CYCLOOXYTOCIN, AN OXYTOCIN ANALOGUE WITH AN ENHANCED STABILITY OF THE SECONDARY STRUCTURE* **

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By the synthesis both in liquid and solid phase, oxytocin-(2—9)-oic acid was prepared and condensed with benzyloxycarbonyl-S-benzylcysteine; the resulting product was converted to the *p*-nitrophenyl ester, decarbobenzoxylated, and cyclised. Racemisation of the asymmetric centre in the side chain of isoleucine was observed in the cyclisation step. The cyclisation product was deblocked, subjected to oxidative closure of the disulfidic bridge, and purified by a continuous free-flow electrophoresis and gel filtration. The thus-obtained cyclooxytocin displayed neither agonistic nor antagonistic effects.

The knowledge of the stereochemistry of peptides is one of the most important conditions for understanding their chemical nature and behaviour as well as their biological effects. The main obstacle in this respect is in most cases the inherent mobility of the molecule of the substance to be examined. This mobility may be decreased by various modifications, for example by the formation of bridges or by introduction of bulky substituents as mentioned by Ovchinnikov some time ago[1]. In this paper, the aimed stabilization of the secondary structure of peptides by covalent bonds is dealt with.

The stabilisation of secondary structures of peptides by covalent bonds is undoubtedly a very attracting and stimulating topic. Investigations on the spatial arrangement of peptides are thus to a certain extent approached by chemical means. This is of course neither the only reason nor the main reason. In addition to stereochemical implications, the aimed stabilisation involves a number of synthetic aspects and problems of a general importance. Such an approach could be particularly useful with biologically active peptides, especially peptide hormones, in which field inter-

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** Aside from usual symbols and abbreviations, HOBt stands for N^1-hydroxybenzotriazole and DCC designates N,N'-dicyclohexylcarbodiimide.

esting consequences may be expected. However, potential complications of principle cannot be *a priori* excluded in this case.*

In the set of conformations of the particular peptide some are more probable than the other depending on the primary structure of the peptide. The spatial arrangement of the peptide governs the spatial relations of the functional groups present and consequently, the mutual reactivity of these groups. When a stable (covalent) bond is introduced between groupings brought into proximity to each other by the spatial arrangement of the peptide, a certain conformation is stabilised (in the optimum case, similar to the starting one). In this manner, the number of degrees of freedom and the number of potential conformers of the peptide in question is reduced. Furthermore, when data on the conformation of the peptide are available, the successful realisation of the cyclisation reaction constitutes a direct chemical argument in favour of the conformation proposed. In the opposite case, the realised modification supplies a partial information on the stereochemistry of the molecule. In either case, the properly selected cyclisation reactions make possible to collect useful information on the position of side chains (both amino acid and peptide chains) which are otherwise accessible only with difficulty.

In the first attempt on an aimed stabilisation of the secondary structure of the peptide, oxytocin was used. The chemistry, stereochemistry, and biological properties of this hormone have been investigated in detail (for example[3,4]). It appeared of interest to connect the amine end and the carboxylic end of the molecule, *i.e.*, to form a bond between the amino group of Cys^1 and the carboxylic group of Gly^9, namely, to realize the preparation of cyclo-(1–9)-oxytocin (*XI*). The peptide side chain of oxytocin which forms one of the two loops in its spatial arrangement, is situated above the cyclic portion of the molecule and extends into the proximity of the cystine residue. This arrangement favours formation of a bond between positions 1 and 9. The presence of such a bond should not interfere with the formation of a disulfidic bridge. It could be inferred from the relationship between the chemical structure of oxytocin and the biological effects that the biological activity of the cyclooxytocin *XI* should be low or that the substance should be inactive at all. The carboxy-terminal amide of glycine is very important for the high activity of oxytocin and vasopressins[5,6]. In view of the obvious interaction with the tissue receptor, the peptide side chain should exhibit a sufficient mobility as the necessary prerequisite.

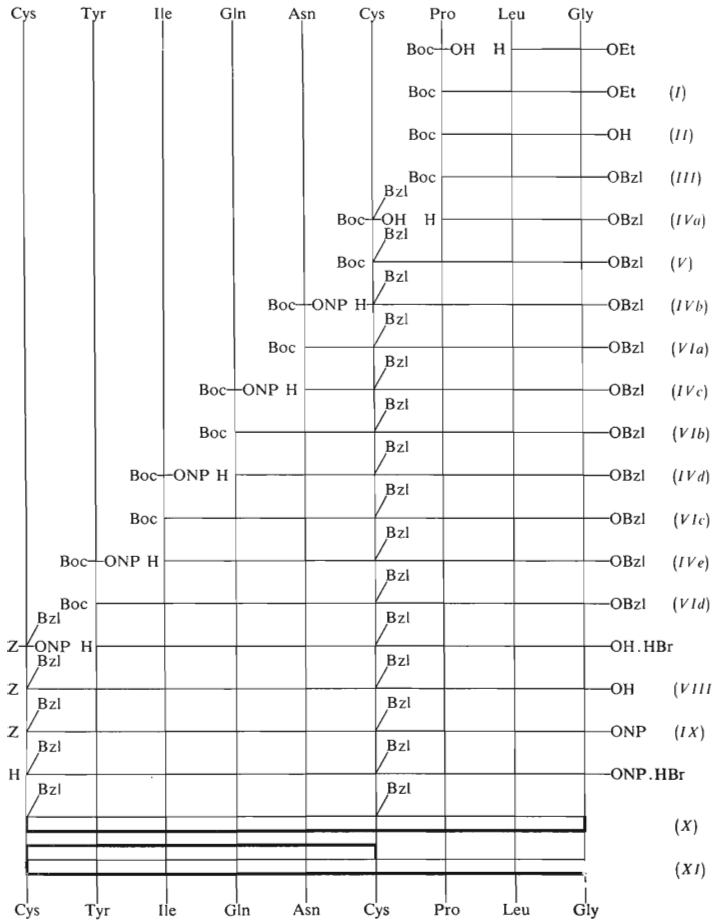
The preparation of intermediates in the synthesis of the oxytocin *XI* was performed both by the classical route (in the liquid phase) and by the solid phase process[7]. The partially protected oxytocin-(2–9)-oic acid was the key intermediate

* A partial answer in this respect is supplied by the paper of Sarantakis and coworkers[2] which was published during the preparation of our communication and which reports on the synthesis of the bicyclic analogue of somatostatin. This analogue is a significant inhibitor of secretion of the growth hormone *in vivo*.

in either case. In the classical synthesis, the acid was prepared by condensation of leucyl-glycine ethyl ester[8] with Boc-proline[9] with the use of the mixed anhydride method[10]. The thus-obtained tripeptide ester was saponified and the acid converted[11] to the benzyl ester. The Boc group was removed with a mixture of trifluoroacetic acid and dichloromethane (this mixture was applied to all subsequent removals of Boc groups). The tripeptide benzyl ester was condensed with Boc-Cys(Bzl)-OH according to the modified method of mixed anhydrides[12,13] (REMA). The peptide chain was then repeatedly lengthened with the use of *p*-nitrophenyl ester of the corresponding Boc-amino acids. In the state of the octapeptide, both the Boc group and the benzyl ester group were removed by the action of hydrogen bromide in acetic acid[14] (Scheme 1). The solid phase synthesis was in principle effected according to the reported scheme[15]. All condensation steps were repeated twice. In condensations of Boc-Asn-OH and all further Boc-amino acids, N^1-hydroxybenzotriazole was added to the reaction mixture in order to suppress dehydration of the amide groups[16] and esterification of the hydroxylic function of tyrosine. The octapeptide was liberated from the resin by the action of hydrogen bromide on a suspension of the resin in a mixture of trifluoroacetic acid and dichloromethane (Table II). The partially protected oxytocin-(2–9)-oic acid was condensed with benzyloxycarbonyl-S-benzylcysteine *p*-nitrophenyl ester[17], the resulting nonapeptide converted[18] by the action of bis (*p*-nitrophenyl) sulfite to the *p*-nitrophenyl ester, the benzyloxycarbonyl residue removed by means of hydrogen bromide in acetic acid[14], and the thus-obtained amino-ester cyclised in pyridine. The cyclisation step is accompanied by racemisation of the asymmetric centre in the side chain of isoleucine but this effect does not complicate the investigations in question. The cyclisation product was deblocked by the action of sodium in liquid ammonia[19]. The disulfide ring was closed by the action of potassium ferricyanide[20]. The crude oxidation product was desalted[21] on a column of Amberlite IRC-50 ion exchange resin and purified[22] by a continuous free-flow electrophoresis[23] and gel filtration on Sephadex G-15.

The purified cyclooxytocin *XI* was homogeneous on thin-layer chromatography, neutral on electrophoresis, and negative towards ninhydrin. In the polarography, the usual reduction wave of the disulfide ($E_{1/2}$ −0·42 V) was recorded. The UV spectrum was of the usual shape with a maximum at 275 nm and a minimum at 256 nm. The CD spectrum (in water and hexafluoroacetone hydrate) confirmed the presence of an acylated amino group at position 1 but was otherwise different from spectra of most oxytocins and vasopressins. Features of similarity may be found in spectra of some vasopressin analogues[24]. In uterotonic[25,26], pressor[27], and galactogogous[28] assays, the cyclooxytocin *XI* was inactive both as agent and inhibitor up to the dose of 5×10^{-2} mg.

The preparation of the cyclooxytocin *XI* indicates that it is possible to form a covalent bond between the NH_2^1 and $COOH^9$ groups of oxytocin and that the existence of this bond does not interfere with the formation of a disulfide bridge. The present


Cys Tyr Ile Gln Asn Cys Pro Leu Gly
Boc OH H OEt
Boc OEt (I)
Boc OH (II)
Boc OBzl (III)
Bzl
Boc OH H OBzl (IVa)
Bzl
Boc OBzl (V)
Bzl
Boc ONP H OBzl (IVb)
Bzl
Boc OBzl (VIa)
Bzl
Boc ONP H OBzl (IVc)
Bzl
Boc OBzl (VIb)
Bzl
Boc ONP H OBzl (IVd)
Bzl
Boc OBzl (VIc)
Bzl
Boc ONP H OBzl (IVe)
Bzl
Boc OBzl (VId)
Bzl
Bzl
Z ONP H OH.HBr
Bzl
Bzl
Z OH (VIII)
Bzl
Bzl
Z ONP (IX)
Bzl
Bzl
H ONP.HBr
Bzl
Bzl
(X)
(XI)
Cys Tyr Ile Gln Asn Cys Pro Leu Gly

SCHEME 1

results could be explained as follows. The oxytocin-receptor bond appears to involve both the tripeptide side chain and the cyclic moiety of oxytocin. The inactivity of the cyclooxytocin *XI* indicates that neither of these two centres is operative in the case of the analogue *XI*. An interaction between the side chain and the tissue receptor requires a certain degree of freedom of the side chain and the absence of strong interactions with the cyclic portion of the molecule. Similar requirements hold for the corresponding centres in the cyclic moiety. The tripeptide side chain of the cyclooxytocin *XI* which forms a covalent bond with the cyclic portion of the molecule, is not able to interact with the tissue receptor. The centres of the cyclic moiety are apparently hindered by this chain.

The above mentioned results justify the stabilisation of peptide molecules as one of the methods in examination of their stereochemistry and furthermore, in investigations on the relationship between chemical structure and biological effects of peptides.

EXPERIMENTAL

Melting points (uncorrected) were taken on a heated microscope stage (Kofler block). Unless stated otherwise, analytical samples were dried over phosphorus pentoxide for 24 h at 0·1 Torr and temperatures fifty degrees centigrade below their melting point value. Analyses and measurements were performed on an Amino-Acid Analyzer Type 6020 (Developmental Workshops, Czechoslovak Academy of Sciences, Prague), a Perkin-Elmer Type 141 apparatus, a VSU-2P apparatus (Carl Zeiss, Jena, German Democratic Republic), and an ORD-UV5 apparatus (Jasco, Japan). The LP-7 polarograph was used (Laboratorní přístroje, Prague). The preparative electrophoretical purification was performed on a modified apparatus according to Hannig[23] (developed and made in workshops of our Institute). The purity of substances was checked by chromatography on ready-for-use Silufol (Kavalier Glassworks, Votice, Czechoslovakia) silica gel foils in solvent systems 1-butanol–acetic acid–water (4 : 1 : 1 and 4 : 1 : 5, systems A and B), chloroform–methanol (9 : 1, system C), ethyl acetate (system D), and 1-butanol–acetic acid–pyridine–water (15 : 3 : 10 : 12, system E) and by electrophoresis on paper Whatman No 3 MM at 700 V (about 50 V/cm) in aqueous acetic acid (pH 2·5).

Tert-butyloxycarbonylprolyl-leucyl-glycine Ethyl Ester (*I*)

To a solution of Boc-Pro-OH (7·4 g; 34 mmol) and N-ethylpiperidine (4·9 g; 34 mmol) in chloroform (25 ml), ethyl chloroformate (3·24 ml; 34 mmol) was added at −10°C, followed after 5 min by introduction (at 0°C) of a solution of H-Leu-Gly-OEt[8] (7·4 g; 34 mmol) in chloroform (15 ml). The whole mixture was kept at room temperature for 3 h and evaporated under diminished pressure. The residue was dissolved in ethyl acetate (100 ml) and water (30 ml), the solution washed with 3% aqueous hydrochloric acid and saturated aqueous sodium hydrogen carbonate (three portions each), dried over anhydrous sodium sulfate, and evaporated under diminished pressure to afford 12·5 g (89%) of the crude product. Crystallisation from ether–light petroleum yielded 10·5 g (76%) of compound *I*, m.p. 95–97°C, $[\alpha]_D^{20}$ −84·8° (*c* 1·0, methanol), $[\alpha]_{436}^{20}$ −110·1° (*c* 1·0, dimethylformamide), homogeneous on chromatography in system C. Reported[29], m.p. 93–94°C, $[\alpha]_{436}^{22}$ −112·10° (*c* 1·0, dimethylformamide), and[30] m.p. 105–106°C, $[\alpha]_D^{20}$ −98·5° (*c* 0·54, acetic acid).

Tert-butyloxycarbonylprolyl-leucyl-glycine (*II*)

A mixture of compound *I* (8·0 g; 19·4 mmol), 1M-NaOH (19·4 ml), and ethanol (15 ml) was stirred at room temperature for 45 min, adjusted to pH 3—4 with dilute hydrochloric acid, and the oil extracted with ethyl acetate. The extract was dried over anhydrous sodium sulfate, evaporated under diminished pressure, and the residue (7·6 g) crystallised from ethyl acetate–light petroleum. Yield, 6·4 g (86%) of compound *II*, m.p. 98—99°C, $[\alpha]_D^{20}$ −79·9° (*c* 1·0, methanol), homogeneous on chromatography in system D. Reported[29], m.p. 99—100°C, $[\alpha]_{546}^{22}$ −76·5° (*c* 1·0, dimethylformamide).

Tert-butyloxycarbonylprolyl-leucyl-glycine Benzyl Ester (*III*)

Methanolic sodium methoxide (6·5 ml of 2M solution) was added to the acid *II* and the mixture evaporated under diminished pressure. The residue was dissolved in dimethylformamide (30 ml) and the solution heated at 90°C with benzyl chloride (2·24 ml; 19 mmol) for 90 min. The solvent was evaporated under diminished pressure, the residue dissolved in ethyl acetate (100 ml), the solution washed with saturated aqueous sodium hydrogen carbonate and water (three portions of each), dried over anhydrous sodium sulfate, and evaporated under diminished pressure. The residue (5·9 g) was recrystallised from ether–light petroleum to afford 5·0 g (81%) of compound *III*, m.p. 105—107°C; this sample was used in the next step. The analytical sample was recrystallised once more from ether–light petroleum; m.p. 113—114°C, $[\alpha]_D^{20}$ −74·1° (*c* 1·0, methanol); the substance was homogeneous on chromatography in systems C and D. For $C_{25}H_{37}N_3O_6$ (475·6) calculated: 63·14% C, 7·84% H, 8·84% N; found: 62·95% C, 7·86% H, 9·08% N.

Removal of the Boc Residue

The Boc peptide derivatives (*cf.* Scheme 1; 10 mmol each) were kept at room temperature in a 80% solution of trifluoroacetic acid in dichloromethane (25 ml) for 1 h and the mixture was then evaporated under diminished pressure. The residues were processed according to the behaviour in trituration with ether. The sticky residues (*IVa*, *IVb*) were triturated with additional three portions of ether, treated with a moderate excess of saturated aqueous sodium hydrogen carbonate, the mixture extracted with ethyl acetate, the extract dried over anhydrous sodium sulfate, evaporated under diminished pressure, and the residue used in the next step. The solid residues (*IVc*—*IVe*) were triturated with ether, collected with suction, dried under diminished pressure over a mixture of phosphorus pentoxide and potassium hydroxide pellets, and used in the next step.

Compounds *VIa*—*VId*

A solution of the appropriate *p*-nitrophenyl ester (12 mmol), the corresponding amino ester or amino ester trifluoroacetate (10 mmol each), N-methylmorpholine (10 mmol), and dimethylformamide (50 ml) was kept at room temperature for 24 h and evaporated under diminished pressure. The residue was triturated with ether (400 ml), the precipitate collected with suction, triturated with three portions of dilute hydrochlorid acid and with saturated aqueous sodium hydrogen carbonate, collected with suction, dried, and recrystallised. For the yields and properties of thus-obtained substances see Table I.

Tert-butyloxycarbonyl-S-benzylcysteinyl-prolyl-leucyl-glycine Benzyl Ester (*V*)

Ethyl chloroformate (2·17 ml; 22·3 mmol) was added at −15°C to a solution of Boc-Cys(Bzl)-OH (7·3 g; 23·5 mmol) and N-methylmorpholine (2·54 ml; 22·3 mmol) in dimethylformamide (15 ml).

After 15 min at −15°C, a precooled (−15°C) solution of compound *IVa* (6·4 g; 17 mmol) in dimethylformamide (15 ml) was added and the mixture kept at −15°C for 3 h. The temperature was then raised to 0°C and the mixture stirred at this temperature with saturated aqueous sodium hydrogen carbonate (10 ml) for 30 min. Aqueous sodium chloride (50 ml; saturated by 90% at room temperature) was then added and the mixture extracted with ethyl acetate. The extract was washed with 3% aqueous hydrochloric acid and saturated aqueous sodium hydrogen carbonate (three portions each) and water, dried over anhydrous sodium sulfate, evaporated under diminished pressure, and the residue crystallised from ethyl acetate–ether. Yield, 7·9 g (71%) of compound *V*, m.p. 131–133°C, $[\alpha]_D^{20}$ −65·5° (*c* 0·5, methanol), homogeneous on chromatography in systems A, B, and C. For $C_{35}H_{48}N_4O_7S$ (668·9) calculated: 62·85% C, 7·23% H, 8·38% N; found: 62·75% C, 7·33% H, 8·35% N.

Benzyloxycarbonyl-S-benzylcysteinyl-tyrosyl-isoleucyl-glutaminyl-asparaginyl-S-benzylcysteinyl-prolyl-leucyl-glycine (*VIII*)

Hydrogen bromide in acetic acid (25 ml of 35% solution) was added at 60°C to a solution of compound *VId* (3·0 g; 2·5 mmol) and anisol (7 ml) in glacial acetic acid (25 ml). After 15 min, the mixture was cooled down and precipitated with ether (100 ml). The flakes were washed with three 100 ml portions of ether, collected with suction, washed with ether again, and dried. The product (2·5 g) which was homogeneous on electrophoresis (pH 2·5 and 5·7) and chromatography (systems A and B), was dissolved in dimethylformamide (20 ml). Z-Cys(Bzl)-ONP (1·2 g; 2·5 mmol) and N-methylmorpholine (0·51 ml; 4·6 mmol) were then added, the whole mixture kept at room temperature for 24 h, poured into 1% aqueous hydrochloric acid (50 ml), and the precipitate collected with suction. Yield, 3·0 g (90%), m.p. 195–204°C. After crystallisation from ethanol–water, the yield was 2·7 g (78%) of compound *VIII*, m.p. 224–226°C, $[\alpha]_D^{20}$ −45·5° (*c* 1·0, di-

TABLE I

Yields and Properties of Substances Prepared by the *p*-Nitrophenyl Ester Method

Compound	Yield, % solvent system	M.p., °C $[\alpha]_D^{20}$	Formula (m.v.)	Calculated/Found		
				% C	% H	% N
VIa	81	156–158[a]	$C_{39}H_{54}N_6O_9S$	59·83	6·95	10·73
	A, C	−57·3°,[b]	(783·0)	59·73	7·17	10·94
VIb	82	204–206[c]	$C_{62}H_8N_8O_{11}S$	58·01	6·86	12·30
	A, B	−85·7°,[b]	(911·2)	58·22	7·01	12·12
VIc	85	223–225[d]	$C_{50}H_{73}N_9O_{12}S$	58·63	7·18	12·31
	A, B	−62·6°,[e]	(1 024·25)	58·44	7·15	12·10
VId	80	224–228[d]	$C_{59}H_{82}N_{10}O_{14}S$	59·68	6·96	11·80
	A, B	−38·1°,[e]	(1 187·4)	59·94	7·24	12·10

[a] From ethyl acetate; [b] methanol, c 0·5; [c] from 1 : 1 ethanol–methanol; [d] from aqueous ethanol; [e] dimethylformamide, *c* 1·0.

methylformamide), homogeneous on chromatography in solvent systems A and B. For $C_{65}H_{85}$. $N_{11}O_{15}S_2$ (1 324·6) calculated: 58·94% C, 6·47% H, 11·63% N; found: 59·37% C, 6·21% H, 10·99% N. Amino acid analysis: Cys(Bzl) 2·07, Tyr 0·91, Ile 1·04, Glu 1·03, Asp 1·03, Pro 1·01, Leu 0·99, Gly 0·94.

Benzyloxycarbonyl-S-benzylcysteinyl-tyrosyl-isoleucyl-glutaminyl-asparaginyl-S-benzyl-cysteinyl-prolyl-leucyl-glycine *p*-Nitrophenyl Ester (*IX*)

The nonapeptide *VIII* (1·32 g; 1·0 mmol) was dissolved in dimethylformamide (5 ml). To the solution, pyridine (10 ml) and bis(*p*-nitrophenyl) sulfite (3·2 g; 1·0 mmol) were added, the whole mixture kept at room temperature for 24 h, and evaporated under diminished pressure. The sticky residue was triturated with ether (50 ml), the solid collected with suction, triturated with three portions of ethyl acetate, collected again, and dried. Yield, 1·0 g (70%), m.p. 150—155°C. After crystallisation from ethanol, the m.p. value of compound *IX* was 183—186°C. Content of the active ester in the product, 94% (determined according to Iselin and coworkers[31]. For C_{71}. $H_{88}N_{12}O_{17}S_2$ (1 445·7) calculated: 58·99% C, 6·13% H, 11·63% N; found: 58·84% C, 5·91% H, 11·08% N. Optical rotation: $[\alpha]_D^{20}$ −50·2° (*c* 0·2, dimethylformamide).

Solid Phase Preparation of Tyrosyl-isoleucyl-glutaminyl-asparaginyl-S-benzylcysteinyl--prolyl-leucyl-glycine

The synthesis was performed with the use of a chloromethylated polystyrene resin crosslinked by 2% with divinylbenzene (Calbiochem, Los Angeles, U.S.A.; Cl content, 5·9%). The resin was esterified with Boc-Gly-OH in ethyl acetate in the presence of triethylamine for 72 h at the reflux temperature. The content of glycine was 0·83 mmol/g of the resin. The synthesis was carried out in a hand-manipulated synthesizer. The procedure was in principle that of ref.[15] (Table II).

TABLE II
Scheme of Solid Phase Synthesis

Step	Process	Agent	Time, min	Repetitions
1	deblocking	CF_3COOH/CH_2Cl_2 (80%)	30	1[a]
2	washing	CH_2Cl_2	3	3
3	neutralisation	$(C_2H_5)_3N$/DMF (10%)	5	3
4	washing	CH_2Cl_2	3	11[b]
5	condensation	DCC, HOBT	120[d]	2[c]
6	washing	CH_2Cl_2	3	3
7	washing	CF_3COOH/CH_2Cl_2 (5%)	5	1[a]
8	washing	CH_2Cl_2	3	3

[a] In the presence of Tyr, 5% of anisole is added. [b] Every neutralisation process is followed by a threefold washing with CH_2Cl_2; the last neutralization is followed by a fivefold washing with CH_2Cl_2. [c] HOBt was not used in condensations of Boc-Leu-OH, Boc-Pro-OH, and Boc--Cys(Bzl)-OH. The condensation of Boc-Ile-OH and Boc-Tyr-OH was repeated three times. [d] Reaction time 1 hour in condensations of Boc-Leu-OH and Boc-Pro-OH.

A three-molar excess of Boc amino acid derivatives, N,N′-dicyclohexylcarbodiimide, and N^1--hydroxybenzotriazole was used in condensations. The peptide was removed from the resin by introduction of hydrogen bromide into a suspension of the resin in the solvent mixture trifluoroacetic acid–dichloromethane–anisol (80 : 20 : 10) for 1 h. The resin was filtered off and washed with a 8% solution of trifluoroacetic acid in dichloromethane. The filtrate and washings were combined and evaporated under diminished pressure. The residue was dissolved in a little ethanol and the solution poured into ether. The hygroscopic precipitate was collected with suction to afford 4·3 g (80% with respect to the first amino acid attached to the resin) of the crude product, m.p. 156–160°C, $[\alpha]_D^{20}$ −57·5° (*c* 0·5, methanol). Amino acid analysis: Tyr 1·09, Ile 0·90, Glu 0·90, Asp 1·1, Cys(Bzl) 0·92, Pro 1·07, Leu 1·05, Gly 0·97. Chromatography in solvent systems A and B and electrophoresis on paper indicated the presence of one main component and about three contaminants (the total content of contaminants was estimated as 10 to 15% of the main product). This crude product was used directly in the condensation with benzyloxycarbonyl-S-benzylcysteine *p*-nitrophenyl ester.

Cyclooxytocine *XI*

A solution of compound *IX* (0·5 g; 0·35 mmol) in glacial acetic acid (3 ml) was combined with hydrogen bromide in acetic acid (4 ml of 35% solution), the whole heated at 60°C for 5 min and cooled down. Ether was then added (100 ml), the precipitate washed by three decantations with ether, and collected with suction. The solid (0·5 g) was dissolved in ethanol (8 ml) and the solution added dropwise over 2 h at 70°C into 400 ml of dry pyridine containing 0·2 ml of acetic acid. The mixture was kept at 70°C for 4 h and evaporated under diminished pressure. The residue was dissolved in a little methanol and the solution injected into 200 ml of ether. The precipitate (0·4 g) was collected with suction and dried. A portion of this product (200 mg) was dissolved in 500 ml of liquid ammonia (freshly distilled over sodium). Sodium was then portionwise added to the solution until the deep-blue colour persisted for 30 s. Liquid ammonia was evaporated under diminished pressure and 2·5% aqueous acetic acid (100 ml) was added to the residue. A small amount of an insoluble solid was removed by filtration. The filtrates were washed with five portions of ether and adjusted to pH 6·75 with aqueous ammonia. The oxidation step was carried out with 0·01M potassium ferricyanide. After the oxidation, the solution was adjusted to pH 4·5 with acetic acid and applied to a column of Amberlite IRC-50 ion exchange resin (30 × 1 cm). The column was washed with 0·25% aqueous acetic acid (250 ml) and the peptide material eluted with 50% aqueous acetic acid. The peptide effluent (50 ml) was diluted with water (30 ml) and freeze-dried to afford 70 mg of a material which was purified on a continuous free-flow electrophoresis (1M-CH_3COOH as carrier electrolyte, 1·5M-CH_3COOH as electrode electrolyte, 2700 V, circulating air temperature −2°C) the sample (5% solution of the freeze-dried material in 20% acetic acid) being added by the rate of 1 ml per h. The course of purification and the purity of the particular fractions was checked by colorimetry at 275 nm, paper electrophoresis, and thin-layer chromatography in solvent systems A and E. Yield, 15 mg of a product which was subjected to a further purification by gel filtration on Sephadex G-15 column (150 × 1 cm) in 1M-CH_3COOH (flow rate, 10 ml per h; the course was checked by colorimetry at 275 nm). The effluents were freeze-dried. Yield, 8·0 mg, $[\alpha]_D^{20}$ −34·5° (*c* 0·1, 2M-CH_3COOH). The elemental analysis (the sample was dried over phosphorus pentoxide at 110°C/0·1 Torr for 10 h) indicated the presence of three molecules of acetic acid and 2·5 molecules of water. For $H_{43}H_{63}N_{11}O_{12}S_2 \,.\, 3\, CH_3COOH \,.\, 2{\cdot}5\, H_2O$ (1215) calculated: 48·42% C, 6·63% H, 12·67% N; found: 48·29% C, 6·21% H, 12·95% N. Amino acid analysis: Cys 1·29, Tyr 0·96, Ile (+alle) 1·02, Glu 1·09, Asp 1·02, Pro 1·03, Leu 0·94, Gly 0·93.

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