SYNTHESIS OF OXYTOCIN ANALOGUES MODIFIED IN THE TRIPEPTIDE SIDE-CHAIN BY CONDENSATION OF AMINOTERMINAL LINEAR HEXAPEPTIDE WITH THE CARBOXYTERMINAL TRIPEPTIDE*

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For the synthesis of oxytocin (*Ia*) analogues modified in the carboxyterminal part of the molecule, a method based on the condensation of protected aminoterminal hexapeptide with tripeptides by the action of dicyclohexylcarbodiimide and pentafluorophenol in the presence of 1-hydroxybenzotriazole was devised. Using this method [7-[U-^{13}C]proline]oxytocin (*Ib*), des-9-glycine-oxytocin (*Ic*) and methyl ester of oxytocinoic acid ([9-glycine methyl ester]oxytocin) (*Id*) were prepared.

It is known that in the case of vasopressin and its analogues** the azide condensation proceeds without great difficulty according to the scheme 6 + 3. In the oxytocin series similar condensation will not proceed or only at a negligible rate even when proline in the carboxy terminal tripeptide is substituted by an amino acid with primary amino group[2]. The difficulty persists even if the rearrangement of hexapeptide azide into its amide is strongly suppressed[3]. It seems that the amino acids by which differentiates oxytocin from vasopressin (isoleucine and leucine in position 3 and 8, resp.) have a negative effect on the conformation of one and/or the other fragment during the condensation of protected hexapeptide azide with tripeptide. Some authors succeeded in overcoming the above-mentioned difficulty by acylation of the 50% excess of tripeptide with the cyclic hexapeptide with a preformed disulfide bond[4,5] (it is most probable that the conformation of cyclic hexapeptide differs from that of linear hexapeptide). It is also possible to perform the condensation of protected hexapeptide acid with the tripeptide using such a condensation agent and selecting such reaction conditions that the danger of racemisation in this step is decreased to a minimum. In this way, *i.e.* by using dicyclohexylcarbodiimide and 1-hydroxysuccinimide, the desired protected nonapeptide with the oxytocin sequence was prepared. However, oxytocin obtained by the conventional way from this protected nonapeptide had only 75% of uterotonic activity[2].

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** Nomenclature and symbolics of amino acids and peptides follow the published proposals[1]. Amino acids used in this study are of L configuration.

In this work we studied the possibility of preparing the protected nonapeptide possessing the oxytocin sequence according to the scheme 6 + 3, by the use of various condensation agents and under different experimental conditions. We started with benzyloxycarbonylhexapeptide acid *IIa* which has both cysteine residues protected by benzyl groups and is prepared from the corresponding tert-butyl ester *IIb* by treatment with trifluoroacetic acid. For the synthesis of the protected hexapeptide *IIb* we used tert-butyl ester of S-benzylcysteine which, after acylation with 2,4,5-trichlorophenyl ester of 2-nitrobenzenesulfenylasparagine, produced protected dipeptide *III*.

Cys-Tyr-Ile-Gln-Asn-Cys-R

Ia, R = Pro-Leu-Gly-NH_2
Ib, R = [U-^{13}C]Pro-Leu-Gly-NH_2
Ic, R = Pro-Leu-NH_2
Id, R = Pro-Leu-Gly-OMe

R^1-Cys(Bzl)-Tyr(R^2)-Ile-Gln-Asn-Cys(Bzl)-R^3

IIa, R^1 = Z, R^2 = H, R^3 = OH
IIb, R^1 = Z, R^2 = Bu^t, R^3 = OBu^t
IIc, R^1 = Tos, R^2 = H, R^3 = OH
IId, R^1 = Tos, R^2 = H, R^3 = OBu^t
IIe, R^1 = Z, R^2 = H, R^3 = Pro-Leu-Gly-NH_2
IIf, R^1 = Z, R^2 = H, R^3 = [U-^{13}C]Pro-Leu-Gly-NH_2
IIg, R^1 = Tos, R^2 = H, R^3 = Pro-Leu-NH_2
IIh, R^1 = Tos, R^2 = H, R^3 = Pro-Leu-Gly-OCH_3

The aminoprotecting group was split off by treatment with HCl in ether and the peptide chain was prolonged by gradual acylation using 2,4,5-trichlorophenyl ester of 2-nitrobenzenesulfenylglutamine, N-hydroxysuccinimide ester of N-2-nitrobenzenesulfenylisoleucine and N-hydroxysuccinimide ester of N-2-nitrobenzenesulfenyl-O-tert-butyltyrosine; substances *IV*, *V* and *VI* were thus formed. The 2-nitrobenzenesulfenyl group was again split off by HCl in ether and acylation with 4-nitrophenyl ester of N-benzyloxycarbonyl-S-benzylcysteine resulted in protected hexapeptide *IIb*.

Nps-Asn-Cys(Bzl)-OBu^t

III

Nps-Gln-Asn-Cys(Bzl)-OBu^t

IV

Nps-Ile-Gln-Asn-Cys(Bzl)-OBu^t

V

Nps-Tyr(Bu^t)-Ile-Gln-Asn-Cys(Bzl)-OBu^t

VI

We also prepared aminoterminal hexapeptide acid *IIc* with a 4-toluenesulfonyl protecting group on the amino group of cysteine by treating the corresponding tert-butyl ester *IIb* with trifluoroacetic acid. In this case the compound *IId* was obtained by condensing N-4-toluenesulfonyl-S-benzylcysteinyltyrosyl-isoleucine azide[6] with tert-butyl ester of glutaminyl-asparaginyl-S-benzylcysteine which we prepared by splitting off the 2-nitrobenzenesulfenyl protecting group from *IV* using HCl in ether. Prolyl-leucyl-glycine amide (*VIIa*), the model substance for the condensation reactions, was synthesized according to recorded methods[7,8]. The coupling of both the peptidic fragments was either effected by dicyclohexylcarbodiimide in the presence of 1-hydroxybenzotriazole[9] (method *A*) or by the action of the dicyclohexylcarbodiimide–pentafluorophenol complex[10,11], in some cases in the presence of 1-hydroxybenzotriazole (method *B*).

Using a 20% excess of amino component *VIIa* and equivalent amounts of the other reaction components (hexapeptide *IIa*, dicyclohexylcarbodiimide and 1-hydroxybenzotriazole) we obtained protected nonapeptide *IIe*, contaminated by a large amount of unreacted hexapeptide (according to amino-acid analysis the yield was only 40%). Using equivalent amounts of both peptidic fragments (*IIa* and *VIIa*), a tenfold excess of dicyclohexylcarbodiimide and twentyfold excess of 1-hydroxybenzotriazole we obtained pure nonapeptide *IIe*. The optical rotation data of the product corresponded to those found for the nonapeptide prepared by other procedures.

X-Leu-Gly-NH_2

VIIa, X = Pro
VIIb, X = [U-^{13}C]Pro
VIIc, X = Z-[U^{13}C]Pro

R^1-Pro-Leu-Gly-R^2

VIIIa, R^1 = Z, R^2 = OMe
VIIIb, R^1 = Z, R^2 = OH

Using the complex pentafluorophenol–dicyclohexylcarbodiimide we substantially reduced the amount of condensation agent. When the equivalent amount of both peptides and a 10% excess of complex were used the product *IIe* was obtained approximately in 80% yield, but contamined by hexapeptide which we did not succeed in separating by gel filtration on Sephadex LH-20 in dimethylformamide. In further experiments we therefore increased the excess of complex to 50% and prolonged the reaction time three times. To exclude racemisation as much as possible we performed the condensation in the presence of an excess of 1-hydroxybenzotriazole. Under such conditions we succeeded in isolating nonapeptide *IIe* with parameters corresponding to those of the substance prepared by method *A*. We checked the applicability of the methods, both in the case of N-benzyloxycarbonyl-hexapeptide *IIa* and in the case of N-4-toluenesulfonyl-hexapeptide *IIc* by synthesizing oxytocin (*Ia*) analogues with a modified side chain (*Ib–d*). For the synthesis of compound *Ib*

a corresponding protected nonapeptide *IIf* was prepared by the condensation of compounds *IIa* and *VIIb* by method *B*. Labelled tripeptide amide *VIIb* was obtaincd by the hydrogenation of its benzyloxycarbonyl derivative *VIIc*, which was prepared by the condensation of [U-^{13}C]benzyloxycarbonylproline and leucyl-glycine amide[12] by dicyclohexylcarbodiimide and 1-hydroxybenzotriazole. The protected nonapeptide *If* was reduced by sodium in liquid ammonia, oxidized by air oxygen and the product *Ib* was isolated and purified by countercurrent distribution and free-flow electrophoresis. Protected octapeptide *Ig* used for the synthesis of analogue *Ic* was prepared by the condensation of N-4-toluenesulfonyl-hexapeptide *IIc* with prolyl-leucine amide[12] by both methods *A* and *B*. Each method resulted in a comparable yield of the product. Octapeptide was reduced by sodium in liquid ammonia, the disulphide bond was closed by oxidation with air oxygen and *Ic* was purified by countercurrent distribution and gel filtration. The protected nonapeptide *IIh* used for the synthesis of *Id* was again prepared by means of N-4-toluenesulfonyl-hexapeptide *IIc*; as in the previous case, both methods *A* and *B* resulted in a product of comparable properties in nearly the same yield. The desired benzyloxycarbonylprolyl-leucyl-glycine methyl ester (*VIIIa*) was prepared by esterification of the acid *VIIb* (ref.[13]) by diazomethane and the benzyloxycarbonyl group was split off by catalytic hydrogenation. Protected nonapeptide *IIh* was reduced and oxidized in the same way as previously indicated and the analogue *Id* was purified by countercurrent distribution and gel filtration. Under conditions of reduction by sodium in liquid ammonia the glycine ester grouping was probably not completely stable; after partial purification by countercurrent distribution the amino-acid analysis revealed only 60% of glycine in the product. We succeeded in obtaining pure analogue *Id* after repeated gel filtration.

[7-[U-^{13}C]Proline]oxytocin (*Ib*) was fully biologically active; its uterotonic activity in the assay on the isolated rat uterus was 450 I.U./mg. This synthesis therefore offers the possibility of using compound *Ib* for conformational studies by means of heteronuclear magnetic resonance. Des-9-glycine-oxytocin (*Ic*) has been already described by Swiss authors[12] who used a somewhat different synthetic approach. This analogue is interesting mainly due to its very high natriferic activity[14] one of the highest activities so far detected for the type of compound derived from neurohypophysial hormones. As for the uterotonic, galactogogic and antidiuretic activities of *Ic*, these were very similar to the data published[12] (in brackets, the data published in paper[12]): isolated rat uterus 3·9 I.U./mg (4·2 ± 0·7), rat galactogogic activity 8·6 I.U./mg (approx. 3·0 I.U./mg in the rabbit) and 0·15 I.U./mg (approx. 0·02 I.U./mg) in the antidiuretic assay. No natriuretic activity[15] was found for the analogue *Ic*.

Another analogue we prepared, namely *Id*, was intended to contribute to our knowledge of the significance of the amide group in position 9. The significance of individual functional groups in the molecule of oxytocin (*Ia*) was studied for a longer period by synthesizing analogues having the corresponding functional groups replaced by

hydrogen or some other, if possible isosteric, group. In this way (for review see[16-18]), the functional insignificance of the amino group of cysteine in position 1, the hydroxyl group of tyrosine in position 2, the disulfide bond, and amide group of glutamine in position 4 was demonstrated. The replacements of amide (or carboxamide) groups of asparagine in position 5 or of glycine amide in position 9 resulted in almost inactive analogues. The published data indicated that inappropriate replacement might lead to a strong decrease of activity even if the functional group concerned is not by itself prerequisite for the activity of the hormone. For example, the replacement of cysteine residues by alanine[19] results in the appearance of the linear peptide without biological activity[20], although the substitution of one or both atoms of sulphur by methylene groups (maintaining the cyclic arrangement) preserved the high degree of biological activities[21-25]. Similarly the replacement of the amino group of cysteine in position 1 by a hydrogen atom yielded a highly active analogue[26-28], whereas its acetylation resulted in a pronounced decrease of activities[29]. Oxytocinoic acid ([9-glycine]oxytocin) was described several times[30-33]; its biological activities are lower by three orders of magnitude than those of oxytocin. The replacement of the carboxamide group by a carboxyl one induces such dramatic changes in the molecule that one can hardly judge the significance of the amide group in position 9 on the basis of this one analogue. We therefore prepared the methyl ester of oxytocinoic acid (*Id*) which, however, also had distinctly decreased biological activities: 0·6 I.U./mg of uterotonic, 0·5 I.U./mg galactogogic and 0·9 I.U./mg pressoric activities. It is rather difficult to decide whether the low activities are the intrinsic properties of *Id* or whether hydrolysis of the ester proceeds so quickly that the measured activities are those of oxytocinoic acid. The value of the pressoric potency (the assay is performed under conditions *in vivo* which would strongly favour hydrolysis) speaks for the former possibility. The pressoric potency of the ester was found to be equal to 30% of the oxytocin pressoric potency, whereas oxytocinoic acid had no activity[30]. Howerer, these experiments cannot exclude (*cf.*[34]) hydrolysis of methyl ester in the blood circulation or in the receptor compartment; if hydrolysis does occur the actual potency of analogue *Id* will be even higher.

EXPERIMENTAL

Melting points were determined on a Kofler block. Samples for elemental analysis were dried for several hours at room temperature over phosphorus pentoxide at 150 Pa. Reaction mixtures were taken down under diminished pressure on a rotary evaporator (water pump, bath temperature 35—40°C). The dimethylformamide-containing mixtures were evaporated with the use of an oil pump (150 Pa). Optical rotations were measured on the Perkin–Elmer 141 MCA apparatus. Analytical electrophoresis was performed in a moist chamber on Whatman 3 MM paper with the use of 1M acetic acid (pH 2·4) and pyridine–acetic acid (pH 5·7) as buffers at 20 V/cm for 45 min. Thin-layer chromatography was carried out on silica gel plates (Kieselgel G, Merck)

in the solvent systems 2-butanol–25% aqueous ammonia–water, 85 : 7·5 : 7·5 (S1), 2-butanol–98% formic acid–water, 75 : 12·3 : 12·7 (S2), 1-butanol–acetic acid–water, 4 : 1 : 1 (S3), and 1-butanol–pyridine–acetic acid–water, 15 : 10 : 3 : 6 (S4). The all-glass, fully automatic Steady State Distribution Machine (Quickfit and Quartz, Stone, Staffordshire, England) with possibilities of transfers of both upper and lower phases, was used for countercurrent distribution. Continuous free-flow electrophoresis was carried out on a modified version[34,35] of the Hannig apparatus[36]. Samples for amino-acid analysis were hydrolysed for 20 h at 105°C in 6M-HCl (in ampoules sealed at 150 Pa) and analyses were carried out on an automatic analyser (Developmental Workshops, Czechoslovak Academy of Sciences, type 6020 A).

[U-^{13}C]Proline, 85% labelling, was supplied by Dr P. Fromageot, Service de Biochimie, Centre d'Études Nucléaires de Saclay, France.

2-Nitrobenzenesulfenylasparaginyl-S-benzylcysteine Tert-butyl Ester (*III*)

To a solution of S-benzylcysteine tert-butyl ester (10·7 g) in dimethylformamide (100 ml) 2,4,5-trichlorophenyl ester of 2-nitrobenzenesulfenylasparagine (17·8 g) was added. After 24 h of stirring at room temperature a further portion of active ester (8·9 g) was added and after 24 h the solvent was evaporated. The residue was triturated with light petroleum, ether, 0·05% H_2SO_4, 5% $NaHCO_3$, and water (14·6 g, m.p. 155—158°C). Crystallization from dimethylformamide and ether afforded 13·8 g (65%) of a product with m.p. 157—158°C. R_F 0·75 (S1), 0·86 (S2), 0·86 (S3), 0·83 (S4); $[\alpha]_D^{22}$ −69·7° (*c* 0·5; dimethylformamide). For $C_{24}H_{30}N_4O_6S_2$ (534·7) calculated: 53·91% C, 5·66% H, 10·47% N, 11·99% S; found: 53·67% C, 5·70% H, 10·61% N, 12·05% S.

2-Nitrobenzenesulfenylglutaminyl-asparaginyl-S-benzylcysteine Tert-butyl Ester (*IV*)

To a solution of the protected dipeptide *III* (10·7 g) in dimethylformamide (20 ml) 3·5M-HCl in ether (12 ml) was added and after 6 min the product was precipitated by the addition of ether. The decantation and extraction with ether was repeated several times in order to solidify. The yield was 8·2 g, $E_{2.4}^{Gly}$ 1·06, $E_{2.4}^{His}$ 0·61, $E_{5.7}^{His}$ 0·65.

To the solution of this hydrochloride in dimethylformamide (50 ml), N-ethylpiperidine (2·9 ml) and 2-nitrobenzenesulfenylglutamine 2,4,5-trichlorophenyl ester (14·4 g) were added. After 40 h stirring at room temperature the mixture was worked up in the same way as in the case of the compound *III* (yield, 12·5 g, m.p. 190—193°C). Crystallization from the mixture of dimethylformamide–water afforded 11·8 g (93%) of the product with m.p. 193—194°C. R_F 0·60 (S1), 0·69 (S2), 0·72 (S3), 0·73 (S4); $[\alpha]_D^{22}$ −29·9° (*c* 0·5, dimethylformamide). For $C_{29}H_{38}N_6O_8 \cdot S_2$ (662·8) calculated: 52·55% C, 5·78% H, 12·68% N, 9·68% S; found: 52·29% C, 5·85% H, 13·01% N, 9·61% S.

2-Nitrobenzenesulfenylisoleucyl-glutaminyl-asparaginyl-S-benzylcysteine Tert-butyl Ester (*V*)

To a solution of the protected tripeptide *IV* (6·6 g) in dimethylformamide (50 ml) 3·5M-HCl in ether (6 ml) was added. After 6 min the product was precipitated by the addition of ether and the separated product was triturated and extracted with ether till it turned to the powder state. The yield was 5·4 g, $E_{2.4}^{Gly}$ 0·96, $E_{2.4}^{His}$ 0·55, $E_{5.7}^{His}$ 0·69.

The solution of this hydrochloride in dimethylformamide (20 ml), N-ethylpiperidine (2·5 ml) and 2-nitrobenzenesulfenylisoleucine N-hydroxysuccinimide ester (4·2 g) were added. The mixture was stirred for 30 h at room temperature and then worked up in the same way as in the case of the compound *III* (6·3 g, m.p. 193—197°C). Precipitation from dimethylformamide

and water afforded 5·54 g (71%) of the product with m.p. 196—198°C. R_F 0·69 (S1), 0·77 (S2), 0·75 (S3), 0·79 (S4); $[\alpha]_D^{22}$ −50·1° (*c* 0·5, dimethylformamide). Amino-acid analysis: Asp 1·00, Glu 1·00, Ile 0·94, Cys(Bzl) + Cys 0·7. For $C_{35}H_{49}N_7O_9S_2.0.5\,H_2O$ (785·0) calculated: 53·55% C, 6·42% H, 12·49% N, 8·17% S; found: 53·23% C, 6·47% H, 12·56% N, 8·28% S.

N-2-Nitrobenzenesulfenyl-O-tert-butyltyrosyl-isoleucyl-glutaminyl-asparaginyl--S-benzylcysteine Tert-butyl Ester (*VI*)

To a solution of the protected tetrapeptide *V* (5·4 g) in dimethylformamide (25 ml) 7M-HCl in ether (2·1 ml) was added and after 3 min the product was precipitated by the addition of ether. The solid compound was filtered and washed with ether; yield 4·3 g; $E_{2,4}^{Gly}$ 0·60, $E_{2,4}^{His}$ 0·34, $E_{5,7}^{His}$ 0·48.

To the solution of the hydrochloride in dimethylformamide (120 ml), N-ethylpiperidine (1 ml) and N-2-nitrobenzenesulfenyl-O-tert-butyltyrosine N-hydroxysuccinimide ester (6·8 g) were added. After stirring for 70 h at room temperature the mixture was worked up as in the case of the compound *III* (6·2 g, m.p. 204—206°C). Precipitation from dimethylformamide and ether yielded 5·7 g (82%) of a product with m.p. 205—206°C. R_F 0·66 (S1), 0·80 (S2), 0·80 (S3), 0·82 (S4); $[\alpha]_D^{22}$ +2·4° (*c* 0·3, dimethylformamide). Amido-acid analysis: Asp 1·00, Glu 1·00, Ile 0·95, Tyr 0·94, Cys(Bzl) + Cys 0·72. For $C_{48}H_{66}N_8O_{11}S_2$ (995·2) calculated: 57·93% C, 6·69% H, 11·26% N, 6·44% S; found: 57·81% C, 6·62% N, 10·99%, 6·38% S.

Benzyloxycarbonyl[U-^{13}C]prolyl-leucyl-glycine Amide (*VIIc*)

Benzyloxycarbonyl chloride (0·45 ml) was added to a solution of [U-^{13}C]proline (0·25 g) in 4M--NaOH (1·9 ml) and the mixture was agitated in a test tube at room temperature for 75 min, extracted with ether and acidified with 12M-HCl. The oil that separated was extracted in ethyl acetate, the solution was washed with water, dried (Na_2SO_4) and evaporated. The residue (0·5 g) was dissolved in dimethylformamide (2 ml), the solution was cooled down to −12°C and 1-hydroxybenzotriazole (0·29 g) and dicyclohexylcarbodiimide (0·41 g) were added. To this solution we added, after 30 min stirring, leucyl-glycine amide (0·71 g), prepared by means of hydrogenation of its benzyloxycarbonyl derivative (1·4 g) in ethanol in the presence of 5% Pd/$BaSO_4$. The mixture was stirred for further 3 h at 0°C and 20 h at room temperature. Dicyclohexylurea was collected, the solvent was evaporated, the residue dissolved in ethyl acetate and the solution washed with 5% $NaHCO_3$, 1M-HCl, water, and dried and evaporated. The foam which was obtained crystallized under ether. Yield, 0·64 g (84%, calculated for [U-^{13}C]proline), m.p. 159—162°C. Amino-acid analysis: Pro 1·00, Leu 1·01, Gly 1·01. The electrophoretic behavior, after the removal of the benzyloxycarbonyl group, was same as that of the nonlabelled compound.

Benzyloxycarbonylprolyl-leucyl-glycine Methyl Ester (*VIIIa*)

Benzyloxycarbonylprolyl-leucyl-glycine (0·77 g) in dioxane solution (10 ml) was esterified by means of diazomethane. The solvent was evaporated and the residue was crystallized from benzene; yield was 0·79 g (92%) of the product with a m.p. 128—129°C; $[\alpha]_D^{22}$ −89·5° (*c* 0·2, methanol). For $C_{22}H_{31}N_3O_6$ (433·5) calculated: 60·96% C, 7·21% H, 9·70% N; found: 61·11% C, 7·24% H, 9·48% N.

N-Benzyloxycarbonyl-S-benzylcysteinyl-O-tert-butyltyrosyl-isoleucyl-glutaminyl-asparaginyl-
-S-benzylcysteine Tert-butyl Ester (*IIb*)

To the solution of the protected peptide *VI* (5·5 g) in dimethylformamide (20 ml) 7M-HCl in ether (1·7 ml) was added and after 3 min the product was precipitated by the addition of ether, filtrated and dried. This yielded 5·3 g of the hydrochloride, $E_{2.4}^{Gly}$ 0·65, $E_{2.4}^{His}$ 0·38, $E_{5.7}^{His}$ 0·51.

To the solution of this hydrochloride in dimethylformamide (50 ml) N-ethylpiperidine (0·8 ml) and N-benzyloxycarbonyl-S-benzylcysteine 4-nitrophenyl ester (5·1 g) were added. After 40 h stirring at room temperature the mixture was worked up in the same way as in the case of the compound *III* (yield, 5·9 g, m.p. 217—222°C). Precipitation from dimethylformamide and water yielded 5·7 g (87%) of the title compound, m.p. 220—223°C. R_F 0·75 (S1), 0·81 (S2), 0·85 (S3), 0·85 (S4); $[\alpha]_D^{22}$ —36·9° (*c* 0·5, dimethylformamide). Amino-acid analysis: Asp 1·08, Glu 1·00, Ile 1·00, Tyr 0·97, Cys(Bzl) + Cys 1·74. For $C_{60}H_{80}N_8O_{12}S_2$ (1 170) calculated: 61·62% C, 6·89% H, 9·58% N, 5·48% S; found: 61·46% C, 7·03% H, 9·68% N, 5·76% S.

N-Benzyloxycarbonyl-S-benzylcysteinyl-tyrosyl-isoleucyl-glutaminyl-asparaginyl-
-S-benzylcysteine (*IIa*)

The solution of the protected hexapeptide *IIb* (1·17 g) in trifluoroacetic acid (8 ml) was set aside for 2 h at room temperature, the solution was evaporated, the residue dissolved in benzene and evaporated (this operation was repeated five times) and after the last evaporation the residue crystallized under ether (0·96 g, m.p. 222—226°C). The product was suspended in hot methanol (10 ml), filtered, washed with methanol (5 ml) and ether. Precipitation from dimethylformamide and water yielded 0·83 g (78%) of the product with m.p. 226—227°C. $[\alpha]_D^{22}$ —32·4° (*c* 0·3, dimethylformamide). The literature[2] records $[\alpha]_D$ —23° (*c* 1·08, dimethylformamide). For $C_{52}H_{64}$·$N_8O_{12}S_2$ (1 057) calculated: 59·07% C, 6·10% H, 10·60% N; found: 58·98% C, 6·10% H, 10·67% N.

N-4-Toluenesulfonyl-S-benzylcysteinyl-tyrosyl-isoleucyl-glutaminyl-asparaginyl-
-S-benzylcysteine Tert-butyl Ester (*IId*)

The solution of the hydrochloride of glutaminyl-asparaginyl-S-benzylcysteine tert-butyl ester (0·74 g; its preparation is described under the compound *V*) in dimethylformamide (4 ml) was added to the solution of N-4-toluenesulfonyl-S-benzylcysteinyl-tyrosyl-isoleucine azide, prepared in turn from the corresponding hydrazide (0·66 g; 2·9 ml of 3·5M-HCl in dioxane; 0·13 ml of butyl nitrite; N-ethylpiperidine). The mixture was stirred 94 h at 0—4°C and during this time its pH was maintained at the value 7 (moist pH paper) by the addition of N-ethylpiperidine. The mixture was evaporated, the residue triturated with water, the solid part filtered and washed on the filter with 1M-HCl, water, 5% $NaHCO_3$, water, and ether; the yield was 1·07 g of the compound with m.p. 218—223°C. Precipitation from dimethylformamide and water afforded 0·97 g (92%) of the product, m.p. 222—224°C. R_F 0·72 (S1), 0·80 (S2), 0·83 (S3), 0·82 (S4); $[\alpha]_D^{22}$ —15·9° (*c* 0·3, dimethylformamide). For $C_{55}H_{72}N_8O_{12}S_3$ (1 133) calculated: 58·29% C, 6·40% H, 9·89% N; found: 57·97% C, 6·43% H, 10·02% N.

N-4-Toluenesulfonyl-S-benzylcysteinyl-tyrosyl-isoleucyl-glutaminyl-asparaginyl-
-S-benzylcysteine (*IIc*)

The same procedure as described under *IIa* was used for the splitting of the ester group from the compound *IId* (1·06 g) with trifluoroacetic acid (4 ml). The yield was 0·84 g (85%) of the title compound, m.p. 228—230°C, $[\alpha]_D^{22}$ —10·9° (*c* 0·5, dimethylformamide). Amino-acid analysis:

Asp 1·02, Glu 0·99, Ile 1·00, Tyr 0·98, Cys(Bzl) + Cys 1·32. For $C_{51}H_{64}N_8O_{12}S_3$ (1077) calculated: 56·86% C, 5·99% H, 10·40% N; found: 56·98% C, 6·12% H, 10·15% N.

N-Benzyloxycarbonyl-S-benzylcysteinyl-tyrosyl-isoleucyl-glutaminyl-asparaginyl-S-benzylcysteinyl-prolyl-leucyl-glycine Amide (*IIe*)

Method A: To the solution of the benzyloxycarbonyl-hexapeptide *IIa* (0·11 g) and 1-hydroxybenzotriazole (0·27 g) in dimethylformamide (2 ml), dicyclohexylcarbodiimide (0·21 g) was added at −12°C, and after 30 min stirring at this temperature prolyl-leucyl-glycine amide (0·03 g) was added. The mixture was stirred for 4 h at 0°C and for 42 h at room temperature. Dicyclohexylurea was removed by suction, dimethylformamide was evaporated and the residue solidified under water. The solid product was filtrated, washed on the filter with 1M-HCl, water, 5% $NaHCO_3$, and with hot water, hot methanol and ether (yield, 0·10 g, m.p. 220−224°C). The precipitation from dimethylformamide and water yielded 0·09 g (68%) of the product with m.p. 224−226°C and R_F 0·35 (S1), 0·40 (S2), 0·53 (S3) and 0·62 (S4); $E_{2.4}^{Gly}$ 0·63, $E_{2.4}^{His}$ 0·46, $E_{5.7}^{His}$ 0·36 (electrophoresis was carried out after splitting off the benzyloxycarbonyl group with HBr in acetic acid). $[\alpha]_D^{22}$ −46·2° (*c* 0·3, dimethylformamide), −57·2° (*c* 0·3, 98% acetic acid). Amino-acid analysis: Asp 0·99, Glu 1·00, Ile 0·99, Tyr 0·98, Cys(Bzl) + Cys 1·83, Pro 0·96, Leu 1·06, Gly 1·04.

Method B: To the solution of benzyloxycarbonyl-hexapeptide *IIa* (0·11 g) and 1-hydroxybenzotriazole (0·03 g) in dimethylformamide (1 ml) we added, at 0°C, a complex of dicyclohexylcarbodiimide–pentafluorofenol (0·12 g) and, after 30 min, prolyl-leucyl-glycine amide (0·03 g). The mixture was stirred 2 h at 0°C and 42 h at room temperature and was worked up as described under method *A*) above. The yield was 0·09 g (68%) of the product which had electrophoretical and chromatographical patterns identical with those of the compound prepared under method *A*. Amino-acid analysis: Asp 1·01, Glu 1·00, Ile 1·02, Tyr 0·97, Cys(Bzl) + Cys 1·65, Pro 1·00, Leu 0·95, Gly 0·96. $[\alpha]_D^{22}$ −46·8° (*c* 0·3, dimethylformamide), −56·2° (*c* 0·3, 98% acetic acid). Literature records the values −43·1°, −50·5°, −46°, and −43° (ref.[37−40]) in dimethylformamide and −60·6°, −64·5°, −51·5°, and −56° (ref.[37−39,41]) in acetic acid.

N-Benzyloxycarbonyl-S-benzylcysteinyl-tyrosyl-isoleucyl-glutaminyl-asparaginyl-S-benzylcysteinyl-[U-^{13}C]prolyl-leucyl-glycine Amide (*IIf*)

Using the method *B*, benzyloxycarbonyl-hexapeptide *IIa* (0·87 g) and [U-^{13}C]prolyl-leucyl-glycine amide (0·87 g; obtained by hydrogenolysis of the compound *VIIc* in methanol in the presence of 5% $Pd/BaSO_4$) were coupled in dimethylformamide solution under the influence of 1-hydroxybenzotriazole (0·23 g) and complex (0·91 g). The yield was 0·89 g of the compound with m.p. 224−226°C. Precipitation from dimethylformamide solution with water afforded 0·86 g (77%) of the title compound, m.p. 225−227°C. The chromatographic and electrophoretic behavior (the latter after splitting off the benzyloxycarbonyl group) was identical with that of the non-labelled nonapeptide *IIe*. Amino-acid analysis: Asp 1·00, Glu 1·00, Ile 1·00, Tyr 0·99, Cys (Bzl) + Cys 1·69, Pro 0·92, Leu 0·98, Gly 1·01. $[\alpha]_D^{22}$ −45·6° (*c* 0·4, dimethylformamide), −58·4° (*c* 0·3, 98% acetic acid). For $C_{65}H_{86}N_{12}O_{14}S_2$ (1319 − calc. for 85% [U-^{13}C]Pro) calculated: 54·65% C, 6·57% H, 12·75% N; found: 54·92% C, 6·49% H, 12·61% N.

[7-[U-^{13}C]Proline]oxytocin (*Ib*)

The protected nonapeptide *IIf* (0·44 g) in liquid ammonia (500 ml) was reduced with sodium until the blue color persisted for 25 s. Ammonium chloride was added until the solution just

turned white. The solution was dried from the frozen state, the residue was taken up in 2% HCl (400 ml; pH of the solution was 3) and the aqueous solution was extracted with ethyl acetate and ether. The pH was then adjusted to the value of 6·9 with 0·01M-NaOH and the solution was stirred for 2 h, the pH was lowered to 3 (2% HCl) and the solution was freeze-dried. Compound *Ib* was purified by countercurrent distribution in the solvent system 2-butanol–0·05% aqueous acetic acid, 1 : 1 (320 transfers of the upper phase and 50 transfers of the lower phase). Peptide material was localized by Folin–Ciocalteau color reaction, the pooled contents of the tubes corresponding to the peak of $K = 0{\cdot}42$ were evaporated to a small bulk and freeze-dried. The compound was further purified by continuous free-flow electrophoresis (6% acetic acid, pH 2·4, 50 min); lyophilisation of the main peak (localized by absorption at 280 nm) afforded 0·09 g of the product; $E^{His}_{2.4}$ 0·41, $E^{His}_{5.7}$ 0·45; R_F 0·35 (S1), 0·37 (S2), 0·49 (S3). Amino-acid analysis: Asp 0·97, Glu 1·00, Ile 1·00, Tyr 1·01, Cys 2·02, Pro 0·99, Leu 1·01, Gly 1·01. $[\alpha]_D^{22}$ −27·4° (*c* 0·2, 1M acetic acid). Literature gives $[\alpha]_D$ −26·2° for isolated[42] and −26·1 ± 1° (*c* 0·53, water)[43], −23·1° (*c* 0·51, 1M acetic acid)[44] and −25·3° (*c* 0·4, 1M acetic acid)[4] for synthetic oxytocin. For $C_{43}H_{66}N_{12}O_{12}S_2.CH_3COOH.H_2O$ (1171) calculated: 45·76% C, 7·05% H, 14·36% N; found: 45·45% C, 6·98% H, 14·53% N. The solid phase synthesis of the title compound has been recently described[45].

N-4-Toluenesulfonyl-S-benzylcysteinyl-tyrosyl-isoleucyl-glutaminyl-asparaginyl-S-benzylcysteinyl-prolyl-leucine Amide (*IIg*)

Using the method *A*, condensation of the hexapeptide *IIc* (0·32 g) and prolyl-leucine amide (0·07 g; obtained from its benzyloxycarbonyl derivative by hydrogenolysis on 5% Pd/$BaSO_4$ in methanol) yielded 0·28 g (73%) of the protected octapeptide *IIg*, m.p. 268–270°C, R_F 0·48 (S2), 0·56 (S3), 0·78 (S4). Amino-acid analysis: Asp 1·04, Glu 1·06, Ile 0·96, Tyr 1·00, Cys(Bzl) + + Cys 1·2, Pro 0·96, Leu 0·98. $[\alpha]_D^{22}$ −30·9° (*c* 0·3, dimethylformamide), −58·1° (*c* 0·3, 98% acetic acid). Literature[12] gives m.p. 270–272 and $[\alpha]_D^{22}$ −28·6° (*c* 2, dimethylformamide) and −65° (*c* 2, acetic acid). For $C_{62}H_{83}N_{11}O_{13}S_3$ (1287) calculated: 57·88% C, 6·50% H, 11·98% N; found: 57·69% C, 6·58% H, 12·11% N.

According to the method *B*, the same amount of peptides afforded 0·26 g (68%) of the product *IIg* with the same values of m.p. and R_F as were obtained under method *A*. Amino-acid analysis: Asp 1·00, Glu 0·94, Ile 0·97, Tyr 0·93, Cys(Bzl) + Cys 1·25, Pro 1·03, Leu 1·07. $[\alpha]_D^{22}$ −29·5° (*c* 0·3, dimethylformamide), −60·5° (*c* 0·3, 98% acetic acid).

Des-9-Glycine-oxytocin (*Ic*)

The protected octapeptide *IIg* (0·23 g) in liquid ammonia (250 ml) was reduced with sodium, the solution was treated with ammonium chloride and the mixture worked up as described for the preparation of *Ib* above. The crude product was subjected to countercurrent distribution (270 transfers of the upper phase and 70 transfers of the lower phase in the solvent system 2-butanol–0·05% aqueous acid, 1 : 1; $K = 0{\cdot}57$) and to gel filtration (Bio-Gel P-4 in 1M acetic acid). The yield was 0·02 g of the title compound, R_F 0·18 (S1), 0·15 (S2), 0·10 (S3), 0·60 (S4); $E^{Gly}_{2.4}$ 0·68, $E^{His}_{2.4}$ 0·39, $E^{His}_{5.7}$ 0·30; $[\alpha]_D^{22}$ −21·2° (*c* 0·05, 1M acetic acid). Amino-acid analysis: Asp 1·00, Glu 0·99, Ile 1·00, Tyr 0·93, Cys 1·74, Pro 1·04, Leu 1·03. For $C_{41}H_{63}N_{11}O_{11}S_2.CH_3COOH.5\,H_2O$ (1082) calculated: 46·93% C, 7·05% H, 14·00% N; found: 46·98% C, 7·05% H, 14·01% N.

N-4-Toluenesulfonyl-S-benzylcysteinyl-tyrosyl-isoleucyl-glutaminyl-asparaginyl-
-S-benzylcysteinyl-prolyl-leucyl-glycine Methyl Ester (*IIh*)

Condensation of the hexapeptide *IIc* (0·32 g) and prolyl-leucyl-glycine methyl ester (0·1 g; obtained from the benzyloxycarbonyl derivative *VIIIa* by hydrogenolysis on 5% Pd/$BaSO_4$ in methanol) according to method *A*, afforded 0·33 g (80%) of the product *IIh*, m.p. 250—252°C, R_F 0·48 (S2), 0·58 (S3), 0·75 (S4); $[\alpha]_D^{22}$ −31·1° (*c* 0·2, dimethylformamide), −65° (*c* 0·2, 98% acetic acid). Amino-acid analysis: Asp 1·07, Glu 1·02, Ile 1·00, Tyr 1·00, Cys(Bzl) + Cys 1·12, Pro 1·02, Leu 1·01, Gly 1·00. For $C_{68}H_{87}N_{11}O_{15}S_3$ (1 359) calculated: 57·46% C, 6·45% H, 11·34% N; found: 57·61% C, 6·52% H, 11·14% N.

Using the method *B*, from the same amount of peptides 0·31 g (76%) of the compound *IIh* was obtained, with the same values of m.p. and R_F as in the preceding paragraph. Amino-acid analysis: Asp 1·04, Glu 0·96, Ile 0·96, Tyr 0·96, Cys(Bzl) + Cys 1·16, Pro 0·96, Leu 1·04, Gly 1·00. $[\alpha]_D^{22}$ −28·1 (*c* 0·2, dimethylformamide), −63·7° (*c* 0·2, 98% acetic acid).

Oxytocinoic Acid Methyl Ester (*Id*)

The protected nonapeptide *IIh* (0·17 g) was reduced in liquid ammonia with sodium and oxidized by aeration in aqueous solution. Analogue *Id* was purified by countercurrent distribution in the solvent system 2-butanol–0·05% aqueous acetic acid, 1 : 1 (270 transfers of the upper phase and 70 transfers of the lower phase). Besides the peak corresponding to the dimer, two more peaks with $K = 0{\cdot}49$ (*A*) and $K = 0{\cdot}64$ (*B*) were obtained. Amino-acid analysis revealed that the material from the first peak (*A*) contained only 30% glycine and from the peak *B* about 60%. This second fraction (*B*) (16 mg) was purified three times by gel-filtration of Bio-gel P—4 in 1M acetic acid. This operation afforded 5·5 mg of the product, R_F 0·15 (S2), 0·14 (S3), 0·60 (S4); $E_{2.4}^{Gly}$ 0·58, $E_{2.4}^{His}$ 0·34, $E_{5.7}^{His}$ 0·25; $[\alpha]_D^{22}$ −53·7° (*c* 0·07, 1M acetic acid). Amino-acid analysis: Asp 1·05, Glu 1·00, Ile 1·00, Tyr 0·97, Cys 1·81, Pro 1·01, Leu 0·96, Gly 0·94. For $C_{44}H_{67}N_{11}O_{13}S_2 . CH_3COOH . 5\,H_2O$ (1 172) calculated: 47·13% C, 6·96% H, 13·14% N; found: 47·02% C, 6·71% H, 13·02% N.

Pharmacological Methods

Antidiuretic activity[46] was estimated on anaesthetized rats (maintained water load of 6—8% of body weight). Pressor activity[47] was determined on despinalized rats. Milk-ejecting activity[48] was determined in lactating rats (5—10 days after parturition). The uterotonic assay was carried out on uterine strips of adult virgin rats[49,50] using Mg^{2+}-free solution.

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