1247) calculated: 57·77% C, 6·95% H, 13·41% N, 5·07% S; found: 57·73% C, 6·75% H, 13·66% N, 5·12% S.

Benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteinyl-O-tert-butyltyrosyl-isoleucyl-glutaminyl-asparaginyl-S-(2,4,6-trimethylbenzyl)cysteinyl-prolyl-leucyl-glycine Amide (*VIa*)

A solution of the protected octapeptide *Va* (0·25 g) in dimethylformamide (2 ml) was mixed with 5M-HCl in ether (1 ml). After standing for 4 min the mixture was diluted with ether and the precipitated hydrochloride collected on filter and washed with ether ($E_{5,7}^{His}$ 0·30, $E_{2,4}^{Gly}$ 0·60). The obtained hydrochloride was dissolved in dimethylformamide (2 ml), mixed with N-ethylpiperidine (1 ml) and treated with a solution of N-benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteine 2,4,5-trichlorophenyl ester (0·56 g) in dimethylformamide (1 ml). After standing for 24 h the same amount of the active ester was added and the mixture was set aside for further 24 h. Dimethylformamide was evaporated, the residue mixed with water and allowed to stand for 1 h at 0°C. The solid portion was washed with saturated solution of sodium hydrogen carbonate, water, 0·5M-HCl and again water. Crystallization from dimethylformamide and ether afforded 0·27 g (92%) of product, m.p. 239—241°C; $[\alpha]_D^{22}$ —54·0° (*c* 0·5, dimethylformamide); R_F 0·56 (S1), 0·58 (S3), 0·78 (S4), 0·80 (S7). Amino acid analysis: 1/2 Cys 2·05, Tyr 1·01, Ile 0·96, Glu 0·95, Asp 0·97, Pro 0·98, Leu 1·03, Gly 1·00. For $C_{75}H_{106}N_{12}O_{14}S_2$ (1463) calculated: 61·54% C, 7·30% H, 11·48% N, 4·38% S; found: 61·32% C, 7·21% H, 11·28% N, 4·29% S.

Oxytocin (*VIIIa*)

A solution of the protected linear nonapeptide *VIa* (50 mg) and anisole (0·6 ml) in liquid hydrogen fluoride (7 ml) was stirred at 0°C for 30 min. Hydrogen fluoride was then evaporated, the residue dried *in vacuo* for 90 min, suspended in water, the formed suspension extracted to pH 6·75 with 0·1M-NaOH and subjected to air oxidation for 1 h. The mixture was then adjusted to pH 3·8 with 0·1M-HCl and freeze-dried. The residue was dissolved in the lower phase (50 ml) of the system 2-butanol–0·05% aqueous acetic acid, equilibrated with the upper phase of the same system and subjected to contercurrent distribution. After 150 transfers of the upper phase, the content of the

tubes 40—60 (K = 0·45) was pooled, taken down and freeze-dried. The resulting material was dissolved in 1M acetic acid and filtered through a column of Biogel P-4, affording 13·2 mg (39%) of the product, $[\alpha]_D^{20}$ —27·8° (*c* 0·2, 1M acetic acid) (reported $[\alpha]_D$ —23·1° (*c* 0·5, 1M acetic acid)[29] and —25·3° (*c* 0·4, 1M acetic acid)[30]). $E_{5,7}^{His}$ 0·41, $E_{2,4}^{Gly}$ 0·58; R_F 0·34 (S1), 0·72 (S2), 0·81 (S3), Amino acid analysis: 1/2 Cys 1·99, Tyr 0·95, Ile 0·99, Glu 1·00, Asp 1·00, Pro 1·00, Leu 1·05, Gly 1·01. For $C_{43}H_{66}N_{12}O_{12}S_2 \cdot 2\,C_2H_4O_2 \cdot 2\,H_2O$ (1163) calculated: 48·53% C, 6·76% H, 14·45% N; found: 48·37% C, 6·82% H, 14·18% N. Uterotonic activity[31,32], determined on an isolated strip of rat uterus in Mg^{2+} — free solution, was 535 I.U./mg, the galactogogic activity[33,34] *in vivo* was 527 I.U./mg.

Benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteinyl-prolyl-N^G-*p*-toluenesulfonylarginyl-glycine Amide (*Ib*)

Benzyloxycarbonylprolyl-N^G-*p*-toluenesulfonylarginyl-glycine amide[27] (4·88 g) was dissolved in acetic acid (15 ml) and decarbobenzoxylated (15 ml of 4M-HBr in acetic acid, 7 min, 60°C). The tripeptide hydrobromide was precipitated by addition of ether, dissolved in dimethylformamide (15 ml), the solution adjusted to pH 10 with N-ethylpiperidine (1 ml) and treated with benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteine *o*-nitrophenyl ester (3.57 g) in dimethylformamide (10 ml). After stirring of the mixture for 24 h at room temperature, the solvent was evaporated, the residue washed with water, saturated solution of sodium hydrogen carbonate, water, 0·5M-HCl and again water. Crystallization from aqueous methanol afforded 4·3 g (72%) of the tetrapeptide amide *Ib*, m.p. 118·5—120°C; $[\alpha]_D^{22}$ —32·8° (*c* 1, dimethylformamide); R_F 0·75 (S1, S3), 0·59 (S2), 0·76 (S4), 0·86 (S7). For $C_{41}H_{54}N_8O_8S_2$ (851·1) calculated: 57·86% C, 6·40% H, 13·17% N, 7·54% S; found: 57·65% C, 6·49% H, 13·14% N, 7·57% S.

o-Nitrobenzenesulfenylasparaginyl-S-(2,4,6-trimethylbenzyl)cysteinyl-prolyl-N^G-*p*-toluenesulfonylarginyl-glycine Amide (*IIb*)

A solution of the protected tetrapeptide *Ib* (4·2 g) in acetic acid (15 ml) was treated with 2M-HBr in acetic acid, kept at room temperature for 30 min, diluted with ether and the precipitated hydrobromide collected with suction. A solution of this tetrapeptide hydrobromide in dimethylformamide (50 ml) was adjusted to pH 10 with N-ethylpiperidine (1 ml) and *o*-nitrobenzenesulfenylasparagine 2,4,5-trichlorophenyl ester (2·33 g) was added. After stirring for 24 h at room temperature, the mixture was taken down, the residue triturated with water, and on the filter washed with a saturated solution of sodium hydrogen carbonate, water, solution of $KHSO_4/K_2SO_4$ (pH 2), water and methanol (10 ml), and crystallized from dimethylformamide–ether to give 3·1 g (64%) of the product, m.p. 169—171°C, $[\alpha]_D^{21}$ —60·1° (*c* 1, dimethylformamide); R_F 0·60 (S1), 0·65 (S3), 0·74 (S4). For $C_{34}H_{57}N_{11}O_{10}S_3$ (984·2) calculated: 52·47% C, 5·83% H, 15·65% N, 9·77% S; found: 52·72% C, 5·91% H, 15·48% N, 9·64% S.

o-Nitrobenzenesulfenylglutaminyl-asparaginyl-S-(2,4,6-trimethylbenzyl)cysteinyl-prolyl-N^G-*p*-toluenesulfonylarginyl-glycine Amide (*IIIb*)

A solution of the protected pentapeptide *IIb* (1·97 g) in dimethylformamide (15 ml) was treated with 3·3M-HCl in ether and kept for 4 min at room temperature. After dilution with ether, the precipitated hydrochloride was collected on filter and washed with ether ($E_{2,4}^{Gly}$ 0·72, $E_{5,7}^{His}$ 0·50). A solution of this hydrochloride in dimethylformamide (7 ml) (pH adjusted to 10 with 0·6 ml of N-ethylpiperidine) was mixed with a solution of *o*-nitrobenzenesulfenylglutamine 2,4,5-trichlorophenyl ester (0·96 g) in dimethylformamide (3 ml) and kept at room temperature for 24 h. The

mixture was again treated with the active ester (0·5 g) and set aside for additional 24 h. After evaporation of the solvent, the residue was triturated with light petroleum and ether, the solid collected and washed on the filter with water, saturated solution of sodium hydrogen carbonate, water and a solution of $KHSO_4/K_2SO_4$ and again water. Crystallization from dimethylformamide and ether gave 1·6 g (72%) of the protected hexapeptide *IIIb*, m.p. 154—156°C; $[\alpha]_D^{20}$ —48·7° (*c* 0·2, dimethylformamide); R_F 0·30 (S1), 0·60 (S4), 0·73 (S7). Amino acid analysis: Glu 0·95, Asp 0·98, 1/2 Cys 0·98, Pro 1·00, Arg 0·99, Gly 1·10. For $C_{48}H_{65}N_{13}O_{12}S_3$ (1112) calculated: 51·83% C, 5·89% H, 16·37% N, 8·64% S; found: 51·97% C, 5·97% H, 16·27% N, 8·44% S.

o-Nitrobenzenesulfenylphenylalanyl-glutaminyl-asparaginyl-S-(2,4,6-trimethylbenzyl)cysteinyl--prolyl-N^G-*p*-toluenesulfonyl-arginyl-glycine Amide (*IVb*)

A solution of the protected hexapeptide *IIIb* (1·22 g) in dimethylformamide (12 ml) was treated with 3·3M-HCl in ether (1 ml), kept at room temperature for 4 min and diluted with ether. The precipitated hydrochloride was collected and washed with ether ($E_{2.4}^{Gly}$ 0·64, $E_{5.7}^{His}$ 0·40). A solution of this hexapeptide hydrochloride in dimethylformamide (10 ml) was adjusted to pH 10 with N-ethylpiperidine (0·3 ml), treated with *o*-nitrobenzenesulfenylphenylalanine 2,4,5-trichlorophenyl ester (0·55 g) and the mixture was stirred at room temperature for 24 h. Another portion of the active ester (0·25 g) was added and the stirring continued for 24 h. The mixture was taken down, the residue triturated with ether, collected on filter and washed successively with water, a solution of $KHSO_4/K_2SO_4$ (pH 2), water, methanol and ether. Crystallisation from dimethylformamide--ether afforded 1·06 g (76%) of the heptapeptide *IVb*, m.p. 169—171°C, $[\alpha]_D^{20}$ —28·4° (*c* 0·2, dimethylformamide); R_F 0·30 (S1), 0·64 (S5), 0·78 (S7). For $C_{57}H_{74}N_{14}O_{13}S_3$ (1259) calculated: 54·35% C, 5·92% H, 15·56% N, 7·64% S; found: 54·18% C, 6·07% H, 15·42% N, 7·86% S.

o-Nitrobenzenesulfenyl-O-tert-butyltyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-(2,4,6-trimethylbenzyl)cysteinyl-prolyl-N^G-*p*-toluenesulfonylarginyl-glycine Amide (*Vb*)

A solution of the protected heptapeptide *IVb* (0·95 g) in dimethylformamide (10 ml) was treated with ethereal 3·3M-HCl (1 ml) and kept for 5 min at room temperature. The hydrochloride was precipitated by addition of ether ($E_{5.7}^{His}$ 0·32, $E_{2.4}^{Gly}$ 0·57). A solution of the heptapeptide hydrochloride was adjusted to pH 10 with N-ethylpiperidine (0·2 ml), combined with a solution of *o*-nitrobenzenesulfenyl-O-tert-butyltyrosine N-hydroxysuccinimide ester (0·37 g) and stirred for 24 h at room temperature. Another portion of the active ester (0·2 g) was added, and the mixture was stirred for 24 h at the same temperature. After evaporation of the solvent, the residue was triturated with light petroleum, collected on filter, washed with water, methanol and ether and crystallized twice from dimethylformamide–ether, yielding 0·75 g (67%) of the octapeptide *Vb*, m.p. 184—186°C; $[\alpha]_D$ +4·0° (*c* 0·5, dimethylformamide); R_F 0·48 (S1), 0·43 (S2), 0·69 (S4), 0·78 (S7). For $C_{70}H_{91}N_{15}O_{15}S_3$ (1478) calculated: 56·86% C, 6·20% H, 14·21% N, 6·50% S; found: 56·74% C, 6·02% H, 14·06% N, 6·58% S.

Benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteinyl-O-tert-butyltyrosyl-phenylalanyl--glutaminyl-asparaginyl-S-(2,4,6-trimethylbenzyl)cysteinyl-prolyl-N^G-*p*-toluenesulfonylarginyl--glycine Amide (*VIb*)

A solution of the octapeptide *Vb* (0·45 g) in dimethylformamide (5 ml) was treated with 2·2M-HCl in ether (1 ml), kept at room temperature for 4 min and diluted with ether. The precipitated hydrochloride was dried, dissolved in dimethylformamide (5 ml) and treated with N-ethylpiperidine (0·2 ml). A solution of N-benzyloxycarbonyl-S-(2,4,6-trimethylbenzyl)cysteine *o*-nitro-

phenyl ester (0·16 g) in dimethylformamide (2 ml) was added and the mixture was stirred at room temperature for 24 h. Another portion of the active ester (0·1 g) was added and stirring was continued for 24 h. After evaporation of dimethylformamide, the mixture was triturated with ethyl acetate, washed with a saturated sodium hydrogen carbonate solution, water, 0·5M-HCl, water and ether, affording 0·32 g (59%) of the protected nonapeptide *VIb*, m.p. 195—197°C, $[\alpha]_D$ —40·3° (*c* 0·5, dimethylformamide); R_F 0·59 (S1), 0·61 (S3), 0·87 (S7). Amino acid analysis: 1/2Cys 2·05, Tyr 0·86, Phe 0·96, Glu 1·00, Asp 1·03, Pro 1·10, Arg 1·08, Gly 1·08. For $C_{85}H_{111} \cdot N_{15}O_{16}S_3$ (1665) calculated: 61·31% C, 6·71% H, 12·61% N, 5·78% S; found: 61·16% C, 7·01% H, 12·65% N, 5·99% S.

Arginine-vasopressin (*VIIIb*)

a) Hydrogen fluoride (10 ml) was distilled into a mixture of the nonapeptide *VIb* (50 mg) and anisole (0·6 ml) and the mixture was stirred at 0°C for 30 min. After evaporation of hydrogen fluoride, the residue was dried for 2·5 h *in vacuo* and dissolved under stirring in water (20 ml). The turbid solution was extracted with ether, passed through a column of Amberlite IR-4B (13 × 1 cm, acetate cycle), adjusted to pH 6·7 with N-ethylmorpholine, stirred for 1 h, adjusted to pH 3·7 with acetic acid and freeze-dried. The residue was dissolved in 1M acetic acid (2 ml) and applied on a column of Biogel P-4 in 1M acetic acid. Fractions, containing the pure vasopressin, were pooled and freeze-dried, affording 15 mg (40%) of the product, $[\alpha]_D^{20}$ —21·9° (*c* 0·2, 1M acetic acid). (Reported values are $[\alpha]_D$ —23° (*c* 0·23, acetic acid)[12], —22° (*c* 0·22, 1M acetic acid)[35] and —26° (*c* 0·5, 1M acetic acid)[36]. $E_{2,4}^{His}$ 0·63, $E_{5,7}^{His}$ 0·62; R_F 0·03 (S1), 0·63 (S4), 0·20 (S7). Amino acid analysis: 1/2Cys 1·95, Tyr 0·93, Phe 1·03, Glu 0·98, Asp 1·00, Pro 1·06, Arg 1·06, Gly 1·05. For $C_{46}H_{65}N_{15}O_{12}S_2 \cdot 2\,C_2H_4O_2 \cdot 2\,H_2O$ (1240) calculated: 48·42% C, 6·26% H, 16·95% N; found: 48·18% C, 6·33% H, 16·87% N. Pressoric activity, determined[37] on a despinalized rat, was 480 I.U./mg.

b) Trifluoromethanesulfonic acid (0·4 ml) was added at 0°C to a solution of the compound *VIb* (50 mg) and anisole (40 µl) in trifluoroacetic acid (0·6 ml). After standing for 30 min at 0°C, the mixture was diluted with ether, the precipitate collected by centrifugation, triturated with ether and dissolved in water (150 ml). The solution was adjusted to pH 6·8 and oxidized with air oxygen for 1 h. After adjusting the pH to 4·4, the solution was applied on a column of Amberlite CG-50 (H^+; 25 ml). After washing with 0·2% acetic acid, the peptidic material was eluted with 50% acetic acid, diluted with water and freeze-dried. The residue was dissolved in 1M acetic acid and purified by gel filtration on Bio-Gel P-4, affording 15 mg (40%) of the product which was further purified by free-flow electrophoresis and was electrophoretically as well as chromatographically identical with that prepared by action of hydrogen fluoride; $[\alpha]_D$ —22·6° (*c* 0·2, 1M acetic acid). Pressoric activity 428 I.U./mg.

S-(β-2,4,6-Trimethylbenzyl)mercaptopropionic Acid

3-Mercaptopropionic acid (5·3 g) was alkylated in liquid ammonia (250 ml) with 2,4,6-trimethylbenzyl chloride (10·6 g). After evaporation of ammonia, the residue was dissolved in water, the solution extracted with ether and the aqueous layer acidified with conc. hydrochloric acid (to pH 4). The mixture was extracted with ethyl acetate, the organic layer taken down, yielding 7·75 g (65%) of an oil, R_F 0·82 (S3), 0·20 (S6), 0·68 (S13), 0·17 (S14). Dicyclohexylammonium salt: m.p. 149—151°C; for $C_{25}H_{41}NO_2S$ (419·7) calculated: 71·55% C, 9·84% H, 3·34% N; found: 71·38% C, 9·96% H, 3·26% N.

p-Nitrophenyl S-(β-2,4,6-Trimethylbenzyl)mercaptopropionate

Dicyclohexylcarbodiimide (1·28 g) was added at 0°C to a solution of S-(β-2,4,6-trimethylbenzyl)-mercaptopropionic acid (1·1 g) and *p*-nitrophenol (0·86 g) in ethyl acetate (30 ml), the mixture was stirred at 0°C for 1 h and then set aside for 3 days at the same temperature. The separated dicyclohexylurea was removed by filtration, the solution taken down and the residue crystallized from methanol, affording 1·4 g (84%) of the ester, m.p. 114—116°C; R_F 0·85 (S6), 0·65 (S1). For $C_{19}H_{21}NO_4S$ (359·4) calculated: 63·49% C, 5·89% H, 3·90% N; found: 63·28% C, 5·78% H, 3·72% N.

S-(β-2,4,6-Trimethylbenzyl)mercaptopropionyl-O-tert-butyltyrosyl-phenylalanyl-glutaminyl--asparaginyl-S-(2,4,6-trimethylbenzyl)cysteinyl-prolyl-N^G-*p*-toluenesulfonylarginyl-glycine Amide (*VII*)

The protected octapeptide *Vb* (149 mg) was converted into its hydrochloride in the same way as described for the preparation of the nonapeptide *VIb*. A solution of the hydrochloride in dimethylformamide (2 ml) was adjusted to pH 10 with N-ethylpiperidine and treated with *p*-nitrophenyl S-(2,4,6-trimethylbenzyl)mercaptopropionate (36 mg). After stirring for 24 h, another portion of the active ester (18 mg) was added. After further 24 h the mixture was taken down, the residue triturated with 0·5M-HCl, washed on the filter with water, saturated solution of sodium hydrogen carbonate, again water, and ether. Crystallization from aqueous dimethylformamide afforded 140 mg (91%) of the product, m.p. 210—212°C, $[\alpha]_D$ —21·4° (*c* 0·5, dimethylformamide); R_F 0·42 (S2), 0·62 (S3), 0·75 (S4), 0·82 (S7). For $C_{77}H_{104}N_{14}O_{14}S_3$ (1 546) calculated: 59·82% C, 6·78% H, 12·68% N; found: 59·80% C, 6·88% H, 12·66% N. Amino acid analysis: Tyr 1·03, Phe 1·03, Glu 0·99, Asp 1·00, Pro 0·99, Arg 0·99, Gly 0·98.

Deamino-arginine-vasopressin (*VIIIc*)

Hydrogen fluoride (7 ml) was distilled into a mixture of the protected peptide *VII* (50 mg) and anisole (0·6 ml) and the mixture was stirred for 30 min at 0°C. After evaporation of hydrogen fluoride, the residue was dried *in vacuo* (150 Pa) for 90 min and dissolved in water (20 ml). The suspension was extracted with ether, the clear solution made up to 150 ml, adjusted to pH 6·75 with 0·1M-NaOH and oxidized with a 0·01M potassium ferricyanide solution for 1 h. The reaction mixture was adjusted to pH 4·2 with 0·1M-HCl and applied on a column of Amberlite CG-50. After washing the column with 0·25% acetic acid, the crude analogue was eluted with 50% acetic acid. The effluents were freeze-dried and the residue purified by free-flow electrophoresis (U = = 2 700 V, I = 99 mA). Fractions containing the pure compound were freeze-dried and afforded 14·6 mg (37%) of the product, $[\alpha]_D$ —91·0° (*c* 0·2, 0·1M acetic acid). The reported values are —103° (*c* 1·3, 0·1M acetic acid)[38], —84·8° (*c* 0·5, 1M acetic acid)[39] and —98·8° (*c* 0·5, 1M acetic acid)[40]. $E^{Gly}_{2,4}$ 0·65, $E^{His}_{5,7}$ 0·33; R_F 0·07 (S1), 0·49 (S4). Amino acid analysis: Tyr 1·03, Phe 1·03, Glu 0·99, Asp 1·00, Pro 0·99, Arg 0·99, Gly 0·98. For $C_{46}H_{64}N_{14}O_{12}S_2 \cdot 2\,CH_3COOH \cdot 2\,H_2O$ (1 225) calculated: 49·01% C, 6·25% H, 16·00% N; found: 48·85% C, 6·32% H, 15·86% N. Pressoric activity 365 I.U./mg.

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