SYNTHESIS OF [4-GLUTAMIC ACID]DEAMINO-1-CARBA-OXYTOCIN*

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[4-Glutamic acid]deamino-1-carba-oxytocin (*Ic*) was prepared by the stepwise method of peptide synthesis in solution, with the use of the *o*-nitrobenzenesulfenyl protective group and active esters in the formation of peptide bonds. Two alternatives were examined, with an unprotected carboxylic function and with protection by means of the methyl ester. The cyclisation was carried out in the stage of a linear octapeptide by means of the active esters method again. By reaction with ammonia, the analogue *Ic* afforded deamino-1-carba-oxytocin.

The majority of the hitherto prepared analogues** of neurohypophyseal hormones oxytocin (*Ia*) and vasopressin, modified in the sequence position 4, retains the high degree of biological activities[2]. This also holds in the case of the deamino-oxytocin analogue in which the glutamine residue in sequence position 4 is replaced by glutamic acid[3]. A similar analogue but derived from oxytocin (*i.e.*, possessing the α-amino group) is almost inactive[4]. [4-Glutamic acid]deamino-oxytocin was also attached[3] by means of the free carboxylic function to AH-Sepharose-4B in the hope that the resulting complex could be useful in isolation of tissue fractions containing oxytocin bond sites ("receptors" in the biological tissue). With a similar intention, the analogue *Ic* has been now prepared since a higher metabolical stability (*i.e.*, resistance to tissue homogenates) as well as a higher chemical stability (in introduction and interaction with reactive groups on the carboxylic function of the amino acid in sequence position 4) could be expected owing to the replacement of the —S—S— bond by the $—CH_2—S—$ grouping.

The general approach to the synthesis of the analogue *Ic* was similar to the scheme used[5] in the preparation of compound *Ib*, *i.e.* the stepwise synthesis of the peptide chain in solution by means of active esters and cyclisation of the linear octapeptide by the active esters method again. The temporary protection of α-amino groups was mainly performed with the use of the *o*-nitrobenzenesulfenyl residue, the hydroxylic

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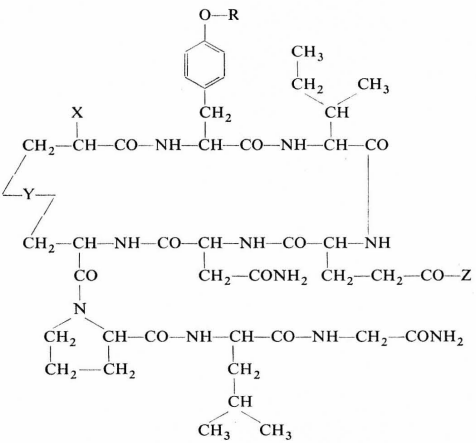
** The amino acids appearing in this paper are of the L-series. The nomenclature and symbols obey the published recommendations[1]. The abbreviation Picr designates the residue of picric acid.

function of tyrosine was protected by the tert-butyl group, and the γ-carboxyl of glutamic acid was blocked in the form of a tert-butyl ester. Final products were purified by countercurrent distribution and gel filtration.

The *o*-nitrobenzenesulfenylglutamic acid γ-tert-butyl ester[6] was liberated from its dicyclohexylammonium salt and converted in the presence of N,N′-dicyclohexylcarbodiimide to the N-hydroxysuccinimide ester. This active ester was used in



Ia, X = NH_2, Y = S—S, Z = NH_2, R = H
Ib, X = H, Y = CH_2—S, Z = NH_2, R = H
Ic, X = H, Y = CH_2—S, Z = OH, R = H
Id, X = H, Y = CH_2—S, Z = OBu^t, R = Bu^t
Ie, X = H, Y = CH_2—S, Z = OH, R = Bu^t

Nps-Asn-Cys($C_3H_6CO_2R$)-Pro-Leu-Gly-NH_2

IIa, R = CH_3, *IIb*, R = H

Nps-Glu(OBu^t)-Asn-Cys($C_3H_6CO_2R$)-Pro-Leu-Gly-NH_2

IIIa, R = CH_3, *IIIb*, R = H

X-Ile-Glu(OBu^t)-Asn-Cys($C_3H_6CO_2R$)-Pro-Leu-Gly-NH_2

IVa, X = Nps, R = CH_3; *IVb*, X = Nps, R = H
IVc, X = H, R = CH_3; *IVd*, X = H, R = H

X-Tyr(Bu^t)-Ile-Glu(OBu^t)-Asn-Cys($C_3H_6CO_2H$)-Pro-Leu-Gly-NH_2

Va, X = Nps; *Vb*, X = Boc

acylation of the pentapeptide amide with the formation of compound *IIIa*; the pentapeptide amide was obtained by removal of the *o*-nitrobenzenesulfenyl protecting group from compound *IIa* by the action of ethereal hydrogen chloride. The lengthening of the peptide chain with an isoleucine residue with the formation of compound *IVa* was performed analogously. After removal of the N-protecting group with ethereal hydrogen chloride, the resulting peptide *IVc* was subjected to alkaline hydrolysis to the peptide *IVd* under optimum conditions. The lowest amount of by-product was formed in water in the presence of sodium hydroxide (the reaction is of first order with the rate constant $2{\cdot}5 \cdot 10^{-3}\ s^{-1}$ at 21°C). In the presence of organic solvents (methanol, dimethylformamide, dioxane), the rate of hydrolysis is rapidly decreased and the tert-butyl ester group begins to undergo fission. An addition of tert-butyl alcohol also decreases the rate of hydrolysis but on the other hand, the extent of the fission of the tert-butyl ester is considerably decreased. This fission also occurs on a sulfonate cation exchange resin; it is therefore necessary to use a short contact time and to work at a low temperature.

In the parallel route, the carboxylic function was unprotected. Both protecting groups of benzyloxycarbonyl-S-(γ-methoxycarbonylpropyl)cysteinyl-prolyl-leucyl--glycine amide were removed (by the action of hydrogen bromide in acetic acid and by alkaline hydrolysis) and the resulting free tetrapeptide amide was acylated with *o*-nitrobenzenesulfenylasparagine 2,4,5-trichlorophenyl ester to afford a mixture of substances which was purified by chromatography on a column of silica gel; in this manner, the pure peptide *IIb* was obtained in 46% yield. The protecting group was then removed and the residue of γ-tert-butyl *o*-nitrobenzenesulfenylglutamate was attached to the peptide sequence. The yield of the hexapeptide *IIIb* was 80%. The isoleucine residue was attached analogously to the peptide chain with the formation of compound *IVb*. Removal of the protecting group and application of the resulting hydrochloride to Amberlite IR-4B (acetate cycle) ion exchange resin afforded the free peptide *IVd*, identical in every respect with the heptapeptide prepared by the former route. The present route is however more laborious and the yields are lower.

With the use of the N-hydroxysuccinimide ester of an appropriately protected tyrosine, the free heptapeptide *IVd* was converted to octapeptides *Va* and *Vb*. In one case, the cyclisation was effected in the usual manner[5] by means of the *p*-nitrophenyl ester after a partial removal of the *o*-nitrobenzenesulfenyl protecting group and the resulting partially protected peptide was subjected to countercurrent distribution and gel filtration. The thus-obtained compound *Id* was converted by the action of trifluoroacetic acid to the analogue *Ic* which was purified by countercurrent distribution and gel filtration. The required product *Ic* was observed to be accompanied by compound *Ie*. A 60-min action of trifluoroacetic acid is therefore insufficient for a quantitative removal of protecting groups. The by-product *Ie* may be converted into the required analogue *Ic* by a 10 min treatment with hydrogen bromide in acetic acid at room temperature. In another case, all the protecting groups of the octapeptide

Vb were simultaneously removed; the cyclisation was carried out analogously to case one to obtain the same product; an incomplete removal of the tert-butyl group from tyrosine by the action of trifluoroacetic acid has not been observed. Contrary to the cyclic peptide *Id*, the protecting group of the lineary peptide is thus not hindered.

A detailed pharmacological analysis of the analogue *Ic* will be published separately[7]. In a preliminary uterotonic assay *in vitro*[8], the value of 1 I.U./mg was found. It is the first case when a monocarba analogue of deamino-oxytocin exhibits a lower activity than the parent disulfide. It is not excluded that this unusually decreased activity is due to an interaction of the free carboxylic function with the amino-terminal portion of the molecule.

The identity of the synthesised analogue *Ic* was established by its reaction with ammonia affording the known[5] deamino-1-carba-oxytocin *Ib* of a high biological activity. This reaction was effected in the presence of a 20 molar excess of both N,N'-dicyclohexylcarbodiimide and N-hydroxybenzotriazole. In the presence of N,N'-dicyclohexylcarbodiimide alone or 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline alone, the reaction failed. The analogue *Ib* can also be obtained by reaction of the product of the first cyclisation *Ie* with ammonia and the subsequent removal of the tert-butyl protecting group by the action of hydrogen bromide in acetic acid.

EXPERIMENTAL

Samples for elemental analysis were dried at 1 Torr and room temperature for 24 h. The stated chromatographical (R_F) and electrophoretical (E) values were determined with the use of pure substances. Spots were detected by means of the ninhydrin reaction or by chlorination (in the case of ninhydrin-negative substances). Thin-layer chromatography was performed on ready-for-use Silufol (Kavalier Glassworks, Votice, Czechoslovakia) silica gel sheets in the following solvent systems: S_1, 2-butanol–98% formic acid–water (75 : 13·5 : 11·5); S_2, 2-butanol–25% aqueous ammonia–water (85 : 7·5 : 7·5); S_3, 1-butanol–acetic acid–water (4 : 1 : 1); and S_4, pyridine–1-butanol–acetic acid–water (10 : 15 : 3 : 6). Electrophoresis was performed in a moist chamber on paper Whatman 3 MM with the use of 1M-CH_3COOH (pH 2·4) and pyridine-acetic acid (pH 5·7) as buffer solutions at 20 V/cm for 60 min in most cases. Amino-acid analyses were performed after hydrolysis of samples (20 h in 6M-HCl at 105°C/1 Torr) in an Automatic Amino-Acid Analyser Type 6020 (Developmental Workshops, Czechoslovak Academy of Sciences, Prague). Reaction mixtures were taken down under diminished pressure on a rotatory evaporator at the bath temperature of 30–40°C. The dimethylformamide-containing mixtures were evaporated with the use of an oil pump. Melting points (uncorrected) were taken on a heated microscope stage (Kofler block). Countercurrent distributions were performed in an all-glass apparatus making possible the shift of both upper and lower phases (Countercurrent Distribution Machine Quickfit & Quartz, Stone, Staffordshire, England). Gel filtration was carried out on a column of Bio-Gel P-4 (Bio-Rad Laboratories, Richmond, California, USA).

o-Nitrobenzenesulfenylglutamic Acid α-N-Hydroxysuccinimide γ-Tert-Butyl Ester

The γ-tert-butyl ester of *o*-nitrobenzenesulfenylglutamic acid (5·1 g) was liberated from the dicyclohexylammonium salt[6] on a column of Dowex 50 ion exchange resin in aqueous methanol.

Usual processing yielded a substance, m.p. 130—132°C. For $C_{15}H_{20}N_2O_6S$ (356·4) calculated: 50·55% C, 5·65% H, 7·86% N; found: 50·39% C, 5·84% H, 8·00% N. The free γ-tert-butyl ester was dissolved in dichloromethane (150 ml) and then N-hydroxysuccinimide (1·8 g) was added. The mixture was cooled down to −10°C, treated with N,N′-dicyclohexylcarbodiimide (3·25 g), kept at −10°C for 1 h and in a refrigerator for 11 h, and finally stirred at room temperature for 5 h. The precipitate of N,N′-dicyclohexylurea was filtered off and washed with dichloromethane. The filtrate and washings were evaporated and the residue was dissolved in ethyl acetate. The organic solution was washed with water, 0·5M-$NaHCO_3$, and water again, dried over anhydrous sodium sulfate, and evaporated. The residual oil solidified when triturated with warm ethanol (20 ml). The crystals were collected with suction, washed with light petroleum, and dried. Yield, 6·02 g (93%); m.p. 118—121°C (does not change on crystallisation from ethanol and light petroleum). R_F values: 0·75 (S_1), 0·83 (S_2), 0·46 (98 : 2 benzene–ethanol). Optical rotation: $[\alpha]_D$ −50·1° (*c* 0·3, dimethylformamide). For $C_{19}H_{23}N_3O_8S$ (453·1) calculated: 50·33% C, 5·11% H, 9·27% N; found: 50·59% C, 5·32% H, 9·27% N.

o-Nitrobenzenesulfenyl-γ-tert-butyl Glutamoyl-asparaginyl-S-(γ-methoxycarbonylpropyl)-cysteinyl-prolyl-leucyl-glycine Amide (*IIIa*)

To a solution of the protected pentapeptide[5] *IIa* (4·3 g) in methanol (250 ml), 2M ethereal hydrogen chloride (6·5 ml) was added and the mixture was concentrated to a small volume. The concentrate was precipitated by the addition of ether and the solid substance was reprecipitated from methanol with ether. The precipitate was repeatedly triturated with ether, filtered, and dried under diminished pressure. The dry material was dissolved in dimethylformamide (56 ml), the solution adjusted to pH 8—9 with N-ethylpiperidine, and then treated with the α-N-hydroxysuccinimide ester of *o*-nitrobenzenesulfenyl-γ-tert-butylglutamic acid (3·15 g). The mixture was kept at room temperature for 76 h, evaporated, and the residue triturated with light petroleum and ether in order to solidify. The solid was collected with suction and washed with water, 0·5M-$NaHCO_3$, water again, and ether. Yield, 5·0 g (93%) of compound *IIIa*, m.p. 168—173°C (this value did not change on crystallisation from a mixture of dimethylformamide, ethyl acetate, and ether); $[\alpha]_D$ −64·6° (*c* 0·2, dimethylformamide). R_F values: 0·54 (S_1), 0·40 (S_2), 0·54 (S_3), 0·68 (S_4); $E_{2.4}^{Gly}$ 0·88, $E_{5.7}^{His}$ 0·52 (after removal of the *o*-nitrobenzenesulfenyl protecting group with methanolic hydrogen chloride). For $C_{40}H_{61}N_9O_{13}S_2$ (940·1) calculated: 51·11% C, 6·54% H, 13·41% N; found: 51·08% C, 6·50% H, 13·26% N.

o-Nitrobenzenesulfenyl-isoleucyl-γ-tert-butyl Glutamoyl-asparaginyl-S-(γ-methoxycarbonyl-propyl)cysteinyl-prolyl-leucyl-glycine Amide (*IVa*)

A solution of the protected hexapeptide *IIIa* (5·0 g) in dimethylformamide (35 ml) was treated with 2M ethereal hydrogen chloride (12·2 ml), the mixture kept at room temperature for 4 min, and precipitated with ether (500 ml). The precipitate of the hexapeptide hydrochloride was washed with ether, dissolved in dimethylformamide (90 ml), and the solution precipitated with ether again. $E_{5.7}^{His}$ 0·50, $E_{2.4}^{Gly}$ 0·89. The solid dissolved in dimethylformamide (90 ml), the solution adjusted to pH 8 by the addition of N-ethylpiperidine (1·1 ml), and treated with the N-hydroxysuccinimide ester of *o*-nitrobenzenesulfenylisoleucine (3·1 g). After 65 h at room temperature, the solution was still ninhydrin-positive. Additional N-ethylpiperidine (0·8 ml) was introduced and after 90 h, another portion (1 g) of the active ester was added. The overall reaction time was 200 h. The solution was then evaporated and the residual oil triturated with ether in order to solidify. The crystals were collected with suction and washed with ether, ethyl acetate, water, and ether again. Yield of crude compound *IVa*, 5·1 g; m.p. 207—213°C. Crystallisation from

dimethylformamide and ethyl acetate yielded 4·7 g (84%) of compound *IVa*, m.p. 216–220°C. R_F values: 0·41 (S_1), 0·44 (S_2), 0·52 (S_3), 0·81 (S_4). The electrophoresis was performed after removal of the amino-protecting group with methanolic hydrogen chloride: $E_{5.7}^{His}$ 0·40, $E_{2.4}^{Glv}$ 0·68. The analytical sample of compound *IVa* was obtained by crystallisation from dimethylformamide and ethyl acetate; m.p. 219–221°C; $[\alpha]_D$ −67·4° (*c* 0·2, dimethylformamide). For $C_{46}H_{72}N_{10}O_{14}S_2.H_2O$ (1071) calculated: 51·57% C, 6·96% H, 13·07% N; found: 51·71% C, 6·67% H, 12·90% N. Amino acid analysis: Asp 1·03, Glu 1·00, Pro 1·00, Gly 1·03, Ile 1·01, Leu 1·02, Cys(C_3H_6COOH) 0·94.

S-(γ-Carboxypropyl)cysteinyl-prolyl-leucyl-glycine Amide

To a solution of benzyloxycarbonyl-S-(γ-methoxycarbonylpropyl)cysteinyl-prolyl-leucyl-glycine amide (3·4 g) in acetic acid (17 ml), 35% hydrogen bromide in acetic acid (34 ml) was added. The mixture was kept at room temperature for 10 min and precipitated with ether. The hydrobromide was collected with suction, washed with ether, dried, dissolved in methanol (50 ml), the solution treated with 1M-NaOH (22 ml), and the whole kept at room temperature for 1 h. The methanol was evaporated and the residue applied to Dowex 50 (H^+) ion exchange resin (180 ml). The column was washed with water until the washings were bromide-negative and then eluted with 10% aqueous pyridine. The eluates were evaporated and the residue was precipitated from methanol with ether. Yield, 1·7 g (65%) of the title amide, m.p. 97–102°C; $[\alpha]_D$ −35·9° (*c* 0·4, dimethylformamide). R_F values: 0·15 (S_1), 0·00 (S_2), 0·11 (S_3), 0·43 (S_4). $E_{5.7}^{His}$ 0·20, $E_{2.4}^{Gly}$ 0·83. For $C_{20}H_{35}N_5O_6S$ (473·6) calculated: 50·72% C, 7·45% H, 14·49% N; found: 50·64% C, 7·61% H, 14·51% N.

o-Nitrobenzenesulfenyl-asparaginyl-S-(γ-carboxypropyl)cysteinyl-prolyl-leucyl-glycine Amide (*IIb*)

The free tetrapeptide amide (1·65 g) was dissolved in dimethylformamide (16 ml), the solution made alkaline with N-ethylpiperidine (0·5 ml), and treated with *o*-nitrobenzenesulfenylasparagine 2,4,5-trichlorophenyl ester (1·6 g). The mixture was kept at room temperature for 24 h and treated with another portion of the active ester (0·8 g) and N-ethylpiperidine (0·2 ml). After 90 h, a further portion of the active ester (1 g) was added. The whole was kept additional 45 h, evaporated, the residue triturated with ether, the solid collected with suction, and washed with ether and ethyl acetate. Crystallisation from methanol and ethyl acetate yielded 2 g of a substance, m.p. 149–166°C. As indicated by chromatography, several by-products were present. A portion of the mixture (1·5 g) was therefore chromatographed on a column of silica gel (400 ml) in 4 : 1 benzene–methanol to afford 0·9 g of the chromatographically homogeneous compound *IIb*; R_F values: 0·45 (S_1), 0·09 (S_2), 0·48 (S_3), 0·64 (S_4); $E_{5.7}^{Pier}$ 0·35, $E_{2.4}^{His}$ 0·00 (without removal of the protecting group). The analytical sample of compound *IIb* was recrystallised from a mixture of dimethylformamide, methanol, and ether; 169–172°C; $[\alpha]_D$ −77·1° (*c* 0·2, dimethylformamide). For $C_{30}H_{44}N_8O_{10}S_2.3\,H_2O$ (794·9) calculated: 45·33% C, 6·34% H, 14·09% N; found: 45·38% C, 6·02% H, 14·37% N. Amino acid analysis: Asp 1·00, Pro 1·05, Gly 1·00, Leu 0·98, Cys(C_3H_6COOH) 0·99.

The chromatography also afforded 83 mg of an additional substance, m.p. 146–148°C; R_F values: 0·40 (S_1), 0·02 (S_2), 0·34 (S_3), 0·65 (S_4); $E_{5.7}^{Pier}$ 0·63, $E_{2.4}^{His}$ 0·00 (without removal of the protecting group). The elemental analysis (found: 46·60% C, 6·06% H, 14·07% N) does not indicate deamidation of asparagine or glycine amide. Amino-acid analysis: Asp 1·00, Pro 1·00, Gly 1·00, Leu 1·00, Cys(C_3H_6COOH) 1·02.

o-Nitrobenzenesulfenyl-γ-tert-butyl Glutamoyl-asparaginyl-S-(γ-carboxypropyl)cysteinyl--prolyl-leucyl-glycine Amide (*IIIb*)

To a solution of the protected pentapeptide *IIb* (0·9 g) in dimethylformamide (10 ml), 2M ethereal hydrogen chloride (2·5 ml) was added, the mixture kept at room temperature for 3 min, and precipitated with ether (100 ml). The hydrochloride was collected with suction, washed with ether, and dried; R_F values: 0·10 (S_1), 0·02 (S_2), 0·09 (S_3), 0·45 (S_4); $E_{5.7}^{His}$ 0·17, $E_{2.4}^{Gly}$ 0·65. The salt was dissolved in dimethylformamide (13 ml), the solution made alkaline with N-ethylpiperidine (0·5 ml) and treated with the N-hydroxysuccinimide ester of *o*-nitrobenzenesulfenyl-γ-tert--butylglutamic acid (0·65 g). After 48 h at room temperature, the mixture was evaporated and the residue triturated with ether. The hygroscopic product was kept for several days under ether to pass into a powder which was reprecipitated from dimethylformamide with ether. Yield, 0·92 g (80%) of compound *IIIb*, m.p. 98—103°C. R_F values: 0·56 (S_1), 0·12 (S_2), 0·62 (S_3), 0·67 (S_4). The electrophoresis was carried out after removal of the amino-protecting group by means of methanolic hydrogen chloride: $E_{5.7}^{His}$ 0·17, $E_{2.4}^{Gly}$ 0·62. The analytical sample was reprecipitated from dimethylformamide with ether. Optical rotation: $[\alpha]_D$ −44·2° (*c* 0·4, dimethylformamide). For $C_{39}H_{59}N_9O_{13}S_2.H_2O$ (944·1) calculated: 49·78% C, 6·51% H, 13·35% N; found: 49·78% C, 6·79% H, 13·02% N.

o-Nitrobenzenesulfenylisoleucyl-γ-tert-butyl Glutamoyl-asparaginyl-S-(γ-carboxypropyl)-cysteinyl-prolyl-leucyl-glycine Amide (*IVb*)

The protected hexapeptide amide *IIIb* (0·90 g) was dissolved in dimethylformamide (10 ml), the solution treated with 2·15M ethereal hydrogen chloride, kept at room temperature for 4 min, and precipitated with ether (180 ml). The precipitate was repeatedly triturated with ether and then thoroughly dried. R_F values: 0·19 (S_1), 0·06 (S_2), 0·21 (S_3), 0·58 (S_4). The hygroscopic hydrochloride was dissolved in dimethylformamide (15 ml), the solution made alkaline with N-ethylpiperidine (0·45 ml) and then treated with the N-hydroxysuccinimide ester of *o*-nitrobenzenesulfenylisoleucine (0·70 g). After 225 h at room temperature, the mixture was evaporated and the residue repeatedly triturated with ether. The solid was collected with suction, washed with ether, dried, and crystallised from dimethylformamide and ethyl acetate. Yield, 1·0 g (99%) of compound *IVb*, m.p. 186—190°C (did not change on recrystallisation from dimethylformamide). R_F values: 0·58 (S_1), 0·18 (S_2), 0·61 (S_3), 0·67 (S_4). Optical rotation: $[\alpha]_D$ −55·3° (*c* 0·4, dimethylformamide). For $C_{45}H_{70}N_{10}O_{14}S_2.2\,H_2O$ (1039) calculated: 50·27% C, 6·93% H, 13·02% N; found: 49·98% C, 6·92% H, 12·81% N.

Isoleucyl-γ-tert-butyl Glutamoyl-asparaginyl-S-(γ-methoxycarbonylpropyl)cysteinyl-prolyl--leucyl-glycine Amide Hydrochloride (*IVc*)

The protected heptapeptide *IVa* (4·1 g) was dissolved in dimethylformamide (50 ml), the solution treated with 2·1M ethereal hydrogen chloride (4·4 ml), kept at room temperature for 4 min, and precipitated with ether. The precipitate was collected with suction and washed with ether to afford 3·56 g (99%) of the hydrochloride *IVc*, m.p. 221—223°C (decomp.). R_F-values: 0·28 (S_1), 0·29 (S_2); $E_{5.7}^{His}$ 0·44, $E_{2.4}^{Gly}$ 0·64. For $C_{40}H_{70}ClN_9O_{12}S.2\,H_2O$ (972·6) calculated: 49·48% C, 7·67% H, 12·99% N; found: 49·73% C, 7·46% H, 13·17% N.

Isoleucyl-γ-tert-butyl Glutamoyl-asparaginyl-S-(γ-carboxypropyl)cysteinyl-prolyl-leucyl--glycine Amide (*IVd*)

A. A solution of the heptapeptide hydrochloride *IVc* (3·2 g) in water (220 ml) was treated with 1M-NaOH (7·5 ml; pH 12·5), then 0·5 ml (after 5 min) and finally 1 ml (after 15 min). The mixture was kept at room temperature for 25 min, adjusted to pH 7 with the use of 1M-HCl (5·0 ml), cooled down to 0°C, and applied to a column of Dowex 50 (H^+) ion exchange resin (120 ml). The column was washed with water (0°C) until the washings did not give a positive reaction on chloride ions. The product was then eluted with 10% aqueous pyridine (0°C) and the eluates were evaporated. The residue was purified by precipitation from methanol with ether. Yield, 2·52 g (83%) of compound *IVd*, m.p. 165–167°C (did not change on reprecipitation of the analytical sample); $[\alpha]_D$ −50·2° (*c* 0·2, dimethylformamide); R_F values: 0·19 (S_1), 0·02 (S_2), 0·27 (S_3), 0·67 (S_4), $E_{5.7}^{His}$ 0·08, $E_{2.4}^{Gly}$ 0·58. For $C_{39}H_{67}N_9O_{12}S.H_2O$ (904·1) calculated: 51·81% C, 7·69% H, 13·94% N; found: 51·99% C, 7·78% H, 14·03% N.

B. A solution of the heptapeptide hydrochloride *IVc* (0·15 g) in methanol (5 ml) was treated with 1M-NaOH (0·7 ml) and kept at room temperature for 1 h. The methanol was evaporated and the residual solution was diluted with water (3 ml). The whole was applied to a column of Dowex 50 (H^+) ion exchange resin (8 ml) and the column washed with water to the negative reaction on chloride ions. The product was then eluted with 10% aqueous pyridine, the eluate concentrated under diminished pressure to a small volume, and the concentrate freeze-dried. The resulting product (116 mg) was precipitated from methanol with ether to afford a mixture of compounds (100 mg) containing not only the unreacted methyl ester and the required acid but also the diacid. It is not possible to exclude the formation of the diacid by the use of a prolonged reaction time and a lower temperature even in the stage when a considerable amount of the methyl ester remains unreacted. The following procedures were used in the separation of the components of the mixture ($E_{5.7}^{His}$ 0·44, $E_{5.7}^{His}$ 0·08, and $E_{5.7}^{Picr}$ 0·06). *1*. Separation on Amberlite IR-4B ion exchange resin. The head aqueous eluates mainly contained the methyl ester but a pure substance was not obtained. *2*. Countercurrent distribution in the solvent system 2-butanol–-0·05% aqueous acetic acid. After 100 transfers of the upper phase, only a single peak ($K = 0·56$) was obtained. The content of tubes 20–55 was pooled, concentrated to a small volume, and the concentrate freeze-dried. *3*. Column chromatography on silica gel (8 g of the adsorbent per 60 mg of the substance) in methanol. The required pure substance *IVd* was obtained in a very poor yield. *4*. Countercurrent distribution in the solvent system 1-butanol–pyridine–benzene–0·1% aqueous acetic acid (6 : 1 : 2 : 9). After 100 transfers of the upper phase, the peptidic material was located by the Folin–Ciocalteau reaction and the content of the particular set of tubes was determined. The required product *IVd* was present in the peak of $K = 0·06$ (tubes 3–23), the K value of the unreacted ester was 0·85 (tubes 30–65), and the distribution coefficient of the diacid was approximately zero (tubes 1–2). The content of the corresponding tubes was pooled, concentrated, and the concentrates were freeze-dried.

C. The protected heptapeptide amide *IVb* (0·90 g) was dissolved in dimethylformamide (14 ml), the solution treated with 2·1M ethereal hydrogen chloride (0·9 ml), kept at room temperature for 4 min, and precipitated with ether (150 ml). The precipitate was collected with suction, washed with ether, and dried. Yield, 0·67 g (84%) of the hydrochloride, m.p. 166–174°C. R_F values: 0·20 (S_1), 0·02 (S_2), 0·28 (S_3), 0·68 (S_4). $E_{5.7}^{His}$ 0·09, $E_{2.4}^{Gly}$ 0·56. The salt was dissolved in water and the solution passed through a column of Amberlite IR-4B ion exchange resin in the acetate cycle (10 ml). The effluent was freeze-dried and the resulting material precipitated from methanol with ether. Yield, 0·54 g (85%) of compound *IVd*, identical with the above substance; $[\alpha]_D$ −53·4° (*c* 0·2, dimethylformamide). Amino acid analysis: Asp 1·01, Glu 0·96, Pro 1·03, Gly 0·99, Ile 0·96, Leu 1·00, Cys($C_3H_6CO_2H$) 1·05.

Tert-Butyloxycarbonyl-O-tert-butyltyrosyl-isoleucyl-γ-tert-butyl Glutamoyl-asparaginyl-S-(γ-carboxypropyl)cysteinyl-prolyl-leucyl-glycine Amide (*Vb*)

To a solution of the free heptapeptide amide *IVd* (300 mg) in dimethylformamide (20 ml), N-ethylpiperidine (0·2 ml) and N-hydroxysuccinimide ester of tert-butyloxycarbonyl-O-tert-butyltyrosine (350 mg) were added. The mixture was stirred at room temperature for 62 h, evaporated, and the residue triturated with light petroleum and ether. The crystals were collected with suction and washed with ether and water. Yield, 360 mg; m.p. 205–215°C. Crystallisation from dimethylformamide and ether afforded 324 mg (80%) of compound *Vb*, m.p. 216–219°C. R_F values: 0·78 (S_1), 0·63 (S_2), 0·70 (S_3), 0·90 (S_2). The electrophoresis was performed after removal of protecting groups by the action of trifluoroacetic acid: $E^{Gly}_{2.4}$ 0·66, $E^{Pier}_{5.7}$ 0·01. The analytical sample was recrystallised from dimethylformamide and ether; the m.p. value did not change. Optical rotation: $[\alpha]_D$ −36·3° (*c* 0·2, dimethylformamide). For $C_{57}H_{92}N_{10}O_{16}S.H_2O$ (1223) calculated: 56·00% C, 7·69% H, 11·48% N; found: 56·02% C, 7·34% H, 11·61% N. Amino acid analysis: Asp 1·03, Glu 1·00, Pro 1·00, Gly 1·00, Leu 1·03, Ile 1·00, Tyr 0·96, Cys($C_3H_6CO_2H$) 0·95.

o-Nitrobenzenesulfenyl-O-tert-butyltyrosyl-isoleucyl-γ-tert-butyl Glutamoyl-asparaginyl-S-(γ-carboxypropyl)cysteinyl-prolyl-leucyl-glycine Amide (*Va*)

To a solution of the free heptapeptide amide *IVd* (637 mg) in dimethylformamide (10 ml), N-ethylpiperidine (0·23 ml) and N-hydroxysuccinimide ester of *o*-nitrobenzenesulfenyl-O-tert-butyltyrosine (0·8 g) were added. The mixture was kept at room temperature for 21 h and treated with additional 0·05 ml of N-ethylpiperidine. After 69 h, the solution was evaporated and the residue triturated with ether. The crystals were collected with suction, washed with ether, ethyl acetate and water, and reprecipitated from a dimethylformamide solution by the addition of ether. Yield, 680 mg (78%) of compound *Va*, m.p. 208–210° (decomp.). R_F values: 0·60 (S_1), 0·46 (S_2), 0·75 (S_3), 0·81 (S_4). The electrophoresis was performed after removal of the protecting group by the action of methanolic hydrogen chloride: $E^{Gly}_{2.4}$ 0·68, $E^{His}_{5.7}$ 0·12. The analytical sample of compound *Va* was recrystallised from dimethylformamide and ether, m.p. 212–213°C (decomp.); $[\alpha]_D$ −0·2° (*c* 0·2, dimethylformamide). For $C_{58}H_{87}N_{11}O_{16}S_2.2\,H_2O$ (1294) calculated: 53·81% C, 7·08% H, 11·90% N; found: 53·70% C, 6·82% H, 11·79% N. Amino acid analysis: Asp 1·00, Glu 0·97, Pro 1·02, Gly 0·99, Ile 0·98, Leu 1·02, Tyr 0·97, Cys($C_3H_6CO_2H$) 1·04.

Lactam of O-Tert-butyltyrosyl-isoleucyl-γ-tert-butyl Glutamoyl-asparaginyl-S-(γ-carboxypropyl)cysteinyl-prolyl-leucyl-glycine Amide (*Ie*)

Under nitrogen, a solution of the protected octapeptide amide *Va* (650 mg) in dimethylformamide (23 ml) and pyridine (23 ml) was treated with bis-*p*-nitrophenyl sulfite (2·1 g) and the mixture stirred at room temperature for 3 h. Additional sulfite (1 g) was then introduced followed by another 1 g (after 14 h). The whole was stirred for 5 h more, evaporated, the residue triturated with ether, the solid collected with suction, and washed with ether and water. Yield, 1·7 g of the substance, m.p. 120–123°C. Reprecipitation from dimethylformamide by the addition of water and trituration with ether afforded 510 mg of the substance, m.p. 168–185°C (did not improve on a further reprecipitation). The protected active ester was dissolved in dimethylformamide (8·5 ml), the solution treated with 2M ethereal hydrogen chloride (1·1 ml), kept at room temperature for 7 min, and precipitated with ether (300 ml). The precipitate was collected with suction, washed with ether, and dried. Yield, 380 mg of the substance, m.p. 138–144°C (decomp.). The substance was dissolved in dimethylformamide (10 ml) and the solution was added at 50°C

with stirring under nitrogen (rate, 2·7 ml per h) to N-ethylpiperidine (0·1 ml) in pyridine (450 ml). The whole was kept at 50°C for 4 h and at room temperature for 12 h, evaporated, the residue triturated with ether, dried (360 mg), and dissolved in the upper phase (25 ml) of the solvent system 2-butanol–0·05% acetic acid. The solution was introduced into the second tube of the countercurrent distribution apparatus (100 transfers of the upper phase and 160 transfers of the lower phase). The peptidic material was located by the Folin–Ciocalteau reaction. The peak of $K = 25$ (content of tubes 80—102) was concentrated and the concentrate was freeze-dried to afford 305 mg of the substance. The peak of $K = 0{\cdot}15$ contained a very small amount of an unknown substance and was not therefore worked up.

A portion (80 mg) of the product was passed through a column of Bio-Gel P-4 (100 × 1 cm) in 3M acetic acid. The peptide-containing fractions (detected by absorption at 280 nm) were freeze-dried to afford 51 mg of a solid which still contained traces of a ninhydrin-positive contaminant. The material was therefore dissolved in the upper phase (25 ml) of the solvent system 1-butanol–benzene–pyridine–0·1% aqueous acetic acid (6 : 1 : 2 : 9) and the solution was introduced into the second tube of the countercurrent distribution apparatus (272 transfers of the lower phase and 98 transfers of the upper phase). The peak of $K = 45$ (content of tubes 90—100) yielded 38 mg of the freeze-dried material. R_F values: 0·37 (S_1), 0·35 (S_2), 0·51 (S_3), 0·71 (S_4). Optical rotation: $[\alpha]_D$ −55·2° (*c* 0·1, 20% aqueous acetic acid). The analytical sample of compound *Ie* was precipitated from methanol with ether. For $C_{52}H_{82}N_{10}O_{13}S.2\,H_2O$ (1123) calculated: 54·72% C, 7·59% H, 12·27% N; found: 54·40% C, 7·31% H, 12·27% N (corrected for the ash content of 1·8%). Amino-acid analysis: Asp 1·02, Glu 1·01, Pro 1·03, Gly 1·05, Ile 0·97, Leu 1·04, Tyr 1·00, Cys($C_3H_6CO_2H$) 0·89.

Lactam of Tyrosyl-isoleucyl-glutamoyl-asparaginyl-S-(γ-carboxypropyl)cysteinyl-prolyl--leucyl-glycine Amide (*Ic*)

A. A portion (100 mg) of the freeze-dried cyclisation product after the first countercurrent distribution was dissolved in trifluoroacetic acid (3·5 ml), the solution kept at room temperature for 1 h, diluted with toluene (3·5 ml), and evaporated. The residue was triturated with ether, dried, and subjected to countercurrent distribution (2-butanol–0·05% aqueous acetic acid; 120 transfers of the upper phase and 100 transfers of the lower phase). Peaks of distribution coefficients 1·6, 2·5, 4·5, and 14·8 were detected. The peak of $K = 2{\cdot}5$ (content of tubes 45—75) was concentrated and the concentrate freeze-dried (55 mg). The material was then subjected to a further purification by gel filtration (Bio-Gel P-4, 3M acetic acid) to afford 30 mg of the freeze--dried material. R_F values: 0·33 (S_1), 0·07 (S_2), 0·29 (S_3), 0·63 (S_4); $E_{5.7}^{His}$ 0·27. Optical rotation: $[\alpha]_D$ −57·5° (*c* 0·1, water). The analytical sample of compound *Ic* was precipitated from methanol with ether. For $C_{44}H_{66}N_{10}O_{13}S.2\,H_2O$ (1001) calculated: 51·27% C, 6·84% H, 13·58% N; found: 51·17% C, 6·59% H, 13·32% N (corrected for the ash content of 2%). Amino-acid analysis: Asp 1·02, Glu 0·99, Pro 1·06, Gly 1·01, Ile 0·97, Leu 0·99, Tyr 0·94, Cys($C_3H_6CO_2H$) 1·04.

The peak of $K = 1{\cdot}6$ (content of tubes 14—44) was concentrated, the concentrate freeze-dried, and the resulting material purified by gel filtration (Bio-Gel P-4, 3M acetic acid) to afford 5·7 mg of the freeze-dried material. R_F values: 0·20 (S_1), 0·02 (S_2), 0·17 (S_3), 0·63 (S_4); $E_{5.7}^{His}$ 0·27. Optical rotation: $[\alpha]_D$ −59·8° (*c* 0·1, water). The analytical sample was precipitated from methanol with ether. For $C_{48}H_{74}N_{10}O_{13}S.5\,H_2O$ (1121) calculated: 50·67% C, 7·44% H, 12·31% N; found: 50·41% C, 6·82% H, 12·56% N (corrected for the ash content of 1·6%). Amino-acid analysis: Asp 1·03, Glu 1·01, Pro 1·03, Gly 1·03, Ile 1·00, Leu 1·03, Tyr 0·97, Cys($C_3H_6CO_2H$) 0·90.

A sample (1 mg) of the latter substance was dissolved in acetic acid (30 μl), the solution treated with 35% hydrogen bromide in acetic acid (100 μl), the whole kept at room temperature for

10 min, and precipitated with ether. The resulting material was reprecipitated from methanol with ether. The product was identical on chromatography and electrophoresis with the above described peptide *Ic*.

Another portion (50 mg) of the freeze-dried cyclisation product after the first countercurrent distribution was dissolved in trifluoroacetic acid (1·5 ml) and the solution kept at room temperature for 1 h. Toluene (1·5 ml) was then added, the whole evaporated, and the residue triturated with ether. The solid was purified on a column of Bio-Gel P-4 in 3M acetic acid. The separated fractions were subjected to an additional purification on Bio-Gel P-4 to obtain both the above mentioned substances in a pure state but in a very poor yield.

B. The protected octapeptide amide *Vb* (290 mg) was dissolved in a mixture of dimethylformamide (12 ml) and pyridine (12 ml) and the solution was treated under nitrogen with bis-*p*-nitrophenyl sulfite (1 g). The mixture was stirred at room temperature for 8 h, treated with additional 1 g of the sulfite, stirred for 12 h, treated with 0·5 g of the sulfite, and stirred for 5 h. The mixture was finally evaporated, the residue triturated with ether, the solid collected with suction, washed on the filter with ether and water, and dried. Yield, 286 mg of a substance, m.p. 198–205°C (decomp.). The substance was dissolved in trifluoroacetic acid (10 ml), the solution kept at room temperature for 1 h, diluted with toluene (10 ml), and evaporated. The residue was triturated with ether, dried, and dissolved in dimethylformamide (9 ml). The solution was added (rate, 2·7 ml per h) at 50°C to 250 ml of pyridine. After 12 h at room temperature, the solution was evaporated, the residue triturated with ether, dried, and purified by countercurrent distribution (2-butanol–0·05% acetic acid; 168 transfers of the upper phase and 201 transfers of the lower phase). The peak of $K = 2{\cdot}42$ (content of tubes 45–78) was concentrated, the concentrate freeze-dried (70 mg), and subjected to gel filtration. Yield, 35 mg of a product identical with substance *Ic* obtained by the above mentioned procedure. In this case, the compound of $K = 1{\cdot}6$ was absent.

Lactam of Tyrosyl-isoleucyl-glutaminyl-asparaginyl-**S**-(γ-carboxypropyl)cysteinyl-prolyl--leucyl-glycine Amide (*Ib*)

To a solution of the cyclopeptide-acid *Ic* of $K = 2{\cdot}5$ (4·8 mg) in dimethylformamide (200 µl), 1M ammonia in dimethylformamide (50 µl) and N-hydroxybenzotriazole (13·5 mg) were added. The mixture was cooled down (0°C), treated with N,N′-dicyclohexylcarbodiimide (20·6 mg), and kept at 0°C for 2 h and at room temperature for 20 h. The solvent was evaporated, the residue shaken for 30 min with 3M acetic acid (3 ml), and the insoluble portion filtered off. The filtrate was applied to a column of Bio-Gel P-4 and the effluent freeze-dried. Yield, 2·6 mg of a substance identical on chromatography and electrophoresis with an authentic sample of deamino-1-carba--oxytocin. R_F values: 0·18 (S_1), 0·17 (S_2), 0·16 (S_3), 0·63 (S_4); $E^{His}_{5.7}$ 0·02. In an assay on isolated rat uterus[7], the activity of 1225 I.U./mg was found in the absence of Mg^{2+} ions.

The other product ($K = 1{\cdot}6$) was subjected to an analogous reaction to afford a substance differing on chromatography from the authentic sample. R_F values: 0·12 (S_1), 0·04 (S_2), 0·08 (S_3), 0·64 (S_4); $E^{His}_{5.7}$ 0·02. A sample (1 mg) of this substance was dissolved in acetic acid (30 µl), the solution treated with 35% hydrogen bromide in acetic acid (100 µl) kept at room temperature for 15 min, and precipitated with ether. The thus-obtained precipitate exhibited the same properties as the earlier prepared deamino-1-carba-oxytocin.

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