AMINO ACIDS AND PEPTIDES. CIV.*

SYNTHESIS OF TWO ANALOGUES OF DEAMINO-OXYTOCIN WITH A DIMINISHED RING NOT CONTAINING A DISULPHIDE BOND

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Received September 11th, 1970

Two analogues of deamino-oxytocin were synthesized in which the original 20-membered cyclic structure of the oxytocin molecule is replaced by a 19-membered ring. In one of these analogues, the disulphide bond is replaced by a single sulphur atom, in the other by a methylene group. Both analogues showed oxytocin-like activity, but far less than that of deamino-oxytocin.

In previous reports in this series[1,2] we have demonstrated that the disulphide bond in the molecule of oxytocin *Ia* is not necessary for a high degree of biological activity as long as the cyclic structure of the molecule is retained. It was demonstrated that analogues in which one or both of the sulphur atoms are replaced by methylene groups have the same, or even higher, activities than deamino-oxytocin[3]** *Ib* (analogues described in the present work, just as the carba analogues described in previous reports, are not derived from oxytocin (*Ia*) but from its deamino-derivative *Ib*); in the case of deamino-dicarba-oxytocin (*Ic*) there was a decrease in biological activity, but even this analogue can be considered as highly active. It was thought to be of interest in further modifying the oxytocin molecule to alter the size of the ring structure, since this aspect apparently plays an important role in determining activity.

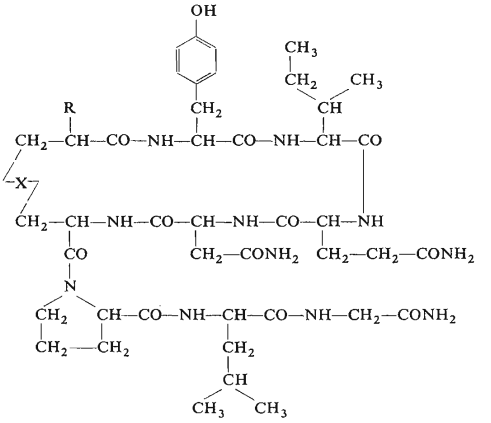
To the present, only two analogues of oxytocin with a diminished ring size have been reported. The first of these, referred to in review article[5], is de-Ile[3]-oxytocin, with 17 atoms in the ring and without oxytocin-like activity. The second is an analogue of deamino-oxytocin with 19 atoms in the ring, in which the residue of β-mercaptopropionic acid in position 1 is replaced

* Part CIII: This Journal *36*, 2480 (1971).

** All amino acids used in this work are L-configuration. Terms and symbols concur with published rules[4]. For the term "carba" analogues, see ref.[2]. Apim = residue of α-aminopimelic acid.

by a residue of mercaptoacetic acid[6]. The latter substance has oxytocin-like activity, although much decreased in comparison with deamino-oxytocin[3].

In the present work we describe the synthesis and some biological activities of two analogues derived from deamino-oxytocin, containing 19 atoms in the ring. In the first of these (*Id*) the disulphide bond is replaced by a single sulphur atom, in the second (*Ie*) by a single methylene group. The synthesis of both substances was carried out according to the procedure worked out[7] for deamino-carba[1]-oxytocin (*If*) and used successfully for two further carba-analogues[2]. The source material was again the amide of prolyl-leucyl-glycine[8,9] *II*, which was acylated with the 5-chloro-8-quinolyl ester of N-benzyloxycarbonyl-S-β-methoxycarbonylethylcysteine with the formation of protected tetrapeptide *Va*. The required active ester (*cf.*[10]) was prepared by carbodiimide synthesis from the hydrochloride of 5-chloro-8-hydroxyquinoline and the dicyclohexylammonium salt of N-benzyloxycarbonyl-S-β-methoxycarbonylethylcysteine (*III*). Introduction of the benzyloxycarbonyl protecting group in the molecule of S-β-methoxycarbonylethylcysteine (obtained by reduction of cystine with sodium in liquid ammonia and alkylation of the sulphur with methyl acrylate) proved to be rather difficult: acylation using benzyloxycarbonyl chloride gave the required product in high yield, but the reaction was poorly reproducible. The use



Ia, X = S—S, R = NH_2
Ib, X = S—S, R = H
Ic, X = CH_2—CH_2, R = H
Id, X = S, R = H
Ie, X = CH_2, R = H
If, X = CH_2—S, R = H

of sodium benzyloxycarbonylthiosulphate[11] which proved of advantage on other trials[2,7] gave very low yields. The best results were obtained by alkylation of the sulphur of benzyloxycarbonylcysteine obtained by reduction of bis-benzyloxycarbonylcystine with zinc and HCl, basically by the same method as that used by Zervas and Photaki[12] for the preparation of N-benzyloxycarbonyl-S-diphenylmethylcysteine. The benzyloxycarbonyl protecting group was removed from tetrapeptide *Va* using HBr in acetic acid and the peptide chain was extended with asparagine, glutamine, isoleucine and tyrosine by the stepwise method using active esters (in the first two cases the 2,4,5-trichlorophenyl esters, in the other cases the N-hydroxysuccinimid esters) as in previously published work[7]; the final product of these reactions was the fully protected octapeptide acid *Xa*.

The same course of reactions was used also for the synthesis of the second analogue *Ie*. α-Aminopimelic acid[13] was esterified and we introduced into the molecule of its ω-methyl ester a benzyloxycarbonyl protecting group as in previous work[2,7] using Bunte-salt[11] rather than benzyloxycarbonyl chloride. From substance *IVa* we could not, however, obtain crystalline 5-chloro-8-quinolyl ester, nor did dicyclohexylcarbodiimide condensation of substance *IVa* with tripeptide-amide *II* give crystalline tetrapeptide *Vb*. A shift from benzyloxycarbonyl protecting group to *o*-nitrobenzenesulphenyl group was also not successful: the reaction of the crystalline derivative *IVb* and substance *II* gave a non-crystalline product which was chromato-

H-Pro-Leu-Gly-NH_2 *II*

Z-Cys($C_2H_4CO_2Me$)OH *III*

X-Apim(OMe)-OH
IVa, X = Z
IVb, X = NPS

Z-X(OMe)-Pro-Leu-Gly-NH_2 *V*

NPS-Asn-X(OMe)-Pro-Leu-Gly-NH_2 *VI*

NPS-Gln-Asn-X(OMe)-Pro-Leu-Gly-NH_2 *VII*

NPS-Ile-Gln-Asn-X(OMe)-Pro-Leu-Gly-NH_2 *VIII*

H-Ile-Gln-Asn-X(OH)-Pro-Leu-Gly-NH_2 *IX*

BOC-Tyr(Bu^t)-Ile-Gln-Asn-X(OH)-Pro-Leu-Gly-NH_2 *X*

a: X = Cys(C_2H_4CO) *b*: X = Apim

graphically and electrophoretically inhomogeneous after removal of the NPS-group with HCl in ether[14]. We returned, therefore, to the non-crystalline benzyloxycarbonyl derivative *Vb*, which was (without isolation) decarbobenzoxylated with HBr in acetic acid and the chromatographically and electrophoretically pure tetrapeptide hydrobromide was acylated with the 2,4,5-trichlorophenyl ester of *o*-nitrobenzenesulphenylasparagine[15] with formation of substance *VIb*. By stepwise extension of the peptide chain through substances *VIIb*, *VIIIb* and *IXb* we produced protected octapeptide-acid *Xb*. Substances *Xa* and *Xb* were transformed by bis-*p*-nitrophenyl sulphite[16] in an atmosphere of nitrogen to the corresponding bis-*p*-nitrophenyl ester, from which the protecting groups of the tert-butyl type were removed with trifluoroacetic acid and the free octapeptideactive esters were cyclised in pyridine solution. Analogues *Id* and *Ie* were isolated from the reaction mixture in a steady-state distribution apparatus and characterised in terms of amino acid composition, elemental analysis, chromatographic and electrophoretic behaviour and optical rotation.

In Table I are presented some biological activities of the two new analogues, and for comparison activities of some closely related molecules: oxytocin[17–19], deamino-oxytocin[3,20], deamino-carba[1]-oxytocin[2], deamino-dicarba-oxytocin[2] and [mercaptoacetic acid[1]]-oxytocin[6]. Determination of uterotonic activity *in vitro*[21], vasodepressor activity[22], activity on the isolated

TABLE I
Biological Activities (IU/mg)

Substance	Rat uterus isolated	Rat uterus *in situ*	Vaso-depressor	Mammary gland *in vitro*	Antidiuretic
Oxytocin (*Ia*)	486[a]	450[b] (cat)	507[a]	533[c]	2·7[a]
Deamino-oxytocin (*Ib*)	803[d]	900[e]	975[d]	—	19[d]
Deamino-carba[1]-oxytocin (*If*)	1 898[f]	1 251[f]	1127	562[f]	21[h]
Deamino-dicarba-oxytocin (*Ic*)	93[f]	95[f]	25[f]	500[f]	3·2[f]
Analogue *Id*	9·2	20·9	6·8	42	0·17
Analogue *Ie*	2·6	0·54	0	—	0·05
[Mercaptoacetic acid[1]]-oxytocin	25[g]	—	4[g]	—	0·1[g]

[a] Ref.[17]; [b] ref.[18]; [c] ref.[19]; [d] ref.[3]; [e] ref.[20]; [f] ref.[2]; [g] ref.[6]. [h] Note added in proof: In ref.[2] the value was erroneously given as 3·75 IU/mg.

mammary gland[18] and the action on the rat blood pressure[23] were carried out by I. Krejčí of the Research Institute for Pharmacy and Biochemistry in Prague, determination of antidiuretic activity[24] and uterotonic activity *in situ*[25] by T. Barth of this Institute. Detailed results will be published by them separately.

Both analogues *Id* and *Ie* retain a certain degree, though low, of oxytocin-like activity. About the same degree of activity was attributable to [mercaptoacetic acid[1]]-oxytocin[6], an analogue which also has a diminished ring size from 20 to 19 atoms but with a disulphide bond retained. In comparison with deamino-oxytocin, all three of the diminished-ring analogues have uterotonic and vasodepressor activities about two orders of ten less. This illustrates the high steric requirements of the biological "receptor" in tissue, but also shows that the latter requirements are not absolute, at least absolute for a change of one atom in the ring number. In comparing the two analogues *Id* and *Ie* with each other, it would appear that the presence of the one sulphur atom was associated with a slightly higher activity. The same is true for the series of three carba analogues with full ring number – both 1- and 6-monocarba compounds were more active than the dicarba analogue with no sulphur at all [2].

All of the known carba analogues to date provide highly suggestive evidence that the disulphide bond *per se* is not necessary for biological activity; in all cases, however, the presence of at least one atom of sulphur in the ring is associated with higher levels of activity. This may be related to the polarity of the sulphur atom as compared with a methylene group or in the steric result of the presence of sulphur in this portion of the molecule in terms of bond length, bond angle, rotation about these bonds, effective volume, *etc.* Present data do not allow us to differentiate between these possibilities, however.

EXPERIMENTAL

Analytical and General Procedures

Samples for elemental analysis were dried for 8–12 *in vacuo* (1 Torr) at 40–70°C. For thin-layer chromatography we used silica gel plates (Kieselgel G, Merck) and solvent systems: 2-butanol–25% ammonia–water (85 : 7·5 : 7·5) (S_1), 2-butanol–90% formic acid–water (75 : 13·5 : 11·5) (S_2), and pyridine–1-butanol–acetic acid–water (10 : 15 : 3 : 6) (S_3). Electrophoretic analyses were carried out in a moist chamber on Whatman 3 MM paper in solvent systems : 1M acetic acid (pH 2·4) and pyridine–acetic acid (pH 5·7) in an electric field of 20 V/cm for 60 min. For detection we used the ninhydrin reaction or chlorination (on paper[26] or silica gel plates[27]). Where R_F and E values are given, pure substance was used. Amino acid analyses were carried out after 20 h hydrolysis in 6M-HCl at 105°C on an automatic analyser[28].

Evaporation of the reaction mixtures was carried out with a rotatory evaporator at a bath temperature of 30–35°C. When mixtures contained dimethylformamide we used an oil vacuum pump.

S-β-Methoxycarbonylethylcysteine

Cystine (4·8 g) was reduced with sodium (1·5 g) in liquid ammonia (250 ml). The reaction mixture was decolourised with ammonium chloride and ammonia was removed by freeze drying. The remainder was dissolved in water (80 ml) with nitrogen bubbling, the pH was adjusted to 8·1 and methyl acrylate (8 ml) was added over 1 hour. The reaction mixture was bubbled through with nitrogen at room temperature for 24 h, pH was adjusted to 6·0 with acetic acid and the solution was cooled to 0°C. The small amount of crystals which separated out was filtered and the filtrate was put on a column of Dowex 50 (H^+-cycle, 200 ml). The column was washed through with water and the product was eluted with 10% aqueous pyridine. By electrophoretic analysis this contained about 10% of dicarboxylic acid. After dissolving in water the mixture was put onto a column of Amberlite IR-4B. The eluate was evaporated and the residue was crystallised from aqueous methanol. The yield was 5·35 g (64%) of a product with m.p. 205—208°C (Kofler); R_F 0·18 (S_1), 0·53 (S_2), 0·49 (S_3); $E_{2.4}^{Gly}$ 0·37, $E_{5.7}^{Gly}$ 1·00. The sample for analysis was recrystallised in the same manner, m.p. 216—218°C (Kofler), 223—225°C (capillary); $[\alpha]_D$ −22·9° (*c* 0·49, water). For $C_7H_{13}NO_4S$ (207·3) calculated: 40·56% C, 6·32% H, 6·76% N; found: 40·85% C, 6·26% H, 6·95% N. The literature[29] presents for a product produced in a similar manner m.p. of 176—178°C, another source[30] reports "approximately 180°"; optical rotation is not presented in these papers.

S-β-Carboxyethylcysteine

To a solution of S-β-methoxycarbonylethylcysteine (1 g) in methanol (10 ml) we added 1M-NaOH (10 ml). After one hour standing at room temperature the methanol was evaporated and the aqueous solution was filtered through a column of Amberlite IRC-50 (ammonium cycle, 28 × 1·2 cm). The eluate was evaporated and the remainder was dissolved in water and diluted with ethanol. The crystals which separated out were filtered and washed on the filter with ethanol and ether. The yield was 0·85 g (92%) of a product with m.p. 219—221°C (Kofler); R_F 0·03 (S_1), 0·45 (S_2), and 0·31 (S_3); $E_{2.4}^{Gly}$ 0·35, $E_{5.7}^{Asp}$ 0·70; $[\alpha]_D$ −10·4° (*c* 0·50, 1M-HCl), −31·8°C (*c* 0·51, water). For $C_6H_{11}NO_4S$ (193·2) calculated: 37·30% C, 5·74% H, 7·25% N; found: 37·21% C, 5·83% H, 7·03% N. The literature presents m.p. of 227—230°C and $[\alpha]_D$ −7·0° (*c* 1, 1M-HCl)[31], or −8·51° (1M-HCl) with m.p. of 214—216°C[30] or 218—220°C with $[\alpha]_D$ −8·19° or −8·39° (1M-HCl)[32] or m.p. of 218°C and $[\alpha]_D$ −9·33° (*c* 3, 1M-HCl)[33] or m.p. of 217°C and $[\alpha]_D$ −25° (water)[34].

The Dicyclohexylammonium Salt of N-Benzyloxycarbonyl-S-β-methoxycarbonylethylcysteine (*III*)

a) *Using benzyloxycarbonyl chloride*: To a solution of S-β-methoxycarbonylethylcysteine (3·65 g) in 0·5M-$NaHCO_3$ (75 ml) we added in aliquots a total of 5·0 ml benzyloxycarbonyl chloride with constant mixing (Vibromisher) and cooling with ice. The pH of the solution was maintained at 7·0 to 7·5 at a temperature of 0°C for 20 min and then for 1 h at room temperature.

The reaction mixture was shaken with ether and acidified to pH 3 with HCl. An oil which separated out was taken into ether, the ether layer was then extracted with water, dried with sodium sulphate and the ether was evaporated off. The residue was azeotropically dried with benzene, dissolved in benzene, and after addition of dicyclohexylamine (3 ml) the solution was diluted with light petroleum. After cooling to 0°C crystals separated out which were filtered and washed with light petroleum. Crystallisation from ethyl acetate gave a yield of 6·6 g (73%) of a product with m.p. 119—121°C (capillary). The sample for analysis was recrystallised in the same manner, m.p. 121—122°C (capillary); $[\alpha]_D$ +3·6° (*c* 0·49, dimethylformamide). For C_{27}-

$H_{42}N_2O_6S$ (522·7) calculated: 62·04% C, 8·10% H, 5·36% N; found: 62·16% C, 8·13% H, 5·21% N.

b) *Bunte-salt method*[11]: A solution of S-β-methoxycarbonylethylcysteine (4·7 g) and sodium benzyloxycarbonyl thiosulphate (6·2 g) in water (80 ml) and 0·5M-$NaHCO_3$ (105 ml) was mixed at room temperature for 2·5 h. The pH of this solution was maintained at 8·0—8·5 by addition of 1M-NaOH (total 18 ml), then acidified to pH 3 (HCl), the product was taken up into ether, the etherial solution was washed with water, dried with sodium sulphate and evaporated to dryness. The residue was azeotropically dried (benzene), dissolved in benzene, and after addition of dicyclohexylamine (4·45 ml) it was diluted with light petroleum. The yield was 0·98 g, m.p. 110—115°C (capillary). Crystallisation from ethyl acetate gave a yield of 0·67 g (7%), m.p. 120—121°C, without depression on admixture of a sample prepared under *a*) above.

c) *Through alkylation of benzyloxycarbonylcysteine*: Bis-benzyloxycarbonylcystine (5 g) was reduced according to described procedure[12] in a mixture of methanol (20 ml) and conc. HCl (6·5 ml) using powdered Zn (3 g) at 5—10°C. N-Benzyloxycarbonylcysteine was azeotropically dried with benzene, dissolved in benzene (20 ml) and to this solution was added triethylamine (2·8 ml) and methyl acrylate (8 ml). After 48 h standing at room temperature the reaction mixture was evaporated to dryness and the dry powder was dissolved in ethyl acetate. This solution was consecutively washed with 1M-HCl, water, then dried with sodium sulphate and evaporated again. The remainder was dissolved in ethyl acetate and after addition of dicyclohexylamine (4 ml) the solution was diluted with light petroleum. The excluded crystals (4·5 g; m.p. 116 to 119°C) were crystallised again from ethyl acetate. The yield was 3·8 g (75%) of a product with m.p. 120—121°C (capillary), undepressed by admixture of a sample prepared under *a*) above.

5-Chloro-8-quinolyl ester of N-benzyloxycarbonyl-S-β-methoxycarbonylethylcysteine

To a solution of the dicyclohexylammonium salt of N-benzyloxycarbonyl-S-β-methoxycarbonylethylcysteine (3·50 g) and the hydrochloride of 5-chloro-8-hydroxyquinoline (1·60 g) in a mixture of chloroform (200 ml) and dimethylformamide (10 ml) at —20°C we added dicyclohexylcarbodiimide (1·50 g). The reaction mixture was stirred for 1 h at —10°C, overnight at room temperature, evaporated to dryness and the dry powder was dissolved in a mixture of ethylacetate and 1M-HCl. The ethyl acetate layer was successively washed with 1M-HCl, water, 0·5M-$NaHCO_3$ and water, dried with sodium sulphate and evaporated. The remainder was triturated with ether, and the resulting crystals were filtered and washed with ether. The yield was 2·15 g (64%) of a product with m.p. 97—99°C (capillary); this product was used for the next stage of synthesis. The sample for analysis was crystallised from a mixture of ethyl acetate and light petroleum, m.p. 112—115°C (capillary); $[\alpha]_D$ —39·4° (*c* 0·50, dimethylformamide). For $C_{24}H_{23}ClN_2O_6S$ (503·0) calculated: 57·30% C, 4·61% H, 5·57% N, 7·05% Cl; found: 57·27% C, 4·25% H, 5·85% N, 6·95% Cl.

The Amide of N-Benzyloxycarbonyl-S-β-methoxycarbonylethylcysteinyl-prolyl-leucyl-glycine (*Va*)

The amide of prolyl-leucyl-glycine[8,9] (1·32 g) and the chloroxin ester of N-benzyloxycarbonyl-S-β-methoxycarbonylethylcysteine (2·25 g) were dissolved in dimethylformamide (44 ml). After two days standing at room temperature the reaction mixture was evaporated, the remainder was ground with light petroleum, the light petroleum was then decanted and the remainder was again ground up with 1M-HCl. The resulting crystals were filtered and washed with 1M-HCl, then water, 0·5M-$NaHCO_3$, water again and dried. The yield was 2·25 g (84%) of a product of m.p. 143—145°C (capillary). The sample for analysis was crystallised from a mixture of 2-propanol and light petroleum, m.p. 146—148°C (capillary); $[\alpha]_D$ —56·4° (*c* 0·50, dimethylformamide).

For $C_{28}H_{41}N_5O_8S.0{\cdot}5\ H_2O$ (616·7) calculated: 54·53% C, 6·87% H, 11·36% N; found: 54·49% C, 6·80% H, 11·57% N.

The Amide of N-*o*-Nitrobenzenesulphenylasparaginyl-S-β-methoxycarbonylethylcysteinyl-prolyl-leucyl-glycine (*VIa*)

To a solution of protected tetrapeptide *Va* (2·5 g) in acetic acid (12·5 ml) we added a 35% solution of HBr in acetic acid (25 ml). After 10 min standing at room temperature the reaction mixture was diluted with ether, the hydrobromide which separated out was washed several times with ether and dried in an exsiccator. After dissolving in dimethylformamide (25 ml) the solution was neutralised with N-ethylpiperidine (pH about 8·5, wet pH paper) and to this we added 2,4,5-trichlorophenyl ester of *o*-nitrobenzenesulphenylasparagine[15] (2 g). After 24 h standing at room temperature we added a further 1 g of active ester and after a further 24 h standing the reaction mixture was evaporated to dryness, the dry powder was triturated with light petroleum and ether, the crystalline portion was filtered and washed with ether, then water, 0·5M-$NaHCO_3$ and finally water. The yield was 3·0 g (98%) of a product of m.p. 172–175°C (capillary). The sample for analysis was crystallised from a mixture of dimethylformamide and ether, m.p. 177–180°C (capillary); $[\alpha]_D$ −79·0° (*c* 0·51, dimethylformamide). For $C_{30}H_{44}N_8O_{10}S_2.0{\cdot}5\ H_2O$ (749·8) calculated: 48·05% C, 6·05% H, 14·95% N; found: 47·94% C, 6·00% H, 15·02% N.

The Amide of *o*-Nitrobenzenesulphenylglutaminyl-asparaginyl-S-β-carbomethoxyethylcysteinyl-prolyl-leucyl-glycine (*VIIa*)

To a solution of protected pentapeptide *VIa* (2·85 g) in dimethylformamide (14 ml) we added 2·2M-HCl in ether (4·2 ml). After 4 min standing at room temperature the reaction mixture was diluted with ether (250 ml), the excluded hydrochloride was filtered and washed with ether; $E^{Gly}_{2.4}$ 0·84, $E^{His}_{5.7}$ 0·53; R_F 0·26 (S_1), 0·20 (S_2). After drying in an exsiccator the pentapeptide hydrochloride was dissolved in dimethylformamide (35 ml), the solution was alkalinised with N-ethylpiperidine (pH about 8·5, moist pH paper) and we added 2,4,5-trichlorophenyl ester of *o*-nitrobenzenesulphenylglutamine[15] (2·3 g). After 24 h standing we added a further portion of active ester (1·2 g) and after a further 24 h the reaction mixture was evaporated to dryness, the dry powder was triturated with light petroleum and ether, the crystalline portion was filtered and washed with ether, then water, 0·5M-$NaHCO_3$ and then water. Crystallisation from a mixture of dimethylformamide and ether gave a yield of 2·05 g (62%) of a product with m.p. 208–210°C (capillary). The sample for analysis was recrystallised in the same manner, m.p. 210–212°C (capillary); $[\alpha]_D$ −46·6° (*c* 0·25, dimethylformamide). For $C_{35}H_{52}N_{10}O_{12}S_2.0{\cdot}5\ H_2O$ (877·9) calculated: 47·88% C, 6·09% H, 15·96% N; found: 47·80% C, 6·13% H, 15·78% N.

The Amide of *o*-Nitrobenzenesulphenylisoleucyl-glutaminyl-asparaginyl-S-β-carbomethoxyethylcysteinyl-prolyl-leucyl-glycine (*VIIIa*)

The NPS-protective group was split off from the protected hexapeptide *VIIa* (1·85 g) in the usual manner (16 ml dimethylformamide, 2·4 ml 2·2M-HCl in ether, 4 min); $E^{Gly}_{2.4}$ 0·71, $E^{His}_{5.7}$ 0·39; R_F 0·03 (S_1), 0·12 (S_2). After drying in an exsiccator the hexapeptide hydrochloride was dissolved in dimethylformamide (30 ml) and we added N-ethylpiperidine (0·5 ml) and the N-hydroxysuccinimid ester of *o*-nitrobenzenesulphenylisoleucine[7] (1·1 g). After 24 h standing at room temperature a further portion of active ester (0·5 g) was added to the reaction mixture and after a further 24 h the mixture was evaporated and worked up in the same manner as described for substance *VIIa*. The product was extracted with hot ethyl acetate, filtered and washed with ethyl acetate

and ether. The yield was 1·65 g (79%) of a product with m.p. 240—242°C (capillary); the sample for analysis was crystallised from a mixture of dimethylformamide–ether, m.p. 245—247°C (capillary) $[\alpha]_D$ −74·0° (*c* 0·51, dimethylformamide). For $C_{41}H_{63}N_{11}O_{13}S_2.H_2O$ (1 000) calculated: 49·23% C, 6·55% H, 15·41% N; found: 49·33% C, 6·63% H, 15·22% N.

The Amide of Isoleucyl-glutaminyl-asparaginyl-S-β-carboxyethylcysteinyl-prolyl-leucyl-glycine (*IXa*)

To a solution of protected heptapeptide *VIIIa* (1·35 g) in dimethylformamide (27 ml) we added 2·2M-HCl in ether (1·75 ml). After 4 min at room temperature the reaction mixture was diluted with ether (250 ml), the excluded hydrochloride was filtered and washed with ether; $E^{His}_{5.7}$ 0·42, $E^{Gly}_{2.4}$ 0·75; R_F 0·03 (S_1), 0·12 (S_2). After drying in an exsiccator the product was dissolved in a mixture of methanol (20 ml) and 1M-NaOH (10 ml). The reaction mixture was stirred for 1 h at room temperature, the methanol was evaporated off and the aqueous solution was transferred to a column of Dowex 50 (H^+-cycle; 60 ml). The column was washed through with water and the product was eluted with 10% pyridine. The eluate was evaporated to dryness and the dry powder was crystallised from a mixture of methanol and ether. The yield was 0·95 g (82%); $E^{Gly}_{2.4}$ 0·67, $E^{His}_{5.7}$ 0·08; R_F 0·11 (S_1), 0·43 (S_2). Amino acid analysis: Asp 1·00, Glu 1·06, Pro 0·96, Gly 1·00, Cys ($C_2H_4CO_2H$) 1·10, Ile 0·97, Leu 1·04. The sample for analysis was recrystallised from a mixture of dimethylformamide and ether; $[\alpha]_D$ −12·4°, (*c* 0·50, dimethylformamide). For $C_{34}H_{58}.N_{10}O_{11}S\ 2\ H_2O$ (851·0) calculated: 47·98% C, 7·35% H, 16·48% N; found: 47·95% C, 7·29% H, 15·91% N.

The Amide of Tert-butyloxycarbonyl-O-tert-butyltyrosyl-isoleucyl-glutaminyl-asparaginyl-S-β-carboxyethylcysteinyl-prolyl-leucyl-glycine (*Xa*)

To a solution of the free heptapeptide-amide *IXa* (0·80 g) in dimethylformamide (15 ml) we added N-ethylpiperidine (0·3 ml) and N-hydroxysuccinimid ester of N-tert-butyloxycarbonyl-O-tert-butyltyrosine[7] (0·90 g). After 3 days standing at room temperature the reaction mixture was evaporated to dryness, the dry powder was ground up with light petroleum and ether, the crystalline portion was filtered and washed with ether, water, 3% citric acid and then water. The yield was 0·90 g of a product with m.p. 207—211°C (Kofler). Extraction with hot methanol gave 0·50 g of a substance with m.p. 216—218°C (Kofler) and from the mother liquors we had a further yield of 0·23 g of a substance with m.p. 195—197°C (Kofler). The substance with the higher melting point was used to prepare the sample for analysis by crystallisation from a mixture of dimethylformamide and ether, m.p. 219—220°C (Kofler); $[\alpha]_D$ −39·7° (*c* 0·50, dimethylformamide). For $C_{52}H_{83}N_{11}O_{15}S.2\ H_2O$ (1170) calculated: 53·36% C, 7·49% H, 13·17% N; found: 53·58% C, 7·48% H, 13·48% N.

The Lactam of Tyrosyl-isoleucyl-glutaminyl-asparaginyl-S-β-carboxyethylcysteinyl-prolyl-leucyl-glycinamide (*Id*)

To a solution of protected octapeptide-acid *Xa* (307 mg) in a mixture of dimethylformamide (10 ml) and pyridine (10 ml) we added bis-*p*-nitrophenyl sulphite[16] (1 g) with constant stirring and bubbling through of nitrogen gas. The reaction mixture after 12 h was diluted with pyridine (5 ml) and we added a further portion of reagent (1 g). After a further 8 h the mixture received a further 0·5 g bis-*p*-nitrophenyl sulphite and after a further 8 h the mixture was evaporated to dryness, the dry powder was triturated with ether, filtered and washed with ether and water. After drying the product was dissolved in trifluoroacetic acid (10 ml), after 1 h standing at room

temperature the solution was diluted with toluene (10 ml) and evaporated. The remnant was dissolved in dimethylformamide (10 ml) and this solution was added over 4 h to pyridine (250 ml) with constant stirring, nitrogen bubbling and heating to a temperature 50°C. The reaction mixture was left standing for 12 h at room temperature and then evaporated to dryness, the dry powder was triturated with ether, filtered, washed with ether and dried. The product was purified in the Steady State Distribution Machine (Quickfit et Quartz, Ltd., Staffordshire, England) in a system 2-butanol and 0·05% acetic acid (1 : 1). After 110 transfers of the upper phase the peak of the product ($K = 1{\cdot}89$; localisation by the Folin reaction) was isolated by evaporation and freeze-drying. The yield was 122·5 mg (45%); R_F 0·18 (S_1), 0·34 (S_2), 0·64 (S_3). On paper electrophoresis in two different buffers (1M acetic acid pH 2·4 and pyridine–acetic acid pH 5·7) the substance showed no migration. For analysis and biological testing the product was further purified by gel filtration on Biogel P-2 and P-4 in 1M acetic acid and then recrystallised form a mixture of methanol and ether; $[\alpha]_D$ −73·1° (*c* 0·22, 1M acetic acid). Amino acid analysis: Asp 1·00, Glu 1·08, Pro 0·94, Gly 0·99, Ile 0·99, Leu 1·00, Tyr 0·84, Cys($C_2H_4CO_2H$) 1·05. For $C_{43}H_{65}N_{11}O_{12}S.4\,H_2O$ (1032) calculated: 50·04% C, 7·13%H, 14·93% N; found: 50·03% C, 6·60% H, 15·05%N.

The Hydrochloride of the ω-Methyl Ester of α-Aminopimelic Acid

α-Aminopimelic acid[13] (2·7 g) was dissolved in a mixture of methanol (40 ml) and 4·15M-HCl in methanol (12·7 ml). After 30 min standing at room temperature the reaction mixture was evaporated and the remainder was twice evaporated after addition of methanol. Crystallisation from a mixture of methanol (20 ml) and ether (70 ml) gave a yield of 2·2 g (63%) of a product with m.p. 222–225°C (Kofler); $E^{Gly}_{2.4}$ 0·65, $E^{Gly}_{5.7}$ 1·00; R_F 0·14 (S_1), 0·51 (S_2). The sample for analysis was recrystallised in the same manner, m.p. 225–227°C (Kofler). For $C_8H_{16}ClNO_4$ (225·7) calculated: 42·57% C, 7·14% H, 6·20% N; found: 43·08% C, 7·25% H, 5·89% N.

The Dicyclohexylammonium Salt of the ω-Methyl Ester of N-benzyloxycarbonyl-α-aminopimelic Acid (*IVa*)

a) *The Bunte-salt method*[11]: To a solution of the hydrochloride of the ω-methyl ester of α-aminopimelic acid (1·22 g) in 0·5M-$NaHCO_3$(80 ml) we added sodium benzyloxycarbonyl thiosulphate[11] (1·65 g). The reaction mixture was stirred at room temperature for 2 h, pH was maintained at 8·0–8·5 by addition of 1M-NaOH (total added, 8·5 ml). The solution was then acidified with HCl to pH 3, the product was taken into ether, the etheric solution was extracted with water and then dried with sodium sulphate and evaporated. The remainder was azeotropically dried (benzene), dissolved in benzene and to it was added dicyclohexylamine (1·3 ml) and light petroleum. After cooling to 0°C the crystals which separated out were filtered and washed with light petroleum. The yield was 2·2 g (82%) of a product with m.p. 103–106°C (capillary). The sample for analysis was crystallised from a mixture of 2-propanol and light petroleum, m.p. 107–109°C (capillary); $[\alpha]_D$ +4·4° (*c* 0·5, dimethylformamide). For $C_{28}H_{44}N_2O_6$ (504·7) calculated: 66·63% C, 8·79% H, 5·55% N; found: 66·44% C, 8·70% H, 5·63% N.

b) *The benzyloxycarbonyl chloride method*: To a solution of the hydrochloride of the ω-methyl ester of α-aminopimelic acid (1·0 g) in a mixture of 5% $NaHCO_3$ (15 ml) and 5% Na_2CO_3 (15 ml) we added benzyloxycarbonyl chloride (0·8 ml) with stirring and cooling with ice. The reaction mixture was stirred for 30 min at 0°C and 1·5 h at room temperature, then extracted with ether, acidified to pH 3, the product was taken up into ether and the latter solution was washed with 1M-HCl, water, dried with sodium sulphate and evaporated. The remainder was dissolved in ether and after addition of dicyclohexylamine (0·8 ml) the solution was diluted with light petroleum and cooled to 0°C. The crystals which separated out were filtered and washed with

light petroleum. The yield was 0·75 g (33%), m.p. 100—103°C (capillary). Crystallisation from a mixture of 2-propanol and light petroleum gave 0·65 g (29%) of a product with m.p. 103—106°C (capillary), undepressed by admixture of a sample prepared under *a*) above.

The Dicyclohexylammonium Salt of the ω-Methyl Ester of N-*o*-Nitrobenzenesulphenyl-α-aminopimelic Acid (*IVb*)

To a solution of the hydrochloride of the ω-methyl ester of α-aminopimelic acid (0·48 g) in chloroform (5 ml) we added triethylamine (0·48 g) and *o*-nitrobenzenesulphenyl chloride (0·38 g). The solution was stirred at room temperature for 2 h, then three times extracted with water, dried with sodium sulphate and evaporated. The residue was dissolved in ethyl acetate, the solution after the addition of dicyclohexylamine (0·22 ml) was cooled to 0°C, the separated crystals were filtered and washed with ethyl acetate and light petroleum; the yield was 0·55 g (52%) of a product with m.p. 160—162°C (Kofler). A sample for analysis was twice crystallised from methanol, m.p. 163—165°C (Kofler); $[\alpha]_D$ −41·9° (*c* 0·51, dimethylformamide). For $C_{26}H_{41}N_3O_6S$ (523·7) calculated: 59·63% C, 7·89% H, 8·02% N; found: 59·88% C, 7·68% H, 7·90% N.

The Amide of the ω-Methyl Ester of *o*-Nitrobenzenesulphenyl-asparaginyl-α-aminopimelyl-prolyl-leucyl-glycine (*VIb*)

A solution of the dicyclohexylammonium salt of the ω-methyl ester of N-benzyloxycarbonyl-α-aminopimelic acid (*IVa*) (1·95 g) in a mixture of methanol (40 ml) and water (25 ml) was stirred for 30 min at room temperature along with Dowex 50 (H^+-cycle, 30 ml). The ion-exchanger was filtered, washed with methanol and the combined filtrates were evaporated and dried azeotropically with benzene. After dissolving in dimethylformamide (20 ml) we added the amide of prolyl-leucyl-glycine[8,9] (1·28 g) and N-hydroxysuccinimide (0·48 g). The solution was cooled to −20°C and we added with stirring dicyclohexylcarbodiimide (0·84 g). The reaction mixture was stirred for 1 h at −10°C, 2 h at 0°C, 12 h at room temperature, the separated dicyclohexylurea was filtered and the filtrate was evaporated. The remainder was triturated with light petroleum, dissolved in ethylacetate and this solution was successively washed with 1M-HCl, water, 0·5M-$NaHCO_3$, water, dried with sodium sulphate and evaporated. The remainder was dried azeotropically with benzene, dissolved in acetic acid (8 ml) and to this was added 35% HBr in acetic acid (20 ml). After 10 min standing at room temperature the reaction mixture was diluted with ether, the hydrobromide which separated out was washed with ether and dried in an exsiccator; $E^{His}_{5,7}$ 0·72, $H^{His}_{2,4}$ 0·56. The product was dissolved in dimethylformamide (24 ml) and we added N-ethylpiperidine (2 ml) and 2,4,5-trichlorophenyl ester of N-*o*-nitrobenzenesulphenylasparagine[15] (2 g). After 24 h standing at room temperature a further aliquot of active ester (1 g) was added and after a further 2 days the reaction mixture was evaporated, the remainder was ground up with light petroleum and ether, the crystalline portion was filtered and washed with ether, then water, 0·5M-$NaHCO_3$ and again water. The yield was 1·8 g (64%, calculated on the dicyclohexylammonium salt of *IVa*) of a product with m.p. 169—170°C (capillary). The sample for analysis was recrystallised from a mixture of dimethylformamide and ether, m.p. 175—177°C (capillary); $[\alpha]_D$ −67·8° (*c* 0·50, dimethylformamide). For $C_{31}H_{46}N_8O_{10}S.H_2O$ (740·8) calculated: 50·26% C, 6·40% H, 15·13% N; found: 50·57% C, 6·44% H, 15·36% N.

The Amide of the ω-Methyl Ester of *o*-Nitrobenzenesulphenyl-glutaminyl-asparaginyl-α-aminopimelyl-prolyl-leucyl-glycine (*VIIb*)

To a solution of protected pentapeptide *VIb* (1·6 g) in dimethylformamide (17 ml) we added 2M-HCl in ether (2·8 ml). After 4 min standing at room temperature the solution was diluted

with ether and the hydrochloride which separated out was collected and washed with ether; $E^{Gly}_{2,4}$ 0·87, $E^{His}_{5,7}$ 0·32; R_F 0·22 (S_1), 0·24 (S_2). After drying in an exsiccator the product was dissolved in dimethylformamide (28 ml) and N-ethylpiperidine (0·8 ml) was added, along with the 2,4,5-trichlorophenyl ester of N-*o*-nitrobenzenesulphenylglutamine[15] (1·3 g). After 15 h standing at room temperature a further portion of the active ester (0·5 g) was added to the reaction mixture and after a further 24 h the mixture was evaporated to dryness. The remainder was triturated with light petroleum and ether, the crystalline portion was filtered and washed with ether. The product was crystallised from a mixture of dimethylformamide and ether and recrystallised from a mixture of methanol and ether. The yield was 1·45 g (77%) of a product with m.p. 192–195°C (Kofler); a sample for analysis was recrystallised from a mixture of dimethylformamide–ether, m.p. 195–197°C (Kofler); $[\alpha]_D$ −52·1° (*c* 0·50, dimethylformamide). For $C_{36}H_{54}N_{10}O_{12}S.0{\cdot}5\ H_2O$ (859·9) calculated: 50·28% C, 6·45% H, 16·29% N; found: 50·12% C, 6·20% H, 15·81% N.

The Amide of the ω-Methyl Ester of *o*-Nitrobenzenesulphenyl-isoleucyl-glutaminyl-asparaginyl-α-aminopimelyl-prolyl-leucyl-glycine (*VIIIb*)

To a solution of protected hexapeptide *VIIb* (1·45 g) in dimethylformamide (7 ml) we added 2M-HCl in ether (2·20 ml). After 5 min standing at room temperature the reaction mixture was diluted with ether, and hydrochloride which separated out was filtered and washed with ether; $E^{Gly}_{2,4}$ 0·73, $E^{His}_{5,7}$ 0·34; R_F 0·07 (S_1), 0·12 (S_2), 0·45 (S_3). After drying in an exsiccator the product was dissolved in dimethylformamide (25 ml) and to the solution was added N-ethylpiperidine (0·5 ml) and the N-hydroxysuccinimid ester of N-*o*-nitrobenzenesulphenylisoleucine[7] (0·75 g). After 24 h standing at room temperature a further aliquot of active ester (0·4 g) was added to the reaction mixture and after a further 24 h the solution was evaporated to dryness, the residue was triturated with light petroleum and ether, the crystalline portion was filtered and washed with ether, then water, 0·5M-$NaHCO_3$ and again water. Crystallisation from a mixture of dimethylformamide and ether gave a yield of 1·15 g (70%) of a product with m.p. 236–239°C (capillary). The sample for analysis was recrystallised in the same manner, m.p. 242–244°C (capillary); $[\alpha]_D$ −70·3° (*c* 0·50, dimethylformamide). For $C_{42}H_{65}N_{11}O_{13}S.2\ H_2O$ (1000) calculated: 50·44% C, 6·96% H, 15·41% N; found: 50·60% C, 6·98% H, 15·45% N.

The Amide of Isoleucyl-glutaminyl-asparaginyl-α-aminopimelyl-prolyl-leucyl-glycine (*IXb*)

To a solution of the protected heptapeptide *VIIIb* (1 g) in dimethylformamide (25 ml) we added 2M-HCl in ether (1·5 ml). After 4 min at room temperature the reaction mixture was diluted with ether, the hydrochloride which separated out was filtered, washed with ether, dried and hydrolysed in a methanolic solution (12·5 ml) with 1M-NaOH (5 ml) for 1 h. After evaporation off of the methanol the solution was filtered through a column of Dowex 50 (H^+-cycle, 75 ml), the column was washed through with water and the product was eluted with 10% pyridine. Evaporation of the eluate to dryness gave a yield of 0·75 g (89%). Amino acid analysis: Asp 1·02, Glu 0·98, Pro 0·95, Gly 0·93, Ile 0·99, Leu 1·00, Apim 0·90. $E^{Gly}_{2,4}$ 0·77, $E^{His}_{5,7}$ 0·21; R_F = 0·02 (S_1), 0·12 (S_2). The sample for analysis was crystallised from a mixture of dimethylformamide and ether; $[\alpha]_D$ −45·5° (*c* 0·50, dimethylformamide). For $C_{35}H_{60}N_{10}O_{11}.2\ H_2O$ (833·0) calculated: 50·46% C, 7·74% H, 16·82% N; found: 50·51% C, 7·96% H, 16·65% N.

The Amide of Tert-butyloxycarbonyl-O-tert-butyltyrosyl-isoleucyl-glutaminyl-asparaginyl-α-aminopimelyl-prolyl-leucyl-glycine (*Xb*)

To a solution of free heptapeptide *IXb* (0·70 g) in dimethylformamide (15 ml) we added N-ethylpiperidine (0·35 ml) and the N-hydroxysuccinimid ester of N-tert-butyloxycarbonyl-O-tert-butyl-

tyrosine[7] (0·9 g). After 24 h at room temperature further portion of the active ester (0·3 g) was added to the mixture and after a further 24 h the mixture was evaporated to dryness, the residue was triturated with light petroleum and ether, the crystalline portion was filtered and washed with ether, then water, aqueous citric acid (pH 3) and again water. The yield was 0·74 g (78%) of a product with m.p. 210–212°C (Kofler). The sample for analysis was crystallised from a mixture of dimethylformamide and ether, m.p. 211–212°C (Kofler); $[\alpha]_D$ −32·6° (*c* 0·50, dimethylformamide). For $C_{53}H_{85}N_{11}O_{15}.H_2O$ (1134) calculated: 56·13% C, 7·73% H, 13·59% N; found: 55·74% C, 7·50% H, 14·02% N.

The Lactam of Tyrosyl-isoleucyl-glutaminyl-asparaginyl-α-aminopimelyl-prolyl-leucyl-glycinamide (*Ie*)

Preparation of the active ester and its cyclisation were carried out in an atmosphere of nitrogen. To a solution of octapeptide *Xb* (334 mg) in a mixture of dimethylformamide (10 ml) and pyridine (10 ml) we added bis-*p*-nitrophenyl sulphite[16] (1 g). After 8 h stirring at room temperature we added further aliquot of the reagent (1 g) and pyridine (5 ml) and after a further 12 h still further bis-*p*-nitrophenyl sulphite (0·5 g). After 6 h the reaction mixture was evaporated to dryness, the residue was triturated with ether, filtered and washed with ether and water. After drying the active ester was dissolved in trifluoroacetic acid (10 ml) and after 1 h at room temperature the solution was diluted with toluene (10 ml) and evaporated. The remainder was dissolved in dimethylformamide (10 ml) and this solution was added to 300 ml of pyridine with stirring, and heated to 50°C. After 12 h at room temperature the reaction mixture was evaporated to dryness, the residue was ground up with ether, filtered and washed with ether. The product was purified in a Steady State Distribution Machine in the same manner as for substance *Id*. 110 transfers of the upper layer were made and the peak corresponding to a partition coefficient of 1·78 (tubes 63–79) was collected, evaporated and freeze-dried. The yield was 142 mg (47%); R_F 0·23 (S_1), 0·45 (S_2), 0·69 (S_3); on paper electrophoresis in two different buffers (1M acetic acid pH 2·4 and pyridine–acetic acid pH 5·7) the compound did not migrate. The sample for analysis and biological testing was further purified on gel columns of Biogel P-2 and P-4 (1M acetic acid); $[\alpha]_D$ −31·4° (*c* 0·24, 1M acetic acid). The amino acid analysis: Asp 0·98, Glu 1·03, Pro 0·99, Gly 0·98, Ile 0·99, Leu 1·00, Tyr 0·92, Apim 0·91. The sample for elemental analysis was recrystallised from a mixture of methanol and ether. For $C_{44}H_{67}N_{11}O_{12}.4\,H_2O$ (1014) calculated: 52·11% C, 7·44% H, 15·15% N; found: 52·38% C, 6·96% H, 15·17% N.

We wish to thank Mrs J. Němcová for technical assistance, Mrs H. Farkašová for the amino-acid analyses and Mrs J. Větrovská for the optical rotation measurements. Elemental analyses were carried out in the Analytical Laboratory of this Institute.

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Translated by J. H. Cort.