AMINO ACIDS AND PEPTIDES. XCIX.*

AN IMPROVED SYNTHESIS OF DEAMINO-CARBA[1]-OXYTOCIN COMPARISON OF VARIOUS METHODS FOR PEPTIDE CYCLISATION

K. JOŠT

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

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Deamino-carba[1]-oxytocin (*Ib*), an analogue of oxytocin without a disulphide bridge, was used for comparison of several described methods for peptide cyclisation: anhydride and azide methods under various conditions, and the active esters method. In the given case the last named technique proved to be the most effective, using *p*-nitrophenyl ester.

By means of synthesis and evaluation of the biological activities of oxytocin (*Ia*) analogues without a disulphide bridge but with the same basic cyclic arrangement of the molecule ("carba-analogues") it has been shown[1,2] that the disulphide bridge is not functionally indispensable for most of the biological activities typical for oxytocin. Rather the S—S bond would appear to function in terms of maintaining a spatial orientation of the molecule necessary for hormone binding with the receptor, *i.e.* it would function to establish the quantitatively high level of activity of the substance[3,4].

Two methods have been described in the synthesis of this group of substances: the first[1], based on the use of protected octapeptide *IIa*, from which the protective groups are removed by Na in liquid ammonia, and subsequent alkylation of the sulphur atom of the cysteine** residue yielded the linear peptide *IIb* with the required structural characteristics of product *Ib*. The second method, more general in nature[6], was based on azide condensation of two tetrapeptide fragments. Among the advantages of the latter method is not only the fact that it permits the synthesis of a number of analogues[2] using a maximal number of common intermediate products, but also the fact that the unnatural amino acid (in some cases very difficult to

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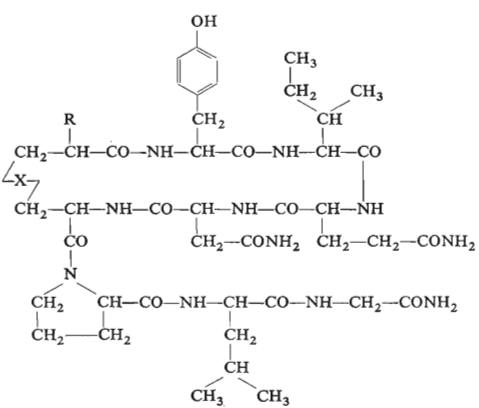
** All amino acids in this work were L-configuration. Terminology and symbols were used according to published suggestions[5].

obtain) in sequence position 6 was put in the peptide chain with a minimum number of synthetic stages. For the last stage, cyclisation of the linear octapeptide (type *IIb*), Woodward reagent[7] (2-ethyl-5-phenyl-isoxazolium 3'-sulphonate) was first used[2] along with the anhydride method[8]. In both cases, however, there was a very low yield of cyclic analogue, on an average (from 30 attempts at cyclisation) 5%, and only very exceptionally were there yields of 25 – 30%. In addition it was observed[2] that the biological activities varied, which might have resulted from the fact that the tested substance was not chemically homogeneous.

The aim of the present work has been to test various cyclisation reactions in the given case of deamino-carba[1]-oxytocin synthesis (*Ib*) in order to find the most productive synthesis for oxytocin analogues with a modified disulphide linkage. In previous work[2] it was quite clear that the products of the cyclisation reaction using Woodward reagent had a lower biological activity than products of anhydride cyclisation, and so the former technique has been left out of the present study. Substance *Ib* was prepared by anhydride and azide synthesis (under various conditions) or cyclisation was carried out using *p*-nitrophenyl ester. The last method was applied by Sakakibara and coworkers in the synthesis of deamino-dicarba-lysine-vasopressin[9], deamino-dicarba-arginine-vasopressin[10] and deamino-dicarba-oxytocin[11].

The starting material for the synthesis of suitable substituted linear octapeptides (*II*) for all these attempts at cyclisation was the protected tetrapeptide derivative *IV*, with a cysteine sulphur atom alkylated with a carboxypropyl residue. Of disadvantage was protection of this carboxylic group with a tert-butyl ester, because this de-



Ia, X = S-S, R = NH_2
Ib, X = CH_2-S, R = H

creased the yields below that from the corresponding methyl ester. Thus the initial S-γ-tert-butoxycarbonylpropylcysteine (*IIIb*) was obtained with a 46% yield, the corresponding NPS derivative *IIIc* with a 48% yield, and tetrapeptide *IVa* finally with only a 21% yield.

X-Tyr-Ile-Gln-Asn-Cys(Y)-Pro-Leu-Gly-NH_2

IIa, X = Z, Y = BZL
IIb, X = H, Y = $C_3H_6CO_2H$
IIc, X = BOC, Y = $C_3H_6CO_2H$
IId, X = BOC, Y = $C_3H_6CO_2C_6H_4NO_2$
IIe, X = BOC, Y = $C_3H_6CON_2H_2BOC$
IIf, X = Z, Y = $C_3H_6CO_2Me$

X-Cys($C_3H_6CO_2R$)

IIIa, X = H R = Me
IIIb, X = H R = Bu^t
IIIc, X = NPS, R = Bu^t
IIId, X = NPS, R = Me
IIIe, X = Z, R = Me

X-Cys($C_3H_6CO_2R$)-Pro-Leu-Gly-NH_2

IVa, X = NPS, R = Bu^t
IVb, X = NPS, R = Me
IVc, X = Z, R = Me

The starting material S-γ-methoxycarbonylpropylcysteine (*IIIa*) was obtained either by alkylation of cysteine with the methyl ester of γ-bromobutyric acid, or as a hydrochloride by partial esterification of S-γ-carboxypropylcysteine. Either *o*-nitrobenzenesulphenyl (*IIId*) or benzyloxycarbonyl (*IIIe*) groups were used to protect the amino group; both of these compounds were isolated in the form of dicyclohexylammonium salts. By condensation with the amide of prolyl-leucyl-glycine[12,13] there resulted tetrapeptides *IVb* and *IVc*. The benzyloxycarbonyl protective group was split off from substance *IVc* by using HBr in acetic acid and the free tetrapeptide was acylated stepwise with *o*-nitrobenzenesulphenylasparagine, *o*-nitrobenzenesulphenylglutamine and *o*-nitrobenzenesulphenylisoleucine, in the first two cases by means of the trichlorophenyl esters[14], in the last case as a hydroxysuccinimide ester. From the protected penta- (*V*), hexa- (*VI*) and heptapeptides (*VIIa*) the *o*-nitrobenzenesulphenyl protective group was split off by means of HCl in ether[15]. The free heptapeptide ester was subjected to alkaline hydrolysis and product *VIIb* was isolated on an ion-exchange column.

NPS-Asn-Cys($C_3H_6CO_2Me$)-Pro-Leu-Gly-NH_2

V

NPS-Gln-Asn-Cys($C_3H_6CO_2Me$)-Pro-Leu-Gly-NH_2

VI

X-Ile-Gln-Asn-Cys($C_3H_6CO_2R$)-Pro-Leu-Gly-NH_2

VIIa, X = NPS, R = Me
VIIb, X = H, R = OH

The tyrosine derivative *VIIIa*, required for further acylation, was obtained by carbodiimide synthesis from hydroxysuccinimide and tert-butyloxycarbonyl-O-tert-butyltyrosine (*VIIIb*) which was in the form of a dicyclohexylammonium salt prepared in two ways: the methyl ester of O-tert-butyltyrosine[16] after transformation to N-tert-butyloxycarbonyl derivative *VIIIc* was subjected to alkaline hydrolysis yielding substance *VIIIb*. The other method started from the methyl ester of benzyloxycarbonyl-O-tert-butyltyrosine[16] which was subjected to alkaline hydrolysis (as the corresponding ethyl ester[17]) and yielded substance *VIIId*[17,18]. This substance after catalytic hydrogenation yielded free O-tert-butyltyrosine[18] (*VIIIe*) which was transformed to substance *VIIIb* by the procedure described by Schnabel[19].

X-Tyr(Bu^t)OR

VIIIa, X = BOC, R = ONSu
VIIIb, X = BOC, R = OH
VIIIc, X = BOC, R = Me
VIIId, X = Z, R = OH
VIIIe, X = H, R = OH

By reaction of the hydroxysuccinimide ester of tert-butyloxycarbonyl-O-tert-butyltyrosine (*VIIIa*) with the free heptapeptide amide *VIIb* we obtained the derivative of octapeptide *IIc*, which was allowed to react either with *p*-nitrophenyl trifluoroacetate[20,21] or with bis-*p*-nitrophenyl sulphite[22] thus being transformed into the corresponding *p*-nitrophenyl ester *IId*. Carbodiimide condensation of substance *IIc* with tert-butyloxycarbonylhydrazine and a subsequent removal of substances with low molecular weight on Sephadex LH-20 yielded the protected hydrazide *IIe*. For synthesis of the free octapeptide amide *IIb* we chose a somewhat different approach, analogous to the preparation of acyclic deamino-dicarba-oxytocin[2]: The tetrapeptide obtained by decarbobenzoxylation of substance *IVc* was acylated with the azide of benzyloxycarbonyltyrosyl-isoleucyl-glutaminyl-asparagine[6] yielding the fully protected octapeptide *IIf* which was decarbobenzoxylated by hydrogen bromide in acetic acid, subjected to alkaline hydrolysis and purified chromatographically on an ion-exchanger using a pH gradient. The free octapeptide amide *IIb* showed a yield of 40% (in comparison with a previous yield of 9%[2]).

Anhydride cyclisation of linear peptide *IIb* was carried out in the same manner as earlier described[2]. By means of a counter-current distribution we isolated from the reaction mixture, along with the required product with a partition coefficient of 2 and the initial linear peptide *IIb* a still further substance (ninhydrin negative with the correct amino acid analysis) with a high partition coefficient, probably an acyclic octapeptide with an α-amino group acylated by the acetyl of a sec-butyloxycarbonyl group. The origin of the latter can be explained by insufficient protection of the amino group (by protonation) during activation of the carboxyl group[23]. The required product *Ib* had a high biological activity, even though the yield was low and the synthesis was not highly

reproducible. If the anhydride synthesis was carried out in the presence of pyridine hydrochloride[23] the final product, even after thorough purification, had a fairly low degree of biological activity. The azide method in the same arrangement as proved efficacious for cyclisation of the carba-fragment of the A-chain of insulin[24] (cyclisation in aqueous solution) gives no product with a partition coefficient near to 2 on processing in the counter-current distribution. Azide cyclisation in a dimethylformamide solution gave a product with an uterotonic activity of only 10 I.U./mg.

Cyclisation of a linear peptide by means of active esters was carried out basically in the same manner as described by Sakakibara and Hase[9]. The protective groups in *IIc* were removed by trifluoroacetic acid and the *p*-nitrophenyl ester of the linear octapeptide was cyclised in pyridine at 50°C. Product *Ib* was obtained in high yield with an activity of 1898 I.U./mg of oxytocic activity on the isolated rat uterus[25].

The analytical values for substance *Ib*, prepared by anhydride or azide cyclisations, did not show the best agreement with theoretical values. However, particularly in the case of azide cyclisation it would be difficult to explain the very low biological activity of the product by the possible presence of side products, as long as the latter did not have a marked inhibitory activity. A low biological activity could also be due to a sulphoxide form of a thio-amino acid. By the methods described in the literature[26] this latter possibility could be neither proved nor disproved. Rotatory dispersion curves in the absorption region of the amide linkage showed significant differences between substances prepared by the above three methods. Such studies of these and other analogues of oxytocin[27] are continuing.

From the fact that analytical data showed the best agreement in the case of *Ib* cyclised by the method of active esters, and from the fact that the latter product also had the highest biological activity, we assume that *Ib* prepared in this manner represents the required oxytocin analogue. In our experience, therefore, for cyclisation of oxytocin analogues with structural alteration in the disulphide linkage the method of active esters is the most advantageous, and has been used in our further syntheses[27]. We are aware, however, that such technical decisions will vary from substance to substance, and the above conclusion cannot be overgeneralised.

EXPERIMENTAL

Samples for elemental analysis were dried for 8—12 h in vacuum of 1 Torr at 70°C. Where R_F and E values are given for chromatography and electrophoresis, such values were obtained with pure substance. Detection was by the ninhydrin reaction or by the chlorination method in the case of ninhydrin-negative substances (paper[28], silica gel plates[29]). 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), pyridine–butanol–acetic acid–water (10 : 15 : 3 : 6) (S_3), 1-butanol–acetic acid–water (4 : 1 : 1) (S_4) and n-heptane–tert-butanol–pyridine (5 : 1 : 1) (S_5)[16,18]. For electrophoresis we used moist chambers, Whatman 3 MM paper and buffers: 1M acetic acid (pH 2·4) and pyridine–acetic acid (pH 5·7). In most cases electrophoresis was carried out for 60 min at a potential drop of 20 V/cm. Amino acid analyses were carried out after 20 h of hydrolysis in 6M-HCl at 105°C on an automatic analyser[30]. Evaporation from the reaction mixtures was carried out

with rotatory evaporator with a bath temperature of 30—35°C. When the mixtures contained dimethylformamide we used an oil vacuum pump for evaporation.

S-(γ-Methoxycarbonylpropyl)cysteine (*IIIa*)

Cystine (10·0 g) was reduced with sodium (4·25 g) in liquid ammonia (250 ml). The colour of excess sodium was removed with ammonium chloride and methyl ester of γ-bromobutyric acid (30·0 g) was added to the reaction mixture and the latter was immediately lyophilised using a water pump. The lyophilisate was dissolved in 1M-HCl (50 ml) at 0°C, the solution was extracted with ether and transferred to a column of Zerolite 225 (H^+-cycle, 250 ml). The column was washed through with water, and the product was then eluted with 10% pyridine at 0°C and evaporated to dryness. (In samples oxidised by performic acid paper electrophoresis did not show the presence of cysteic acid under conditions when 0·2% of added cysteic acid was easily detectable.) The residue after evaporation was dissolved in water (300 ml) and filtered through a column of Amberlite IR-4B (50 ml). The eluate was evaporated to dryness and crystallised from water with the addition of a small amount of pyridine. The yield was 11·2 g (62%) with m.p. 209—212°C (Kofler); $E_{2.4}^{Gly}$ 0·39; R_F 0·10 (S_1), 0·35 (S_2). The sample for analysis was recrystallised from water, m.p. 217—219°C (Kofler); $[\alpha]_D$ −15·5° (*c* 0·50, water); +0·6° (*c* 0·50, 1M-HCl). For $C_8H_{15}NO_4S$ (221·3) calculated: 43·43% C, 6·83% H, 6·33% N; found: 43·55% C, 6·89% H, 5·95% N.

S-(γ-Methoxycarbonylpropyl)cysteine Hydrochloride

S-γ-Carboxypropylcysteine (1·24 g) was dissolved in a mixture of methanol (10·5 ml) and 4·8M-HCl in methanol (7·5 ml). After 30 min at room temperature the reaction mixture was evaporated to dryness and the residue was dissolved 3× in methanol and re-evaporated to dryness, then triturated with ether, filtered and washed with ether (1·45 g). The product was crystallised from a mixture of methanol–ether, with a yield of 0·87 g (56%), m.p. 155—156°C; $[\alpha]_D$ 0° (*c* 0·50, 1M-HCl); −0·4° (*c* 0·50, dimethylformamide). For $C_8H_{16}ClNO_4S$ (257·7) calculated: 37·28% C, 6·26% H, 5·43% N, 13·76% Cl; found: 37·52% C, 6·26% H, 5·43% N, 13·52% Cl.

S-(γ-Tert-butyloxycarbonylpropyl)cysteine (*IIIb*)

Cystine (1·78 g) was reduced with sodium in liquid ammonia in the usual manner. After decolouration of the solution with ammonium chloride we added tert-butyl ester of γ-iodobutyric acid (3·8 g). The ammonia was removed by lyophilisation, the residue was dissolved in water and the pH was adjusted to 6·5 with HCl. Cooling to 0°C resulted in crystal formation, and these were collected on filter paper and washed. Crystallisation from water gave a yield of 0·88 g (46%) of a product, m.p. 206—208°C (capillary). The sample for analysis was recrystallised from water with no change in melting point. $E_{2.4}^{Gly}$ 0·42, $E_{5.7}^{Gly}$ 1·00; R_F 0·11 (S_1), 0·50 (S_2); $[\alpha]_D$ −14·7° (*c* 0·26, water). For $C_{11}H_{21}NO_4S$ (263·4) calculated: 50·16% C, 8·04% H, 5·32% N; found: 50·13% C, 8·07% H, 5·26% N.

S-γ-Carboxypropylcysteine

a) *From the methylester*: A solution of 1·05 g *IIIa* in methanol (10 ml) and 1M-NaOH (15 ml) was stirred at room temperature for 2 h. The solution was then evaporated to dryness, the residue dissolved in water and the pH adjusted to 4·5 with 6M-HCl. After cooling to 0°C crystals formed, were collected on filter, washed with water and recrystallised from water. The yield was 0·95 g

(91%), m.p. 231—233°C (Kofler). The sample for analysis was recrystallised in the same manner, m.p. 235—237°C (Kofler); $E^{Gly}_{2.4}$ 0·58, $E^{Gly}_{5.7}$ 0·42. $[\alpha]_{-D}$ −18·5° (*c* 0·19, water); $[\alpha]_D$ −1·6° (*c* 0·5, 1M-HCl). The literature[31] reports m.p. of 239—242°C and $[\alpha]_D$ of −1·3° (*c* 1, 1M-HCl). For $C_7H_{13}NO_4S$ (207·3) calculated: 40·56% C, 6·32% H, 6·76% N; found: 40·97% C, 6·21% H, 6·91% N.

b) *From tert-butyl ester*: 0·30 g of ester *IIIb* were dissolved in trifluoroacetic acid and after 2 h at room temperature the solution was evaporated to dryness. The residue was dissolved in water and the pH was adjusted to 4·0 with ammonium hydroxide. After cooling to 0°C crystals formed which were collected on filter paper, washed with water and crystallised from water. The product amounted to 0·12 g (52%), m.p. 230—231°C (Kofler). The sample for analysis was recrystallised in the same manner, $[\alpha]_D$ −18·4° (*c* 0·18, water). According to its m.p., electrophoretic behaviour and elementary analysis, the product was identical with the sample prepared in part *a*) above.

c) *By alkylation of cysteine*: Cystine (1·80 g) was reduced with sodium (0·74 g) in liquid ammonia (150 ml) and after decolouration of the solution with ammonium chloride γ-iodobutyric acid (6·4 g) was added to the reaction mixture. The solution was lyophilised *in vacuo* using a water pump, and the lyophilisate was dissolved in water (30 ml). The pH of the solution was adjusted to 5·0 using 6M-HCl. After cooling to 0°C the crystals formed were filtered, washed with water after collection, then washed with ether, and crystallised from water (100 ml); yield 2·68 g (86%), m.p. 232—235°C (Kofler). The sample for analysis was recrystallised in the same manner and $[\alpha]_D$ was −17·7° (*c* 0·20, water). According to the melting point, electrophoretic behaviour and elemental analysis this product was identical with those prepared under *a*) and *b*) above.

The Dicyclohexylammonium Salt of Benzyloxycarbonyl-S-(γ-methoxycarbonylpropyl)-cysteine (*IIIe*. DCHA)

a) *By means of benzyloxycarbonyl chloride*: With constant mixing and cooling benzyloxycarbonyl chloride (0·72 ml) was added to a solution of the hydrochloride of S-(γ-methoxycarbonylpropyl)cysteine (0·65 g) in 0·5M-$NaHCO_3$ (12 ml). The pH of the reaction mixture was maintained at 7·0 by addition of 0·5M-$NaHCO_3$ (6 ml). After 20 min stirring at 0°C and 1 h at room temperature the mixture was extracted with ether, acidified with HCl to pH 3, and the oily fluid which separated out was taken up in ether. The ether solution was washed with water, dried with sodium sulphate and evaporated. The residue was dried azeotropically (benzene), then dissolved in benzene (5 ml) and after addition of dicyclohexylamine (0·46 ml) was diluted with light petroleum, cooled to 0°C, and the product which separated out was filtered and washed with light petroleum (1·16 g, m.p. 112—118°C). Crystallisation from ethyl acetate gave a yield of 1·06 g (76%), m.p. 117—119°C. The sample for analysis was recrystallised in the same manner, m.p. 118—119°C; $[\alpha]_D$ −2·0° (*c* 0·51, dimethylformamide). For $C_{28}H_{44}N_2O_6S$ (536·7) calculated: 62·66% C, 8·26% H, 5·22% N; found: 62·74% C, 8·33% H, 5·44% N.

b) *By means of Bunte salt*[32]: Sodium benzyloxycarbonyl thiosulphate (0·81 g) was added with mixing to a solution of S-(γ-methoxycarbonylpropyl)cysteine (0·66 g) in water (20 ml). The pH of the reaction mixture was maintained at 7·0 by addition of about 15 ml of 0·5M-$NaHCO_3$. After 2 h the pH of the mixture was reduced to 3·0 with HCl, the product was taken up in ether, and the latter solution was then extracted with water, dried with sodium sulphate and evaporated. The remnant was azeotropically dried (benzene), dissolved in benzene, and after addition of 0·55 g dicyclohexylamine was diluted with light petroleum, cooled to 0°C and the separated product was filtered and washed with light petroleum. The yield was 1·05 g (64%), m.p. 114—117°C, with no depression with the sample prepared under *a*) above.

The Dicyclohexylammonium Salt of N-(*o*-Nitrobenzenesulphenyl)-S-(γ-methoxycarbonylpropyl)cysteine (*IIId*. DCHA).

With constant mixing *o*-nitrobenzenesulphenyl chloride (0·76 g) was added to a solution of the hydrochloride of S-(γ-methoxycarbonylpropyl)cysteine (1·02 g) in chloroform (10 ml) and triethylamine (1·7 ml). After 6 h at room temperature the solution was diluted with chloroform, washed with water, 0·2M-H_2SO_4 and again water, dried with sodium sulphate and after addition of dicyclohexylamine (0·8 ml) was evaporated. Crystallisation from a mixture of ethyl acetate and ether resulted in a product of 1·60 g (76%) with m.p. 157—151°C. The sample for analysis was recrystallised in the same manner, m.p. 159—161°C, $[\alpha]_D$ —40·0° (*c* 0·50, dimethylformamide). For $C_{26}H_{41}N_3O_6S_2$ (555·7) calculated: 56·19% C, 7·44% H, 7·56% N; found: 56·42% C, 7·66% H, 7·68% N.

The Dicyclohexylammonium Salt of *o*-Nitrobenzenesulphenyl-S-(γ-tert.butoxycarbonylpropyl)cysteine (*IIIc*. DCHA)

o-Nitrobenzenesulphenylchloride (1·25 g) and 2M-NaOH (4·8 ml) were added in several aliquots and with continual mixing to a solution of S-(γ-tert-butoxycarbonylpropyl)cysteine (2·0 g) in dioxane (10 ml) and 2M-NaOH (4 ml). The reaction mixture was diluted with water, filtered and the filtrate was acidified with 1M-H_2SO_4. The oily liquid which separated out was taken up into ethyl acetate, the latter solution was washed with water, dried with sodium sulphate, and after addition of dicyclohexylamine (1·6 ml) was diluted with light petroleum and cooled to 0°C. The product which separated out was filtered, washed with light petroleum and consisted of 2·5 g, m.p. 154—156°C. It was then crystallised from aqueous ethanol; yield 2·16 g (48%), m.p. 155 to 157°C. The sample for analysis was recrystallised in the same manner, m.p. 156—158°C; $[\alpha]_D$ —44·4° (*c* 0·50, dimethylformamide). For $C_{29}H_{47}N_3O_6S_2$ (597·8) calculated: 58·26% C, 7·93% H, 7·03% N; 10·73% S; found: 58·00% C, 7·92% H, 6·85% N, 10·38% S.

The Amide of *o*-Nitrobenzenesulphenyl-S-(γ-tert.butoxycarbonylpropyl)cysteinyl-prolyl-leucyl-glycine (*IVa*)

To a solution of the dicyclohexylammonium salt of *o*-nitrobenzenesulphenyl-S-(γ-tert-butoxycarbonylpropyl)cysteine (*IIIc*. DCHA) (0·60 g) in dimethylformamide (15 ml) at 0°C was added prolyl-leucyl-glycine amide (0·30 g) and 2·5M-HCl (0·40 ml) in dimethylformamide (3 ml), all at 0°C. The resulting crystals were filtered, washed with dimethylformamide and to the solution was added dicyclohexylcarbodiimide (0·22 g). After 24 h at room temperature the separated dicyclohexylurea was filtered, washed with dimethylformamide, and the combined filtrates were evaporated to dryness. The residue was ground with light petroleum, dissolved in ethyl acetate with the addition of 10% ethanol and washed successively with cooled 0·2M-H_2SO_4 and water, and evaporated. Crystallisation from a mixture of ethyl acetate and light petroleum gave a yield of 146 mg (21%), m.p. 87—90°C (Kofler). The sample for analysis was recrystallised in the same manner, m.p. 88—91°C (Kofler); $[\alpha]_D$ —60·4° (*c* 0·50, dimethylformamide). For $C_{30}H_{46}N_6O_8S_2$ (682·9) calculated: 52·77% C, 6·79% H, 12·31% N; found: 53·13% C, 6·90% H, 12·25% N.

5-Chloro-8-quinolyl Ester of Benzyloxycarbonyl-S-(γ-methoxycarbonylpropyl)cysteine

To a solution of the dicyclohexylammonium salt of benzyloxycarbonyl-S-(γ-methoxycarbonylpropyl)cysteine (*IIIc*. DCHA) (4·8 g) and the hydrochloride of 5-chloro-8-hydroxyquinoline (1·98 g) in a mixture of chloroform (250 ml) and dimethylformamide (6 ml), cooled to —20°C, was added with continuous mixing dicyclohexylcarbodiimide (1·86 g). The reaction mixture was stirred

for 1 h at −10°C, left for 36 h at room temperature, concentrated to half the original volume, the separated dicyclohexylurea was filtered, and the filtrate was evaporated to dryness. The dry powder was dissolved in a mixture of ethyl acetate and water, the organic layer was successively washed with water, 0·5M-$NaHCO_3$ and again water, dried with sodium sulphate and evaporated to dryness. Crystallisation from a mixture of ethyl acetate and light petroleum gave a yield of 4·15 g (90%) of a product, m.p. 105—107°C (capillary). The sample for analysis was recrystallised from ethyl acetate, m.p. 115—116°C (capillary); $[\alpha]_D$ −37·1° (*c* 0·50, dimethylformamide). For $C_{25}H_{25}ClN_2O_6S$ (517·0) calculated: 58·08% C, 4·87% H, 5·42% N, 6·86% Cl; found: 58·06% C, 4·86% H, 5·48% N, 6·59% Cl.

The Amide of Benzyloxycarbonyl-S-(γ-methoxycarbonylpropyl)cysteinyl-prolyl-leucyl-glycine (*IVc*)

a) *Anhydride method*: To a solution of the dicyclohexylammonium salt of benzyloxycarbonyl-S-(γ-methoxycarbonylpropyl)cysteine (3·0 g) in a mixture of ethanol (75 ml) and water (30 ml) was added Dowex 50 W X 4 (H^+-cycle, 30 ml). After 30 min mixing the ion exchanger was filtered off and washed with ethanol and the combined filtrates were evaporated and azeotropically dried (benzene). The residue was dissolved in a mixture of chloroform (21 ml) and N-ethylpiperidine (0·78 ml), the solution was cooled to −5°C and to it was added sec-butyl chloroformate (0·75 ml). After 5 min at 0°C a solution of prolyl-leucyl-glycine amide[12,13] (1·59 g) in dimethylformamide (24 ml) was added to the mixed anhydride, all previously cooled to 0°C. After 1 h at room temperature the reaction mixture was evaporated to dryness, the dry powder was dissolved in ethyl acetate and the latter solution was successively washed with 1M-HCl, water, 0·5M-$NaHCO_3$ and again water, dried with sodium sulphate and evaporated. Crystallisation from a mixture of benzene and light petroleum gave a yield of 2·50 g (72%), m.p. 111—113°C (capillary). The sample for analysis was recrystallised from aqueous dimethylformamide, m.p. 115—117°C (capillary); $[\alpha]_D$ −56·3° (*c* 0·50, dimethylformamide). For $C_{29}H_{43}N_5O_8S$ (621·4) calculated: 56·02% C, 6·97% H, 11·27% N; found: 56·26% C, 7·05% H, 10·97% N.

b) *Active ester method*: The amide of prolyl-leucyl-glycine (1·5 g) and chloroquinolyl ester of benzyloxycarbonyl-S-(γ-methoxycarbonylpropyl)cysteine (2·6 g) were dissolved in dimethylformamide (15 ml). The reaction mixture was left to stand 3 days at room temperature and then evaporated to dryness. The dry powder was triturated with 1M-HCl, filtered and washed on the filter with HCl and water. Crystallisation from aqueous dimethylformamide gave a yield of 2·85 g (91%), m.p. 117—118°C. The sample for analysis was recrystallised in the same manner, m.p. 115—117°C, with no depression with the sample prepared under *a*) above; $[\alpha]_D$ −55·9° (*c* 0·5, dimethylformamide).

The Amide of *o*-Nitrobenzenesulphenylasparaginyl-S-(γ-methoxycarbonylpropyl)cysteinyl-prolyl-leucyl-glycine (*V*)

The solution of protected tetrapeptide *IVc* (2·5 g) in acetic acid (12·5 ml) was treated with 35% hydrogen bromide in acetic acid (25 ml). After 10 min at room temperature the reaction mixture was diluted with ether and the hydrobromide which separated out was washed several times with ether. After drying in an exsiccator the tetrapeptide hydrobromide was dissolved in dimethylformamide (25 ml) and to this solution was added N-ethylpiperidine (2 ml) and 2,4,5-trichlorophenyl ester of *o*-nitrobenzenesulphenylasparagine[14] (2 g). After 24 h at room temperature a further gram of the trichlorophenyl ester of *o*-nitrobenzenesulphenylasparagine was added to the reaction mixture and after a further 48 h the solution was evaporated to dryness and the residue was successively ground with light petroleum and ether. The crystalline substance was

filtered and washed on the filter with ether, water, 0·5M-$NaHCO_3$ and again water. Crystallisation from aqueous dimethylformamide gave 2·5 g (84%), m.p. 185—188°C (capillary). The sample for analysis was recrystallised in the same manner, m.p. 191—193°C (capillary); $[\alpha]_D$ —73·7° (*c* 0·51, dimethylformamide). For $C_{31}H_{46}N_8O_{10}S_2$ (754·9) calculated: 49·33% C, 6·14% H, 14·85% N; found: 49·32% C, 6·15% H, 14·67% N.

The Amide of *o*-Nitrobenzenesulphenylglutaminyl-asparaginyl-S-(γ-methoxycarbonylpropyl)-cysteinyl-prolyl-leucyl-glycine (*VI*)

To a solution of 1·7 g protected pentapeptide *V* (1·7 g) in methanol (75 ml) we added 2M-HCl in ether (2·8 ml). The solution was concentrated down to a small volume and then diluted with ether. The oil which separated out was triturated with ether until it all was transformed into a microcrystalline state. $E^{His}_{5.7}$ 0·45, $E^{Gly}_{2.4}$ 0·83; R_F 0·73 (S_1), 0·82 (S_2). After drying in an exsiccator the pentapeptide hydrochloride was dissolved in dimethylformamide (22 ml), the solution was alkalinised with N-ethylpiperidine (0·5 ml) and 2,4,5-trichlorophenyl ester of *o*-nitrobenzenesulphenylglutamine[14] (1·35 g) was added. After 24 h standing at room temperature a further 0·5 g of active ester were added and after a further 24 h standing the reaction mixture was evaporated to dryness, the residue was ground with light petroleum and ether, filtered, and washed with ether, water, 0·5M-$NaHCO_3$ and again water; yield 1·95 g, m.p. 169—173°C. Crystallisation from a mixture of dimethylformamide and ether gave a yield of 1·65 g (84%), m.p. 176—179°C (capillary). The sample for analysis was recrystallised in the same manner, m.p. 180—182°C (capillary); $[\alpha]_D$ —56·3° (*c* 0·50, dimethylformamide). For $C_{36}H_{54}N_{10}O_{12}S_2.1\,H_2O$ (901·0) calculated: 48·00% C, 6·27% H, 15·55% N; found: 48·32% C, 6·28% H, 15·49% N.

The N-Hydroxysuccinimide Ester of *o*-Nitrobenzenesulphenylisoleucine

To a solution of *o*-nitrobenzenesulphenylisoleucine[15] (13·0 g) and N-hydroxysuccinimide (5·3 g) in tetrahydrofurane (40 ml), cooled to —10°C, we added 10 g dicyclohexylcarbodiimide. The reaction mixture was mixed for 2 h at 0°C and left overnight at room temperature. The separated dicyclohexylurea was filtered, washed with tetrahydrofurane and the solution was evaporated to dryness. The residue was ground with light petroleum, filtered and washed with light petroleum and water. Crystallisation from ethanol gave 11·6 g (67%), m.p. 110—112°C (Kofler). The sample for analysis was recrystallised in the same manner, with no change in melting point; $[\alpha]_D$ —104·0° (*c* 0·50, dioxane). For $C_{16}H_{19}N_3O_6S$ (381·4) calculated: 50·38% C, 5·02% H, 11·02% N; found: 50·65% C, 5·06% H, 10·91% N.

The Amide of *o*-Nitrobenzenesulphenylisoleucyl-glutaminyl-asparaginyl-S-(γ-methoxycarbonylpropyl)cysteinyl-prolyl-leucyl-glycine (*VIIa*)

To a solution of protected hexapeptide *VI* (1·65 g) in dimethylformamide (14 ml) was added 2·2M-HCl in ether (2·1 ml) and after 4 min standing at room temperature the reaction mixture was diluted with ether (300 ml). The oil which separated out was triturated with ether, and its crystalline portion was filtered and washed on the filter with ether, and then was dried in an exsiccator; $E^{His}_{5.7}$ 0·40, $E^{Gly}_{2.4}$ 0·71; R_F 0·05 (S_1), 0·09 (S_2), 0·49 (S_3) and 0·11 (S_4). The hydrochloride was dissolved in dimethylformamide (28 ml), the solution was alkalinised with N-ethylpiperidine (0·30 ml) and to it was added hydroxysuccinimide ester of *o*-nitrobenzenesulphenylisoleucine (1·0 g). After 60 h of standing at room temperature the reaction mixture was evaporated to dryness and the residue was extracted with hot ethyl acetate; yield 1·45 g (79%), m.p. 242 to 245°C (capillary). The sample for analysis was crystallised from a mixture of dimethylformamide and

ether, m.p. 247—249°C (capillary); $[\alpha]_D$ −72·9° (*c* 0·51, dimethylformamide). For $C_{42}H_{65}N_{11}O_{13}S_2$ (996·2) calculated: 50·64% C, 6·58% H, 15·47% N; found: 50·73% C, 6·66% H, 15·67% N.

The Methyl Ester of N-Tert-butyloxycarbonyl-O-tert-butyltyrosine (*VIIIc*)

The methyl ester of benzyloxycarbonyl-O-tert-butyltyrosine[16] (2·0 g) was hydrogenated according to Kinoshita and Klostermeyer[16]. The dried powder from this reaction was dissolved in pyridine (4·5 ml) and freshly distilled tert-butyloxycarbonyl azide (2·0 ml) was added. After 24 h standing at room temperature a further 1 ml of the azide was added and the reaction mixture was evaporated to dryness. The residue was dissolved in ethyl acetate and the latter solution was washed consecutively with 0·5M-$NaHCO_3$, water, 3% citric acid and again water, dried with sodium sulphate and evaporated; yield 3·15 g (94%), m.p. 95—97°C (capillary). R_F 0·54 (S_5; under the same conditions the R_F of the corresponding benzyloxycarbonyl derivative was 0·52, which agrees with the literature[16]). The sample for analysis was crystallised from light petroleum, m.p. 98—100°C (capillary); $[\alpha]_D$ −17·4° (*c* 0·51, dimethylformamide). For $C_{19}H_{29}NO_5$ (351·4) calculated: 64·93% C, 8·32% H, 3·99% N; found: 64·58% C, 8·33% H, 4·42% N.

The Dicyclohexylammonium Salt of Benzyloxycarbonyl-O-tert-butyltyrosine (*VIIId*. DCHA)

Saponification of the methyl ester (3·85 g) was carried out in an analogous fashion to the ethyl ester[17]. The yield was 4·3 g (77%) of the dicyclohexylammonium salt, m.p. 159—162°C (Kofler); $[\alpha]_D$ +32·3° (*c* 0·50, ethanol). The literature gives 161—161·5°C, $[\alpha]_D$ +34·68° (*c* 1, ethanol)[18] or 154—154·5°C (ref.[17]).

O-Tert-butyltyrosine (*VIIIe*)

Benzyloxycarbonyl-O-tert-butyltyrosine (*VIIId*) was released from the dicyclohexylammonium salt (5·5 g) using Dowex 50, and was hydrogenated in an aqueous methanol solution in the presence of Pd-black. The yield was 2·25 g (95%) of a product with $[\alpha]_D$ −25·6° (*c* 0·51, water); R_F 0·18 (S_1), 0·48 (S_2). The literature[18] gives $[\alpha]_D$ −25·77° (*c* 1, water).

The Dicyclohexylammonium Salt of Tert-butyloxycarbonyl-O-tert-butyltyrosine (*VIIIb*)

a) *Hydrolysis of the methyl ester* (VIIIc): To a solution of the methyl ester of tert-butyloxycarbonyl-O-tert-butyltyrosine (4·35 g) in methanol (45 ml) 1M-NaOH (11 ml) was added. After 2 h at room temperature the methanol was evaporated off, the reaction mixture was acidified with 6% citric acid, the product was extracted into ethyl acetate, the latter solution was washed with water, dried with sodium sulphate and evaporated to dryness. The residue was dissolved in light petroleum (10 ml) and dicyclohexylamine (2·4 ml) was added. After cooling to 0°C crystals separated out which were filtered and washed with cold light petroleum; yield 5·7 g (93%), m.p. 129—132°C (capillary). The sample for analysis was crystallised from light petroleum, m.p. 134 to 136°C (capillary); $[\alpha]_D$ +24·4° (*c* 0·5, dimethylformamide). For $C_{30}H_{50}N_2O_5$ (518·7) calculated: 69·46% C, 9·72% H, 5·40% N; found: 69·52% C, 9·58% H, 5·31% N.

b) *Acylation of* O-*tert-butyltyrosine*: To a solution of O-tert-butyltyrosine *VIIIe* (6·4 g) in a mixture of dioxane (30 ml) and water (30 ml) (pH adjusted to 10·5 by addition of 4M-NaOH) was added tert-butyloxycarbonyl azide (6 ml) and the reaction mixture was left to stand with mixing for 6 h; pH was maintained at 10·5 by addition of 4M-NaOH (total consumption 12·5 ml). The reaction mixture was then extracted with ether, acidified to pH 4·5 by addition of 7M-HCl (8 ml) and the separated product was taken up into ethyl acetate. The latter solution was washed

with water, dried with sodium sulphate and evaporated to dryness. The residue was dissolved in light petroleum and dicyclohexylamine (6·5 ml) was added. The solution was cooled to 0°C and crystals separated out and were filtered and washed with cold light petroleum. The yield was 10·8 g (77%) of a product with melting point 134—136°C, with no depression as compared with the sample prepared under *a*) above.

The Hydroxysuccinimide Ester of Tert-butyloxycarbonyl-O-tert-butyltyrosine (*VIIIa*)

To a solution of the dicyclohexylammonium salt *VIIIb* (6·3 g) in 50% ethanol (160 ml) was added Dowex 50 (70 ml) in the H^+ cycle. After 30 min mixing at room temperature the ion exchanger was filtered, the filtrate was concentrated by evaporation and then dried azeotropically with benzene. After dissolving in tetrahydrofurane (50 ml) we added N-hydroxysuccinimide (1·6 g), the solution was cooled to −15°C and dicyclohexylcarbodiimide (3·0 g) was added. The reaction mixture was mixed for 1 h at −10°C, 12 h at 0°C and 24 h at room temperature. The separated dicyclohexylurea was filtered and the filtrate was evaporated to dryness. Crystallisation from ethanol yielded 4·1 g (78%), m.p. 136—138°C (capillary). The sample for analysis was recrystallised in the same manner, m.p. 140—142°C; $[\alpha]_D$ −40·2° (*c* 0·5, dimethylformamide). For $C_{22}H_{31}N_2O_7$ (435·5) calculated: 60·67% C, 7·18% H, 6·43% N; found: 60·62% C, 7·11% H, 6·72% N.

The Amide of Isoleucyl-glutaminyl-asparaginyl-S-[γ-carboxypropyl]-cysteinyl-prolyl-leucyl-glycine (*VIIb*)

To a solution of protected heptapeptide *VIIa* (1·35 g) in dimethylformamide (27 ml) was added 2M-HCl in ether (1·62 ml). After 4 min standing at room temperature the reaction mixture was diluted with ether (270 ml). The hydrochloride which separated out was filtered, washed with ether and dried in an exsiccator. After dissolving in methanol (15 ml) we added 1M-NaOH (5 ml) and after 1 h standing at room temperature the methanol was evaporated off, the aqueous solution was placed on a Dowex 50 (H^+-cycle; 50 ml) column. The column was washed through with water and the product was eluted with 10% pyridine. The eluate was concentrated to a small volume and freeze-dried. The dry powder was dissolved in 90% methanol, diluted with ether and the separated product was filtered and washed with ether. The yield was 1·05 g (96%); $E^{Gly}_{5.7}$ 0·71, $E^{His}_{2.4}$ 0·13; R_F 0·20 (S_2), 0·03 (S_1). The sample for analysis was recrystallised from a mixture of dimethylformamide and ether; $[\alpha]_D$ −50·9° (*c* 0·50, dimethylformamide). Amino-acid analysis: Asp. 1·00, Cys ($C_4H_7O_2$) + Gly 2·00, Glu 1·00, Pro 0·90, Ile 0·91, Leu 1·00. For $C_{35}H_{60}N_{10}O_{11}S.1\,H_2O$ (847·0) calculated: 49·62% C, 7·38% H, 16·54% N; found: (corrected for 0·5% ash) 49·33% C, 7·33% H, 16·49% N.

The Amide of Tert-butyloxycarbonyl-O-tert-butyltyrosyl-isoleucyl-glutaminyl-asparaginyl-S-(γ-carboxypropyl)cysteinyl-prolyl-leucyl-glycine (*IIc*)

To a solution of the free heptapeptideamide *VIIb* (0·85 g) in dimethylformamide (15 ml) we added N-ethylpiperidine (0·3 ml) and the hydroxysuccinimide ester of protected tyrosine *VIIIa* (0·90 g). After 60 h at room temperature the solution was evaporated to dryness, the residue was triturated successively with light petroleum, ether, and washed on the filter with water. The product was extracted with hot methanol; the yield was 0·80 g (68%), m.p. 212—214°C (Kofler). A sample of this product was treated for 1 h with trifluoroacetic acid: $E^{Gly}_{2.4}$ 0·62, $E^{His}_{5.7}$ 0·18; R_F 0·07 (S_1), 0·34 (S_2), 0·79 (S_3). The sample for analysis was crystallised from a mixture of dimethylformamide and ether; m.p. 213—214°C (Kofler); $[\alpha]_D$ −33·9° (*c* 0·50, dimethylformamide). For $C_{53}H_{86}N_{11}O_{15}S.1\,H_2O$ (1 167·4) calculated: 54·55% C, 7·60% H, 13·21% N; found: 54·58%C, 7·77% H, 13·38% N.

The Amide of Benzyloxycarbonyltyrosyl-isoleucyl-glutaminyl-asparaginyl-S-(γ-methoxycarbonylpropyl)cysteinyl-prolyl-leucyl-glycine (*IIf*)

Protected tetrapeptide *IVc* (1·0 g) was decarbobenzoxylated with hydrogen bromide in the usual manner, and then dried in an exsiccator. Hydrazide of benzyloxycarbonyltyrosyl-isoleucyl-glutaminyl-asparagine[6] (1·03 g) was dissolved in dimethylformamide (45 ml) and 6·2M-HCl in tetrahydrofurane (1·5 ml) and cooled to −30°C. Freshly distilled butyl nitrite (0·33 ml) was added and after 5 min at −30°C the tetrapeptide hydrobromide solution in dimethylformamide (15 ml) was added. The reaction mixture was alkalinised with N-ethylpiperidine (2 ml; moist pH indicator paper), warmed to 0°C slowly over 1 h, left standing 24 h at 0°C and then evaporated to dryness. The residue was triturated with dilute HCl and water; yield 1·40 g (81%), m.p. 212–217°C (Kofler). The sample for analysis was crystallised from a mixture of dimethylformamide and water, m.p. 219–222°C (Kofler); $[\alpha]_D$ −37·6° (*c* 0·50, dimethylformamide). For $C_{53}H_{77}N_{11}O_{15}S$ (1140·3) calculated: 55·84% C, 6·81% H, 13·51% N; found: 56·00% C, 6·88% H, 13·33% N.

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

Protected octapeptide *IIf* (1·30 g) was decarbobenzoxylated with 35% hydrogen bromide in acetic acid (20 ml), the hydrobromide was precipitated with ether and after drying was subjected to alkaline hydrolysis (20 ml 1M-NaOH, 2 h at room temperature). The inorganic salts were removed using Dowex 50 in the H^+ cycle (60 ml); the product was eluted from the column with 10% pyridine, concentrated to a small volume and freeze dried. The dry powder was purified chromatographically on Dowex 1 X 2 (200–400 mesh; acetate, pre-equilibrated with 1% pyridine) using a gradient elution of 1% pyridine to 1% acetic acid in a manner previously described[2]. The product was eluted at pH 5·5 and had the same characteristics as the substance previously described[2]. The yield after freeze drying was 0·45 g (40%).

The Amide of Tert-butyloxycarbonyl-O-tert-butyltyrosyl-isoleucyl-glutaminyl-asparaginyl-S-(γ-*p*-nitrophenoxycarbonylpropyl)cysteinyl-prolyl-leucyl-glycine (*IId*)

a) *Using* p-*nitrophenyl trifluoracetate*: To a solution of the octapeptide acid *IIc* (361·8 mg) in a mixture of pyridine (6 ml) and dimethylformamide (4 ml) was added *p*-nitrophenyl trifluoroacetate[20,21] (526 mg). The reaction mixture was stirred and heated to 50°C for 6 h, then cooled to 0°C and diluted with cold water (50 ml). Crystals formed which were filtered and washed on the filter with water; yield 349·1 mg (82%), m.p. 222–225°C (Kofler). The sample for analysis was crystallised from methanol, m.p. 225–227°C (Kofler); $[\alpha]_D$ −34·4° (*c* 0·50, dimethylformamide). For $C_{59}H_{89}N_{12}O_{17}S.H_2O$ (1288·5) calculated: 54·96% C, 7·12% H, 13·05% N; found: 54·66% C, 6·76% H, 13·42% N.

b) *Using bis-*p-*nitrophenyl sulphite*: (preparation of the active ester and its cyclisation were both carried out in an atmosphere of N_2). Octapeptide acid *IIc* (313 mg) was dissolved in a mixture of dimethylformamide (10 ml) and pyridine (10 ml) and bis-*p*-nitrophenyl sulphite[22] (1·0 g) was added. After 7 h stirring at room temperature a further gram of nitrophenyl sulphite and pyridine (5 ml) was added, the mixture was stirred a further 12 h and a further 0·5 g of the reagent was added. After a further 5 h stirring the mixture was evaporated to dryness, the residue was triturated with ether, filtered and washed on the filtre with ether and water. The yield was 329 mg (95%) of a product with the same characteristics as that prepared under *a*) above.

The Amide of Tert-butyloxycarbonyl-O-tert-butyltyrosyl-isoleucyl-glutaminyl-asparaginyl-S-(γ-tert-butyloxycarbonyl-hydrazinocarbonylpropyl)cysteinyl-prolyl-leucyl-glycine (*IIe*)

To a solution of octapeptide-acid *IIc* (471·2 mg) and tert-butyloxycarbonylhydrazine (95·7 mg) in dimethylformamide (5 ml) at −25°C we added dicyclohexylcarbodiimide (150·4 mg) in dimethylformamide (1 ml). The reaction mixture was stirred for 1 h at −15°C, 4 h at 0°C and 12 h at room temperature, placed on a column (100 × 1 cm) of Sephadex LH-20 (in dimethylformamide) and the fractions containing the peak were dried, the dry powder triturated with ether and then filtered and washed on the filter with ether; yield 475·4 mg (92%), m.p. 224–226°C (Kofler). The sample for analysis was crystallised from methanol, m.p. 228–230°C (Kofler); $[\alpha]_D$ −37·5° (*c* 0·50, dimethylformamide). For $C_{58}H_{95}N_{13}O_{16}S$ (1262·5) calculated: 55·11% C, 7·58% H, 14·42% N; found: 55·04% C, 7·59% H, 14·24% N.

The Lactam of Tyrosyl-isoleucyl-glutaminyl-asparaginyl-S-γ-carboxypropylcysteinyl-prolyl-leucyl-glycinamide (Deamino-carba[1]-oxytocin) (*Ib*)

a) *The anhydride method*: To a solution of acyclic peptide *IIb* (151 mg) in dimethylformamide (16·5 ml) at −15°C we added sec-butyl chloroformate (21·5 mg). The reaction mixture was stirred 15 min at −15°C, 5 min at 0°C, then diluted with dimethylformamide (22 ml) at 0°C, and neutralised with N-ethylpiperidine (17·6 mg). After 12 h at room temperature the reaction mixture was evaporated to dryness and the residue was dissolved in water (26 ml). The insoluble material was filtered off and the filtrate was freeze dried. The dry powder was dissolved in 25 ml of the upper phase of the system 2-butanol–0·05% acetic acid. The filtered solution was placed in the second tube of an all-glass Steady State Distribution Machine (Quickfit & Quartz, Ltd., Stone, Staffordshire, England) and 100 transfers of the upper and 100 transfers of the lower phase were carried out. The peptide material was localised with Folin-Ciocalteau reagent. The peak with a partition coefficient of 2·35 (tubes 27–53) was concentrated to a small volume and freeze dried. The yield was 34·6 mg of product. There were two further peaks with partition coefficients 0·17 and 10·3. The substance with coefficient 2·35 (25·3 mg) was dissolved in 1 ml acetic acid and placed on a Biogel P-2 column (100 × 1 cm) filled with 1M acetic acid. The peak of biologically active material (localised by the optical density at 280 nm) was freeze dried; yield 17·9 mg. The sample for analysis was recrystallised from a mixture of methanol and ether; R_F 0·20 (S_1) 0·35 (S_2); $[\alpha]_D$ −58·4° (*c* 0·15, water). Amino-acid analysis: Asp 1·03, Glu 1·00, Pro 0·79, Gly + Cys($C_4H_7O_2$) 2·69, Ile 0·89, Leu 1·05, Tyr 0·89. For $C_{44}H_{67}N_{11}O_{12}S.7\,H_2O$ (1100·3) calculated: 48·04% C, 7·42% H, 14·01% N; found: 48·26% C, 6·30% H, 13·59% N. Test on the isolated rat uterus showed an activity of 1404 I.U./mg.

b) *Anhydride method with the addition of pyridine hydrochloride*: Acyclic peptide *IIb* (88·3 mg) was dissolved in dimethylformamide (10 ml) and pyridine hydrochloride (10·2 mg) was added. Cyclisation was carried out as under *a*) (12·7 mg sec-butyl chloroformate, 13 ml dimethylformamide, 10·4 mg N-ethylpiperidine). After drying the product was filtered through Biogel P-2, the peak freeze-dried and purified in a countercurrent machine as in *a*). The yield was 8·1 mg of a product with biological activity 258 I.U./mg (isolated rat uterus). Amino-acid analysis: Asp 0·94, Glu 1·00, Pro 0·88, Gly + Cys($C_4H_7O_4$) 2·68, Ile 0·84, Leu 1·00, Tyr. 0·75.

c) *Azide method*: Fully protected octapeptide hydrazide *IIe* (94·5 mg) was dissolved in trifluoroacetic acid (10 ml) and after 1 h standing at room temperature we added toluene (10 ml) and the solution was evaporated to dryness. $E_{5.7}^{His}$ 0·39, $E_{2.4}^{Gly}$ 0·90 (detection with ninhydrin and Cl_2). The residue was dissolved in dimethylformamide (2 ml) and 6·5M-HCl in tetrahydrofurane (0·15 ml) was added, the mixture cooled to −40°C and butylnitrite (11 mg) was added. The temperature of the reaction mixture was raised to 0°C over 20 min and the solution was diluted

with dimethylformamide (75 ml) at 0°C. The pH of the reaction mixture was adjusted to 9 with N-ethylpiperidine (moist pH paper indicator) and after 7 days standing at 0°C the solution was evaporated to dryness. The residue was dissolved in 25 ml of the upper phase of the same system as in *a*) above and in all 140 transfers of the upper and 135 of the lower phases were carried out in the counter-current machine. The contents of tubes 45—63 (peak corresponding to $K = 2{\cdot}15$) were pooled, concentrated to a small volume and freeze dried. The dry powder was dissolved in 3M acetic acid (3 ml) and filtered through Biogel P-4 (in 3M acetic acid; 100 × 1 cm). The yield after freeze drying was 28 mg, and this product was further purified on Biogel P-4 and P-2 (both in 1M acetic acid). The yield was 15·8 mg; the sample for analysis was recrystallised from a mixture of methanol and ether; $[\alpha]_D$ −58·6° (*c* 0·11, water); R_F 0·20 (S_1) 0·34 (S_2). Amino-acid analysis: Asp 1·03, Glu 1·00, Pro 0·91, Gly + Cys ($C_4H_7O_2$) 1·75, Ile 0·91, Leu 1·06, Tyr 0·62. For $C_{44}H_{67}N_{11}O_{12}S.6\,H_2O$ (1082·3) calculated: 48·84% C, 7·36% H, 14·24% N; found: 48·98% C, 6·39% H, 14·00% N. The sample had a biological activity of 10·3 I.U./mg on the isolated rat uterus.

d) *The method of active esters*: *p*-Nitrophenyl ester of protected octapeptide *IId* (329 mg) was dissolved in trifluoroacetic acid (10 ml) and after 1 h at room temperature the solution was diluted with toluene (10 ml) and evaporated to dryness. The residue was dissolved in dimethylformamide (10 ml) and at a rate of 2·7 ml/h this solution was added to 200 ml pyridine with constant mixing at a temperature of 50°C. The reaction mixture was then stirred at the same temperature for 4 h and left standing a further 12 h at room temperature, following which it was evaporated to dryness. The residue was triturated with ether, filtered and washed with ether; yield 311·7 mg. 106·2 mg of this product was dissolved in 25 ml of the upper phase of the system for counter-current distribution as in *a*) above and 110 upper phase transfers were carried out. The contents of tubes 66—84 ($K = 2{\cdot}15$) were pooled, concentrated to a small volume and freeze dried. The yield was 55·0 mg (60%) of a product which was chromatographically and electrophoretically pure; R_F 0·20 (S_1), 0·35 (S_2). For analysis this product was further purified by filtration on Biogel P-4 and P-2 (in 1M acetic acid) and recrystallised from a mixture of methanol and ether; $[\alpha]_D$ −71·7° (*c* 0·16, water). The amino acid analysis: Asp 1·00, Glu 0·97, Pro 1·04, Gly + Cys ($C_4H_7O_2$) 1·91, Ile 1·00, Leu 1·04, Tyr 0·98. For $C_{44}H_{67}N_{11}O_{12}S.4\,H_2O$ (1046·2) calculated: 50·52% C, 7·23% H, 14·73% N; found: 50·42% C, 6·62% H, 14·61% N. The isolated rat uterus test in the absence of Mg showed a biological activity of 1898 I.U./mg.

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