SYNTHESIS AND SOME BIOLOGICAL ACTIVITIES OF ANALOGUES OF DEAMINO-VASOPRESSIN WITH THE DISULPHIDE BRIDGE ALTERED TO A THIOETHER BRIDGE*

K.JOŠT, Z.PROCHÁZKA, J.H.CORT, T.BARTH, J.ŠKOPKOVÁ, Z.PRUSÍK and F.ŠORM

Institute of Organic Chemistry and Biochemistry,
Czechoslovak Academy of Sciences, 166 10 Prague 6

Received March 14th, 1974

Stepwise synthesis in solution and cyclisation by means of active esters were used to prepare three analogues of deamino-vasopressin with the disulphide bridge altered to the thioether group —S—CH_2— (referred to as 6-carba-analogues). In sequence position 8 the analogues differed by the presence of L-ornithine, L-arginine or D-arginine. Typical vasopressin-like activities, in particular antidiuretic, were very high in these analogues.

A disulphide bridge is a frequent structural feature of biologically active peptides. Synthesis and biological evaluation of analogues of oxytocin[1] and insulin[2] have shown that replacement of the —S—S— bridge by other groups does not result in a disappearance of biological activities typical for these two hormones. In some cases, structural alterations – including modification of the disulphide bridge – have resulted in marked increases in biological activities in comparison with the parent molecule. In the case of oxytocin such increased activity is observed with removal of the N^{α}-amino group and replacement of the disulphide bridge with —CH_2—S— (ref.[3]) or —S—CH_2— (ref.[4]) groups. Replacement of the disulphide bridge of deamino-oxytocin by an ethylene group[1,4–6] also resulted in an active analogue, but with a lower potency than the parent hormone. It is not surprising that similar relations were observed with analogues of the other neurohypophysial hormone, vasopressin, where removal of the N^{α}-amino group and substitution of the disulphide bridge by an ethylene group resulted in retention of the wide spectrum of vasopressin-like activities, but – with the possible exception of antidiuretic activity – with a loss of potency as compared with both lysine- and arginine-vasopressins or their deamino-analogues[7–9]. Since disulphide replacement by a thioether, as opposed to an ethylene, group in oxytocin resulted in increased activity, similar analogues of vasopressin were prepared. Of the two possibilities: —S—CH_2— and —CH_2—S—, the former (6-carba) was chosen because of the relative activities

* Part CXXII in the series Amino Acids and Peptides; Part CXXI: This Journal *39*, 1290 (1974).

of the two oxytocin analogues. Pressor and antidiuretic activities (also as determined by the modified Burn test[10]) of deamino-6-carba-oxytocin were higher than those of deamino-1-carba-oxytocin[4], whereas the decrease in uterine blood flow produced by the latter two analogues was approximately the same[10]. In the present work the preparation and some of the basic pharmacological activities of analogues of deamino--vasopressin are described, with the disulphide bridge changed to a —S—CH_2— group. In sequence position 8 were residues of ornithine* (*Ie*), L- (*If*) or D-arginine (*Ig*).

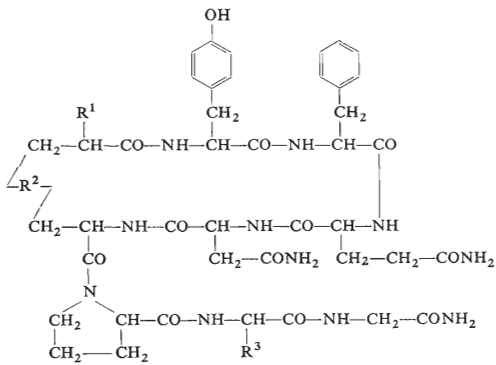
The basic synthetic procedure was similar to that used previously to prepare deamino-dicarba-vasopressins[7–9]. The peptide chain was extended in a stepwise fashion using active esters (*cf.*[12]) and protecting groups easily split off by acidolysis (*e.g.* *o*-nitrobenzenesulphenyl[13], *p*-methoxybenzyloxycarbonyl[14] and tert-butyl-oxycarbonyl[14,15]). Cyclisation of all analogues was carried out in the octapeptide stage using active esters[16]. The first approach was to prepare 8-arginine analogues from 8-ornithine ones by transforming the 8th residue as the final synthetic stage. This approach has been used successfully for other analogues of neurohypophysial peptides[17,18] but failed in the present case, even though in a trial run there was a quantitative transformation of the amide of N^{α}-tert-butyloxycarbonylornithyl--glycine (obtained by catalytic removal of the benzyloxycarbonyl protecting group from substance *II*) by the action of 1-guanyl-3,5-dimethylpyrazole[19] in dimethyl-formamide to yield the corresponding arginine dipeptide. The synthesis of arginine--containing molecules was performed with use of the *p*-toluenesulphonyl protecting group, which can be removed[20] from the guanidino group by the action of liquid hydrogen fluoride. The same protective group has also been used in the synthesis of arginine-containing dicarba-analogues[8,9].

From protected dipeptide *II* (prepared either using dicyclohexylcarbodiimide or active ester) the tert-butyloxycarbonyl protecting group was split off using tri-fluoroacetic acid. Electrophoresis showed that in this case there is no splitting of the benzyloxycarbonyl group (*cf.*[21]). Reaction with *o*-nitrobenzenesulphenylproline N-hydroxysuccinimide ester gave tripeptide amide *IIIa*, from which the *o*-nitrobenzenesulphenyl protecting group was split off by hydrogen chloride in ether and the product was condensed with a derivative of homocysteine, the amino group of which was protected either with an *o*-nitrobenzenesulphenyl (*IVa*) or a *p*-methoxybenzyloxy-carbonyl (*IVb*) group. Derivative *IVa* was prepared by the action of *o*-nitrobenzene-sulphenyl chloride on S-(2-methoxycarbonylethyl)homocysteine[4] either in chloro-form in the presence of a tertiary base, or with a somewhat better yield in aqueous dioxane in the presence of bicarbonate. A similar derivative with a *p*-methoxybenzyl-

* Amino acids used in this work were of L-configuration, except for D-arginine where noted in the text. Nomenclature and symbols are according to published suggestions[11]. The symbol Hcy is used for a residue of homocysteine.

oxycarbonyl group (*IVb*) was prepared by the action of *p*-methoxybenzyloxycarbonyl chloride (in very low yield), *p*-methoxybenzyloxycarbonyl azide or by reduction of N,N'-bis(*p*-methoxybenzyloxycarbonyl)homocystine with zinc and hydrochloric acid with subsequent alkylation of the homocysteine derivative with methyl acrylate[22]. Amide of *o*-nitrobenzenesulphenyl-S-(2-methoxycarbonylethyl)homocysteinyl-prolyl--N^{δ}-benzyloxycarbonylornithyl-glycine and the corresponding *p*-methoxybenzyloxycarbonyl derivative were transformed (without characterisation) into protected peptide *VIa* (after splitting off the α-amino protecting groups) by reaction with *o*-nitrobenzenesulphenylasparagine 2,4,5-trichlorophenyl ester.

Synthesis of arginine-containing analogues made use of known tripeptides, the amide of benzyloxycarbonylprolyl-N^{G}-*p*-toluenesulphonylarginyl-glycine[23] (*IIIc*) and the corresponding D-arginine derivative[24] (*IIId*). After splitting off of the benzyloxycarbonyl group with hydrogen bromide in acetic acid they were condensed with N-benzyloxycarbonyl-S-(2-methoxycarbonylethyl)homocysteine (*IVc*) in the form of the 5-chloro-8-quinolyl ester[4] with the formation of both diastereoisomeric tetrapeptides *Va* and *Vb*. Protecting groups were removed as above and reaction with *o*-nitrobenzenesulphenylasparagine 2,4,5-trichlorophenyl ester gave pentapeptides *VIb* and *VIc*. The next stage was carried out in a similar manner for all three final



Ia, $R^1 = NH_2$, $R^2 = S{-}S$, $R^3 = (CH_2)_3{-}NH{-}C(NH)NH_2$
Ib, $R^1 = H$, $R^2 = S{-}S$, $R^3 = (CH_2)_3{-}NH{-}C(NH)NH_2$
Ic, $R^1 = H$, $R^2 = S{-}S$, $R^3 = (CH_2)_3{-}NH_2$
Id, $R^1 = H$, $R^2 = CH_2{-}CH_2$, $R^3 = (CH_2)_3{-}NH{-}C(NH)NH_2$
Ie, $R^1 = H$, $R^2 = S{-}CH_2$, $R^3 = (CH_2)_3{-}NH_2$
If, $R^1 = H$, $R^2 = S{-}CH_2$, $R^3 = (CH_2)_3{-}NH{-}C(NH)NH_2$ (L-arginine residue)
Ig, $R^1 = H$, $R^2 = S{-}CH_2$, $R^3 = (CH_2)_3{-}NH{-}C(NH)NH_2$ (D-arginine residue)

analogues (ornithine, L- or D-arginine in position 8). The *o*-nitrobenzenesulphenyl protecting group was removed from pentapeptides *VIa–VIc* by hydrogen chloride in ether and the peptide chain was extended by glutamine and phenylalanine residues by reaction with active esters of the corresponding *o*-nitrobenzenesulphenyl amino acids (with formation of protected hexapeptides *VIIa–VIIc* and heptapeptides *VIIIa–VIIIc*). From the latter substances the *o*-nitrobenzenesulphenyl group was again removed in the same manner and the methyl esters were subjected to alkaline hydrolysis with the formation of free peptides *VIIId–VIIIf*. Reaction with the N-hydroxysuccinimide ester of N-tert-butyloxycarbonyl- or N-*o*-nitrobenzenesulphe-

Boc-Orn(Z)-Gly-NH_2
II

R-Pro-X-Gly-NH_2

IIIa, R = Nps, X = Orn(Z)
IIIb, R = H, X = Orn(Z)
IIIc, R = Z, X = Arg(Tos)
IIId, R = Z, X = D-Arg(Tos)

R-Hcy($C_2H_4CO_2Me$)

IVa, R = Nps
IVb, R = Z(OMe)
IVc, R = Z

Z-Hcy($C_2H_4CO_2Me$)-Pro-X-Gly-NH_2

Va, X = Arg(Tos)
Vb, X = D-Arg(Tos)

Nps-Asn-Hcy($C_2H_4CO_2Me$)-Pro-X-Gly-NH_2

VIa, X = Orn(Z)
VIb, X = Arg(Tos)
VIc, X = D-Arg(Tos)

Nps-Gln-Asn-Hcy($C_2H_4CO_2Me$)-Pro-X-Gly-NH_2

VIIa, X = Orn(Z)
VIIb, X = Arg(Tos)
VIIc, X = D-Arg(Tos)

R^1-Phe-Gln-Asn-Hcy($C_2H_4CO_2R^2$)-Pro-X-Gly-NH_2

VIIIa, R^1 = Nps, R^2 = Me, X = Orn(Z)
VIIIb, R^1 = Nps, R^2 = Me, X = Arg(Tos)
VIIIc, R^1 = Nps, R^2 = Me, X = D-Arg(Tos)
VIIId, R^1 = H, R^2 = H, X = Orn(Z)
VIIIe, R^1 = H, R^2 = H, X = Arg(Tos)
VIIIf, R^1 = H, R^2 = H, X = D-Arg(Tos)

R-Tyr(Bu^t)-Phe-Gln-Asn-Hcy($C_2H_4CO_2H$)-Pro-X-Gly-NH_2

IXa, R = Boc, X = Orn(Z)
IXb, R = Nps, X = Orn(Z)
IXc, R = Nps, X = Arg(Tos)
IXd, R = Nps, X = D-Arg(Tos)

Tyr(R)-Phe-Gln-Asn-Hcy(C_2H_4CO)-Pro-X-Gly-NH_2

Xa, R = H, X = Orn(Z)
Xb, R = Bu^t, X = Orn(Z)
Xc, R = Bu^t, X = Arg(Tos)
Xd, R = Bu^t, X = D-Arg(Tos)

nyl-O-tert-butyltyrosine gave protected octapeptides *IXa–IXd*. By the action of bis-(*p*-nitrophenyl) sulphite[25] the latter were transformed into active esters and after removal of amino protective groups (in the case of *IXa* with simultaneous removal of tert-butyl ether) cyclisation was carried out in dilute pyridine solution. Monomeric cyclopeptides *Xa–Xd* were isolated using counter-current distribution. Free analogues were obtained by the action of hydrogen bromide in acetic acid (for ornithine analogue) or liquid hydrogen fluoride (for arginine analogues). Counter-current distribution of analogues *Ie–Ig* did not give pure substances; for this reason we used gel filtration and continuous free-flow electrophoresis.

Biological activities of the analogues presented here, as compared with reference substances, are presented in Table I. Typical vasopressin-like activities were found for all three analogues *Ie–Ig*. Pressor activity, determined both on despinalised[26] and nephrectomised[27] rats, were for both analogues containing L-amino acids in sequence position 8 of the same order of magnitude as in the disulphide parent substance. The marked decrease in pressor activity in analogue *Ig* could be expected from the known effect of a D-amino acid in position 8 in dissociating vasopressin activities[24,28]. It should be stressed, however, that pressor activity is merely an integral expression of the vascular status of many different vascular beds. Of both theoretical and possible practical importance are detailed studies of the actions of these analogues with measurements of regional haemodynamics. A modification of the Sapirstein technique[29] of ^{86}Rb distribution was used for these observations, and the detailed results will be reported elsewhere[30]. Whereas analogue *Ig* had little if any haemodynamic activity, the remaining two substances, and *If* in particular, produced very pronounced decreases in blood flow to the gastrointestinal tract and uterus with minimal cardiac and systemic effects and no action on renal blood flow, and these actions showed a prolonged time course.

The antidiuretic activity of analogues *Ie* to *Ig* reached very high levels. There were quantitative differences, however, between the different tests used, so that even the order of potency varied from test to test. In anaesthetised rats[31] the lowest activity was observed with substance *If* (but this "lowest" value was still three times of the naturally occurring hormone arginine-vasopressin) while the activity of substance *Ig* was so high that it could not be calculated reliably. In a modified Burn assay[32] (unanaesthetised rats) the lowest activity of the three was shown by the ornithine-containing analogue *Ie* and both arginine-containing substances *If* and *Ig* had higher activities than any so far reported in the literature, including [8-D-arginine]deamino-vasopressin[33] and its 4-valine analogue[34]. These activity discrepancies find their counterparts in the literature and possible methodological explanations have been discussed[35]. In addition to the above results, mention should be made of the order-of-ten differences in antidiuretic activities reported for deamino-dicarba-arginine-vasopressin ([1,6-aminosuberic acid, 8-arginine] vasopressin) (*cf.* Table I) using different methods of assaying[8,9].

There are not yet sufficient data to state whether [4-valine, 8-D-arginine]deamino--vasopressin or analogue *Ig* has the more advantageous ratio of antidiuretic to pressor activities, particularly because of the error accruing to accurate measurement of very low pressor values[34]. It is clear, however, that replacement of the disulphide bridge by a thioether one will have to be counted as one of the factors[34] (*cf.*[36]) which increase both the amplitude and duration of antidiuretic activity, along with removal of the N^{α}-amino group, replacement of tyrosine by phenylalanine in sequence position 2, increase in the hydrophobicity of residue number 4 and a change of the L-form for the D- in sequence position 8 (examples are given in ref.[34]). It should be mentioned that these separate structural changes, each with its own functional sequelae, need not be additive if combined, and could even work one against the other (*cf.*[37]).

One explanation of the increased antidiuretic activity of the three analogues herein presented could be related to resistance to enzymatic cleavage. In the modified Burn assay the half-times of antidiuretic response were compared at a constant dosage of 50 pg/100 g (Table I). All of the carba analogues showed values several times those of the parent hormone, arginine-vasopressin. The half-time of the anti-

TABLE I

Biological Activities of Vasopressin Analogues (I.U./mg)

Vasopressin analogue		Rat uterus	Pressor (rat)		Antidiuretic (rat)			References
		isolated	de-spinalised	ne-phrecto-mised	anesthe-tised	un-anesthe-tised	$T\text{-}^1/_2$ min	
[8-Arginine]	*Ia*	12	487	450[b]	503	450[b]	42[b]	55
[8-Arginine]deamino-	*Ib*	27	370	—	1 300	—	—	56
[8-Ornithine]deamino-	*Ic*	15·5	355	—	202	—	—	57
[8-Arginine]deamino-dicarba-	*Id*	15 11·9	37 23	— —	1 274 84·5	— —	— —	9 8
[8-D-Arginine]deamino-		5	0·5	0·1[b]	870	900[b]	180[b]	33
[4-Valine,8-D-arginine]-deamino-		8	0·01	0·1[a]	1 230	1 500[a]	250[a]	34
[8-Ornithine]deamino--6-carba-	*Ie*	27	172	135	4 618	650	120	[c]
[8-Arginine]deamino--6-carba-	*If*	74	223	165	1 569	1 550	220	[c]
[8-D-Arginine]deamino--6-carba-	*Ig*	0·66	2·9	0·2	[c]	2 200	350	[c]

[a] The sample kindly provided by Professor W. H. Sawyer, Dept. Pharmacol., Columbia University, N.Y. [b] The sample kindly provided by Dr J. L. Mulder, Ferring Ltd., Malmö. [c] *cf.* text.

diuretic action of analogue *Ig* was practically twice that of [8-D-arginine]deamino--vasopressin and also longer than that showed by the 4-valine analogue of the latter substance. The thioether bridge would appear to be more metabolically stable than the naturally-occurring disulphide bridge[38]. Most available evidence concerns the stability of analogues exogeneously administered and freely circulating (or partially and weakly adsorbed or bound to larger circulating peptides or proteins) but attention should also be given to the stability of the active peptide complex to its receptor, particularly since the high potency of many carba analogues suggests that the disulphide bridge cannot form a functional part of this complexing process. In other words, the question is raised as to whether the uncomplexed disulphide bridge does not represent a limiting factor for the stability of the complex, a more stable thioether bridge increasing the chemical half-life of the latter. The objection that "fixation" in space of the hypothetical peptide-receptor complex should drastically limit the possibility of interaction with yet another macromolecule – a cleaving enzyme – neglects *a*) the dynamic state of the cell membrane thought to contain the receptor, and *b*) the possibility, expressed already for acetylcholine-receptors and cholinesterase in the membrane of the muscle end-plate[39], that the membrane could be a mosaic of adjacent receptor sites and sites containing enzyme which can cleave either the hormone itself or the receptor-complexed hormone. Such a proposal is not the same thing as stating that prolonged activity could be due to delayed elimination of carba-analogues from the "receptor compartment", an effect which has been argued against in the case of deamino-6-carba-oxytocin in comparison with oxytocin itself[40]. A further factor which might play a role in increasing antidiuretic activity is the fact that carba analogues are sterically altered in the region of the original disulphide bridge and that this steric effect might result in a better receptor "fit" (*cf.*[41]).

EXPERIMENTAL

Standard Procedures

Melting points were determined on a Kofler block and values were corrected. Samples for elemental analysis were dried over phosphorus pentoxide for 12 or more h at room temperature and 1 Torr. Thin layer chromatography was carried out on silica gel plates (Kieselgel G, Merck) in systems 2-butanol–25% ammonia–water (85 : 7·5 : 7·5) (S1), 2-butanol–90% formic acid–water (75 : 13·5 : 11·5) (S2) and 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 6) (S3). Electrophoresis was carried out on Whatman No 3 MM paper for 1 h at a potential drop of 20 V/cm in buffers: 1M acetic acid (pH 2·4) and pyridine–acetate (pH 5·7). Detection was by means of ninhydrin or in some cases by chlorination. In those cases where R_F and E values are presented, pure peptides were used unless otherwise indicated. Samples for amino- acid analysis were hydrolysed for 20 h at 105°C in 6M-HCl (in ampoules sealed at 1 Torr). The analyses were carried out on an automatic two-column analyser (Development Workshops, Czechoslovak Academy of Sciences, Prague, type 6020). For evaporation we used rotatory evaporators (water

pump, bath temperature 30—35°C). Mixtures containing dimethylformamide were evaporated *in vacuo* from an oil pump (1 Torr).

Isolation and Purification Techniques

Counter-current distribution: The all-glass Steady State Distribution Machine from Quickfit & Quartz, Ltd., Stone, Staffordshire, United Kingdom, with independent shifting of both upper and lower phases, was used. In all cases the system employed was 2-butanol–0·05% acetic acid and the peptide material was located by the Folin-Ciocalteau reaction (samples from every other tube).

Continuous free-flow electrophoresis: The instrument used has been described elsewhere[42,43]. Samples were dissolved in 0·5M acetic acid in a concentration of 0·4 to 4·5% (w/v) and processed with a terminal voltage of 2·6 kV (a potential drop of 52 V/cm) in a carrier electrolyte of 0·5M acetic acid. The electrode compartment contained 1M acetic acid. Solutions with samples were injected into the separation chamber through a slit 0·52 mm wide. The separation compartment was 500 mm long in the direction of current flow and 440 mm long from sample input to fraction collection, orthogonal to current flow. Sample injection was 85 mm distant from the ion-exchange membrane dividing the separation chamber from the anode compartment. The entire system was pneumatically cooled and temperature maintained, in individual cases, in the region −2 to +2°C. 48 fractions were collected and maintained at 4°C. Peptide material was located by OD_{280}.

Gel filtration: Columns 100 × 1 cm or 140 × 2·5 cm were used, filled with Bio-Gel (Bio-Rad Laboratories, Richmond, Cal.) P-2 or P-4 in 1M or 3M acetic acid. Filtration was carried out at rates of 7 or 12 ml/h. Peptide material was located by OD_{280}.

Tert-butyloxycarbonyl-N^{δ}-benzyloxycarbonylornithyl-glycinamide (*II*):

a) From benzyloxycarbonylglycinamide (12·2 g) the protecting group was removed by the action of 35% HBr in acetic acid for 30 min. The hydrobromide was precipitated with ether, dissolved in dimethylformamide and to this solution N-ethylpiperidine was added (to pH 6—7 by wet pH paper), 1-hydroxybenzotriazole[44] (8·0 g) and a solution of N^{α}-tert-butyloxycarbonyl-N^{δ}-benzyloxycarbonylornithine[45] (21·4 g) in dimethylformamide (100 ml). The above solution was cooled to −15°C, dicyclohexylcarbodiimide (13·0 g) was added and the mixture was stirred for 1 h at −10°C. It was then left overnight at 0°C, dicyclohexylurea was filtered off, the filtrate was evaporated and the residue dissolved in a mixture of ethyl acetate and 0·5M-$NaHCO_3$. The ethyl acetate phase was sequentially washed with 0·5M-$NaHCO_3$, 1M citric acid, 0·5M-$NaHCO_3$ and water and then dried over sodium sulphate. After evaporation the remaining substance was crystallised from ethyl acetate. The yield was 17·8 g (72%) of a product with m.p. 125—127°C. The sample for analysis was recrystallised in the same manner with no change in m.p.; $[\alpha]_D^{25}$ −2·2° (*c* 0·5, dimethylformamide); the sample after removal of the protecting group with trifluoroacetic acid: $E_{2,4}^{Gly}$ 1·16, $E_{5,7}^{His}$ 0·72; after removal of protecting groups with HBr in acetic acid: $E_{2,4}^{Gly}$ 1·95, $H_{5,7}^{His}$ 1·67. For $C_{20}H_{30}N_4O_6$ (422·5) calculated: 56·90% C, 7·16% H, 13·29% N; found: 57·41% C, 7·30% H, 13·36% N.

b) In the same manner as in *a*) above the benzyloxycarbonylglycinamide (0·83 g) was converted to the hydrobromide and after drying it was dissolved in dimethylformamide and the pH adjusted to 8·0 with N-ethylpiperidine. To this solution N^{α}-tert-butyloxycarbonyl-N^{δ}-benzyloxycarbonylornithine *p*-nitrophenyl ester[45] (1·95 g) was added. After 24 h stirring at room temperature

a further portion of active ester (0·50 g) was added and after a further 48 h the reaction mixture was evaporated. Crystallisation from ethyl acetate and light petroleum gave a yield of 1·06 g (63%) of a product with m.p. 122—125°C with no depression on admixture of the sample prepared according to *a*).

o-Nitrobenzenesulphenylprolyl-N$^{\delta}$-benzyloxycarbonylornithyl-glycinamide (*IIIa*)

Protected dipeptide *II* (1·7 g) was dissolved in trifluoroacetic acid (15 ml) and after 1 h at room temperature the solution was diluted with toluene (15 ml) and evaporated. The residue was dissolved in dimethylformamide (10 ml) and to this solution was added N-ethylpiperidine (pH about 8·5; wet pH paper) and *o*-nitrobenzenesulphenylproline N-hydroxysuccinimide ester[46] (1·5 g). After 24 h stirring at room temperature a further portion of active ester (0·7 g) was added and the mixture was, after 24 h, evaporated. The residue was triturated with light petroleum, ether, water and 0·5M-$NaHCO_3$. The crystals were filtered off, washed with water, dried and recrystallised from methanol and ether. The yield was 1·75 g (76%) of a product with m.p. 148—153°C. The sample for analysis was recrystallised in the same manner, m.p. 152—155°C; $[\alpha]_D^{25}$ −30·0° (*c* 0·5, dimethylformamide). For $C_{26}H_{32}N_6O_7S$ (572·6) calculated: 54·60% C, 5·63% H, 14·68% N; found: 54·67% C, 5·91% H, 15·02% N.

Prolyl-N$^{\delta}$-benzyloxycarbonylornithyl-glycinamide (*IIIb*)

To a solution of protected tripeptide *IIIa* (0·86 g) in dimethylformamide (5 ml) we added 2M-HCl in ether (2·3 ml). After 5 min at room temperature the mixture was diluted with ether, the hydrochloride which separated out was ground with ether, filtered off, dried and dissolved in water (10 ml). The aqueous solution was filtered through a column of Amberlite IR-4B (25 ml, OH^- cycle) and the eluate was evaporated. The resulting syrup was triturated with ethyl acetate and ether and converted to a solid product of 0·43 g (69%); $[\alpha]_D^{25}$ −21·2° (*c* 0·5, dimethylformamide), −35·9° (*c* 0·5, water); $E_{2,4}^{Gly}$ 0·91; $E_{5,7}^{His}$ 0·50. For $C_{20}H_{29}N_5O_5.1{\cdot}5\,H_2O$ (446·5) calculated: 53·85% C, 7·22% H, 15·72% N; found: 54·16% C, 6·97% H, 15·95% N.

o-Nitrobenzenesulphenyl-S-(2-methoxycarbonylethyl)homocysteine Dicyclohexylammonium Salt (*IVa*)

a) To a suspension of S-(2-methoxycarbonylethyl)homocysteine[3] (1·1 g) in chloroform (30 ml) we added triethylamine (0·7 ml) and within 5 min alternately *o*-nitrobenzenesulphenyl chloride (0·95 g) and triethylamine (about 1·3 ml; pH must be alkaline on wet pH paper). After 2·5 h stirring at room temperature the reaction mixture was diluted to twice its volume and then shaken up sequentially with water, 0·1M-H_2SO_4 (0°C) and water, dried with sodium sulphate and evaporated. The residue was dissolved in ethyl acetate (20 ml) and after addition of dicyclohexylamine (1·0 ml) and ether (20 ml) the mixture was cooled to 0°C. The crystalline product was filtered off and washed with ether. The yield was 0·68 g (24%) of a product with m.p. 152 to 157°C. Recrystallisation from methanol yielded 0·50 g (18%) of a product with m.p. 156—162°C. The sample for analysis was recrystallised from methanol: m.p. 157—162°C; $[\alpha]_D^{25}$ −36·9° (*c* 0·5, dimethylformamide). For $C_{26}H_{41}N_3O_6S_2$ (555·7) calculated: 56·25% C, 7·44% H, 7·57% N; found: 55·88% C, 7·34% H, 7·58% N.

b) S-(2-Methoxycarbonylethyl)homocysteine (2·2 g) was dissolved in water (40 ml) and dioxane (40 ml). The pH of the mixture was adjusted to 7·6 by addition of about 2·5 ml 0·5M-$NaHCO_3$.

Over 10 min a solution of *o*-nitrobenzenesulphenyl chloride (2·0 g) in dioxane (40 ml) was added drop by drop. pH was maintained constant at 7·6 by automatic addition of 1M-NaOH (about 14 ml in all). The mixture was stirred for a further 10 min, acidified with 0·25M-H_2SO_4 (0°C) to pH 3·0 and extracted with ethyl acetate. The ethyl acetate extract was washed with water, dried with sodium sulphate and further processed as in *a*) above. After recrystallisation from methanol the yield was 2·3 g (42%) of a product with m.p. 156—161°C with no depression on admixture with the sample prepared under *a*).

p-Methoxybenzyloxycarbonyl-S-(2-methoxycarbonylethyl)homocysteine Dicyclohexylammonium Salt (*IVb*)

a) S-(2-Methoxycarbonylethyl)homocysteine (11·0 g) was dissolved in a mixture of dioxane (200 ml), water (100 ml) and 0·5M-$NaHCO_3$ (15 ml). To this solution we added *p*-methoxybenzyloxycarbonyl azide[47] (10 g) and the pH was maintained at 7·6 (pH-stat) over the 54 h of the reaction by addition of about 40 ml of 2M-NaOH. During this period we added to the mixture after 24 h, and again after 48 h, 2·5 g (each time) of *p*-methoxybenzyloxycarbonyl azide. The mixture was extracted with ether, the pH of the aqueous layer was adjusted to 3·0 with citric acid and the product was extracted into ether. The ether extract was washed with water, dried with sodium sulphate, evaporated, azeotropically dried (benzene) and dissolved in benzene (30 ml). To this solution we added dicyclohexylamine (10·4 ml) and light petroleum to the point of turbidity and after cooling to 0°C the crystalline portion was filtered off and washed with light petroleum. The yield was 14·73 g (63%) of a product of m.p. 136—139°C. Recrystallisation from ethyl acetate yielded 14·03 g (60%) of a product with m.p. 143—146°C. The sample for analysis was recrystallised in the same manner, m.p. 145—146·5°C; $[\alpha]_D^{25}$ +6·0° (*c* 0·5, dimethylformamide). For $C_{29}H_{46}N_2O_7S$ (566·7) calculated: 61·50% C, 8·18% H, 4·94% N; found: 61·80% C, 8·01% H, 4·90% N.

b) A solution of S-(2-methoxycarbonylethyl)homocysteine (1·1 g) in 0·5M-$NaHCO_3$ (20 ml) was cooled to 0°C; over 15 min we added 10 mmol *p*-methoxybenzyloxycarbonyl chloride[48] in tetrahydrofurane (10 ml). The pH of the mixture was maintained at about 7·5 by addition of 0·5M-$NaHCO_3$. The mixture was stirred 20 min at 0°C and one hour at room temperature, and processed as in *a*). The yield was 0·07 g (3%) of a product with m.p. 125—134°C; after recrystallisation from ethyl acetate the m.p. was 142—144°C with no depression on admixture of sample prepared as in *a*) above.

c) To a solution of N,N′-bis(*p*-methoxybenzyloxycarbonyl)homocystine (0·9 g) in methanol (30 ml) and conc. hydrochloric acid (1 ml), cooled to 0°C, we added over 15 min zinc powder (0·55 g) with stirring. After 15 min at 0°C the zinc was filtered off, washed with methanol and the filtrates poured into water (125 ml, bubbled through with N_2 for 30 min). The product was extracted into ether and the ether extract was washed with water (pre-bubbled through with N_2) and evaporated. The residue was dissolved in water (10 ml) and 0·5M-$NaHCO_3$ (pH adjusted to 8·1, bubbled through with N_2), methyl acrylate (0·55 ml) was added and the mixture was stirred for 24 h at room temperature (pH of the mixture maintained at 9·2). After acidification with citric acid to pH 3·0 the mixture was processed as in *a*) above. After recrystallisation from ethyl acetate the yield was 0·82 g (48%) of a product with m.p. 137—141°C without depression on admixture of sample prepared under *a*), but with a lower optical rotation (+3·6°, *c* 0·3, dimethylformamide).

In a parallel experiment the residue of the ether extract after zinc reduction was dissolved in benzene (10 ml) and after addition of triethylamine (0·50 ml) and methyl acrylate (0·55 ml) the mixture was stirred at room temperature for 2 days, evaporated, dissolved in ethyl acetate

(150 ml), washed with citric acid, water, dried with sodium sulphate and processed as in *a*). After recrystallisation the yield was 0·75 g (44%) of a product with m.p. 127—136°C.

N,N'-Bis(*p*-methoxybenzyloxycarbonyl)homocystine

Homocystine[49] (1·61 g) was suspended in dioxane (18 ml) and water (9 ml). pH was adjusted to 10·8 with 2M-NaOH and *p*-methoxybenzyloxycarbonyl azide[46] was added. After 3 h stirring at pH 10·8 (pH-stat, 2M-NaOH) the mixture was extracted with ether and to the aqueous phase we added a suspension of Dowex 50 W (H^+-cycle, 140 ml) in ethyl acetate (100 ml). After 2 h stirring at room temperature the ionex was filtered off, washed with ethyl acetate, the aqueous layer extracted with ethyl acetate, all of the ethyl acetate extracts were pooled, dried with sodium sulphate and evaporated. The residue was crystallised from a mixture of ethyl acetate and light petroleum. The yield was 2·8 g (78%) of a product with m.p. 136—138°C. The sample for analysis was recrystallised in the same manner, m.p. 137—139°C; $[\alpha]_D^{25}$ −39·5° (*c* 0·5, dimethylformamide). For $C_{26}H_{32}N_2O_{10}S_2$ (596·7) calculated: 52·38% C, 5·41% H, 4·70% N; found: 52·30% C, 5·42% H, 4·58% N.

o-Nitrobenzenesulphenylasparaginyl-S-(2-methoxycarbonylethyl)homocysteinyl-prolyl--N^{δ}-benzyloxycarbonylornithyl-glycinamide (*VIa*)

a) To a solution of the protected tripeptide *IIIa* (5·7 g) in dimethylformamide (30 ml) we added 2M-HCl in ether (15 ml). After 5 min at room temperature the mixture was diluted with ether, the hydrochloride which separated out was ground up, filtered and washed with ether. After drying the tripeptide hydrochloride was dissolved in dimethylformamide (100 ml) and alkalinised with N-ethylpiperidine to pH about 8·0 (wet pH paper). To a solution of the dicyclohexylammonium salt of *p*-methoxybenzyloxycarbonyl-S-(2-methoxycarbonylethyl)homocysteine (*IVb*; 5·7 g) in methanol (150 ml) and water (50 ml) we added Dowex 50W (50 ml, H^+-cycle). After 30 min stirring at room temperature the resin was filtered off and washed with methanol and the filtrates were evaporated and azeotropically dried (benzene). The residue was dissolved in dimethylformamide (100 ml) and added to a solution of free tripeptide in dimethylformamide with N-ethylpiperidine. To this solution we added 1-hydroxybenzotriazole (1·4 g) and after cooling to −15°C dicyclohexylcarbodiimide (2·3 g). The mixture was stirred for 1 h at −10°C, left overnight at +3°C and evaporated. The residue was dissolved in ethyl acetate (20 ml), the insoluble portion filtered off and the filtrate washed sequentially with 0·5M-$NaHCO_3$, 2M citric acid, 0·5M-$NaHCO_3$, water, dried with sodium sulphate and evaporated. (After removal of the protecting groups using 35% HBr in acetic acid the sample showed: $E_{2,4}^{Gly}$ 1·64; $E_{5,7}^{His}$ 1·19). After azeotropic drying (benzene) anisol (3 ml) and trifluoroacetic acid (30 ml) were added to the protected tetrapeptide. After 1 h at room temperature the solution was diluted with toluene (50 ml) and evaporated. The residue was dissolved in dimethylformamide (150 ml), pH of the solution adjusted with N-ethylpiperidine to about 8·0 (wet pH paper) and the 2,4,5-trichlorophenyl ester of *o*-nitrobenzenesulphenylasparagine[50] (4·5 g) was added. After 24 h stirring at room temperature further active ester (2·0 g) was added and after a further 24 h the mixture was evaporated. The residue was triturated with light petroleum, ether, 0·5M-$NaHCO_3$ and water. After crystallisation from methanol and ether the yield was 10·95 g (62%) of a product with m.p. 136—139°C. The sample for analysis was recrystallised in the same manner, m.p. 137—140°C; $[\alpha]_D^{25}$ −54·4° (*c* 0·49, dimethylformamide). For $C_{38}H_{51}N_9O_{12}S_2.0{\cdot}5\,H_2O$ (898·9) calculated: 50·75% C, 5.83% H, 14·02% N; found: 50·61% C, 5·66% H, 13·90% N.

b) To a solution of substance *IIIa* (0·57 g) in methanol (10 ml) we added 0·225M-HCl in methanol (4·0 ml; 1·02 equiv) and after 1 h stirring at room temperature (the course of splitting

off of the *o*-nitrobenzenesulphenyl group was followed by TLC in benzene) the mixture was evaporated. The residue was dissolved in chloroform (10 ml) and dimethylformamide (10 ml). To this solution was added the dicyclohexylammonium salt of *o*-nitrobenzenesulphenyl-S-(2-methoxycarbonylethyl)homocysteine (0·56 g) and 1-hydroxybenzotriazole (0·14 g) and, after cooling to −20°C, dicyclohexylcarbodiimide (0·23 g). The mixture was stirred for 1 h at −10°C, left overnight at +3°C and evaporated. The residue was dissolved in dimethylformamide (5 ml) and after 1 h at +3°C the dicyclohexylurea was filtered off. The filtrate was evaporated and the remainder was ground up sequentially with 0·5M-$NaHCO_3$, water, 0·1M-H_2SO_4 and water and azeotropically dried with benzene. The remainder was dissolved in dimethylformamide (4 ml) and 2M-HCl in ether (1·5 ml) was added. After 5 min at room temperature the mixture was diluted with ether. The tetrapeptide-hydrochloride which separated out was filtered off, washed with ether, and dried ($E^{Gly}_{2,4}$ 0·79, $E^{His}_{5,7}$ 0·43) and dissolved in dimethylformamide (10 ml). This solution was alkalinised with N-ethylpiperidine to pH about 8·0 (wet pH paper) and the 2,4,5-trichlorophenyl ester of *o*-nitrobenzenesulphenylasparagine (0·45 g) was added. After 24 h stirring at room temperature further active ester (0·20 g) was added and the mixture was processed as in *a*). After recrystallisation from dimethylformamide and ether the yield was 0·44 g (49%) of a product with m.p. 137—140°C with no depression on admixture of sample prepared as in *a*); $[\alpha]_D^{25}$ −53·0° (*c* 0·50, dimethylformamide). With a similar approach, in which for formation of the peptide bond by means of dicyclohexylcarbodiimide instead of 1-hydroxybenzotriazole we used N-hydroxysuccinimide, or when the reaction was carried out only with dicyclohexylcarbodiimide, just as in cases in which the *o*-nitrobenzenesulphenyl group was removed in the same manner as in *a*), the product was obtained in lower yield and less pure.

o-Nitrobenzenesulphenylglutaminyl-asparaginyl-S-(2-methoxycarbonylethyl)homocysteinyl-prolyl-N^{δ}-benzyloxycarbonylornithyl-glycinamide (*VIIa*)

To a solution of protected pentapeptide *VIa* (5·34 g) in dimethylformamide (40 ml) we added 2M-HCl in ether (12·0 ml). After 5 min at room temperature the mixture was diluted with ether and the hydrochloride which separated out was collected and ground up with ether and filtered; $E^{Gly}_{2,4}$ 0·67, $E^{His}_{5,7}$ 0·42. After drying the pentapeptide hydrochloride was dissolved in dimethylformamide (150 ml), pH was adjusted to about 8·5 (wet pH paper) with N-ethylpiperidine and we added the 2,4,5-trichlorophenyl ester of *o*-nitrobenzenesulphenylglutamine[50] (2·9 g). After 24 h stirring at room temperature we added further active ester (1·4 g) and after a further 24 h the mixture was evaporated. The residue was triturated with light petroleum and ether and thus converted to a solid substance. The product was precipitated from methanol and ether, filtered off, dried on the filter and washed with water, 0·5M-$NaHCO_3$ and water. The yield was 4·6 g (76%) of a product with m.p. 161—165°C. The sample for analysis was recrystallised from methanol and ether with no change in m.p.; $[\alpha]_D^{25}$ −40·0° (*c* 0·2, dimethylformamide). For $C_{43}H_{59}N_{11}O_{14}S_2.H_2O$ (1036) calculated: 49·85% C, 5·93% H, 14·88% N; found: 49·57% C, 5·82% H, 14·92% N.

o-Nitrobenzenesulphenylphenylalanyl-glutaminyl-asparaginyl-S-(2-methoxycarbonylethyl)-homocysteinyl-prolyl-N^{δ}-benzyloxycarbonylornithyl-glycinamide (*VIIIa*)

The *o*-nitrobenzenesulphenyl-protecting group was split off from the protected hexapeptide *VIIa* (3·1 g) in the usual manner (15 ml dimethylformamide, 5·0 ml 2M-HCl in ether, 5 min); $E^{Gly}_{2,4}$ 0·59. After drying the hexapeptide hydrochloride was dissolved in dimethylformamide (100 ml) and N-ethylpiperidine was added until pH reached about 8·5 (wet pH paper) and the 2,4,5-trichlorophenyl ester of *o*-nitrobenzenesulphenylphenylalanine[51] (1·5 g) was added. After 24 h stirring

at room temperature further active ester (0·7 g) was added and after a further 24 h the mixture was evaporated and the residue was triturated with light petroleum and then with ether, filtered and immediately precipitated from ethanol and ether. The product was filtered off, washed with water, 0·5M-$NaHCO_3$ and water, and dried. The yield was 3·0 g (85%) of product. Crystallisation from dimethylformamide and water gave 2·85 g (80%) of product with m.p. 183—185°C. The sample for analysis was recrystallised in the same manner with no change in m.p.; $[\alpha]_D^{25} -14{\cdot}5°$ (*c* 0·25, dimethylformamide). For $C_{52}H_{68}N_{12}O_{15}S_2.H_2O$ (1183) calculated: 52·80% C, 5·96% H, 14·23% N; found: 52·63% C, 5·76% H, 14·47% N.

Phenylalanyl-glutaminyl-asparaginyl-S-(2-carboxyethyl)homocysteinyl-prolyl-N$^{\delta}$-benzyloxy-carbonylornithyl-glycinamide (*VIIId*)

To a solution of protected heptapeptide *VIIIa* (2·12 g) in dimethylformamide (15 ml) we adde d 2M-HCl in ether (4·0 ml). After 5 min at room temperature the mixture was diluted with ether and the hydrochloride which separated out was filtered and washed with ether; $E_{2.4}^{Gly}$ 0·62, $E_{5.7}^{His}$ 0·33 After drying the hydrochloride was dissolved in a mixture of methanol (20 ml) and 1M-NaOH (8·0 ml). After 1 h at room temperature the methanol was evaporated off at 20°C and the aqueous solution was transferred to a column of Dowex 50 (H^+-cycle, 60 ml). The column was washed with water and the product eluted by 10% pyridine. The eluate was acidified to pH 6·0—6·5 with acetic acid, evaporated to a smaller volume and freeze-dried. The lyophilisate was precipitated from methanol and ether, filtered, washed thoroughly with ether, and after drying (1·65 g) dissolved in 50 ml of the lower phase of the system 2-butanol–0·05% acetic acid. The solution was transferred to the second and third tubes of the counter-current distribution machine and 182 transfers of the upper phase were carried out. The contents of tubes 39—75 ($K = 0{\cdot}43$) were pooled, evaporated to a small volume and freeze dried. The yield was 1·21 g (67%) of product. The sample for analysis was crystallised from dimethylformamide and ether; $[\alpha]_D^{25} -34{\cdot}0°$ (*c* 0·2, dimethylformamide); $E_{2.4}^{Gly}$ 0·62, $E_{5.7}^{His}$ 0·12. Amino-acid analysis: Orn 0·97, Asp 1·03, Glu 1·00, Pro 0·99, Gly 1·06, Hcy($C_2H_4CO_2H$) 0·98, Phe 0·99. For $C_{45}H_{63}N_{11}O_{13}S.\ 2{\cdot}5\ H_2O$ (1043) calculated: 51·85% C, 6·58% H, 14·78% N; found: 51·60% C, 6·28%H, 14·90%N.

Tert-butyloxycarbonyl-O-tert-butyltyrosyl-phenylalanyl-glutaminyl-asparaginyl-
-S-(2-carboxyethyl)homocysteinyl-prolyl-N$^{\delta}$-benzyloxycarbonylornithyl-glycinamide (*IXa*)

To a solution of heptapeptide *VIIId* (1·12 g) in dimethylformamide (50 ml) N-ethylpiperidine (0·70 ml) and N-tert-butyloxycarbonyl-O-tert-butyltyrosine N-hydroxysuccinimide ester[3] (0·50 g) were added. After 24 h stirring at room temperature further active ester (0·30 g) was added and the same amount again after a further 24 h. After a day, the mixture was evaporated and ground up with light petroleum and then ether. The crystalline portion was filtered and washed on the filter with ether, water, 3% citric acid and water. Yield 1·20 g (81%), m.p. 192—194°C. The sample for analysis was crystallised from dimethylformamide and ether without change in m.p.; $[\alpha]_D^{25} -33{\cdot}0°$ (*c* 0·2, dimethylformamide); $E_{2.4}^{Gly}$ 1·05, $E_{5.7}^{His}$ 0·35 (after removal of protecting groups with hydrogen bromide in acetic acid). For $C_{63}H_{88}N_{12}O_{17}S.H_2O$ (1335) calculated: 56·60% C, 6·79% H, 12·59% N; found: 56·39% C, 6·62% H, 12·80% N.

o-Nitrobenzenesulphenyl-O-tert-butyltyrosyl-phenylalanyl-glutaminyl-asparaginyl-
-S-(2-carboxyethyl)homocysteinyl-prolyl-N$^{\delta}$-benzyloxycarbonylornithyl-glycinamide (*IXb*)

To a solution of heptapeptide *VIIId* (0·60 g) in dimethylformamide (40 ml) N-ethylpiperidine (0·25 ml) was added along with the N-hydroxysuccinimide ester of *o*-nitrobenzenesulphenyl-

-O-tert-butyltyrosine[52] (0·30 g). After 24 h stirring at room temperature further active ester (0·15 g) was added and after a further 24 h the mixture was evaporated and the residue was triturated with light petroleum and ether. The crystalline portion was filtered off and washed with ether, 0·1N-H_2SO_4 and water. The yield was 0·74 g (90%) of a product with m.p. 181—184°C. The sample for analysis was crystallised from dimethylformamide and ether; m.p. 180—183°C, $[\alpha]_D^{25}$ +13·7° (*c* 0·5, dimethylformamide). For $C_{64}H_{83}N_{13}O_{17}S_2.H_2O$ (1389) calculated: 55·35% C, 6·17% H, 13·13% N; found: 55·40% C, 6·03% H, 13·41% N.

The Lactam of Tyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-(2-carboxyethyl)homocysteinyl--prolyl-N^{δ}-benzyloxycarbonylornithyl-glycinamide (*Xa*)

To a solution of protected octapeptide-acid *IXa* (400 mg) in a mixture of dimethylformamide (15 ml) and pyridine (15 ml) we added with constant mixing and N_2 bubbling bis(*p*-nitrophenyl) sulphite[25] (1·4 g). After 7 h at room temperature further reagent (1·4 g) was added along with 7 ml pyridine and after a 15 h a further 0·7 g of reagent. After a further 5 h stirring and bubbling through of N_2 the mixture was evaporated, the residue was ground up with ether, filtered and washed with ether and water and dried in air; yield 405·7 mg (95%). The active ester was dissolved in trifluoroacetic acid (8·0 ml) and after 1 h at room temperature the mixture was diluted with toluene (10 ml) and evaporated. The residue was dissolved in dimethylformamide (15 ml) and this solution was added over 6 h to 400 ml pyridine with stirring, heating to 50°C and bubbling through of N_2. After 15 h at room temperature the mixture was evaporated, the residue was triturated with ether, filtered, washed with ether and purified by counter-current distribution (184 transfers of the upper phase and 535 of the lower). A peak with a distribution coefficient of 4·21 (tubes 22—67) was concentrated by evaporation and freeze-dried. The yield was 180 mg (55%) of a product which was further purified by gel filtration in 3M acetic acid and precipitated from methanol and ether. The final yield was 112 mg (34%) of an analytically pure product, R_F 0·16 (S1), 0·33 (S2), 0·67 (S3); $[\alpha]_D^{25}$ −52·4° (*c* 0·2, dimethylformamide). Amino-acid analysis: Orn 0·98, Asp 1·05, Glu 1·01, Pro 0·98, Gly 1·02, Tyr 0·87, Phe 0·98, Hcy($C_2H_4CO_2H$) 1·13. For $C_{54}H_{71}$. $.N_{12}O_{14}S.4\,H_2O$ (1216) calculated: 53·30% C, 6·46% H, 13·82% N; found: 53·48% C, 5·93% H, 13·90% N.

The Lactam of O-Tert-butyltyrosyl-phenylalanyl-glutaminyl-asparaginyl--S-(2-carboxyethyl)homocysteinyl-prolyl-N^{δ}-benzyloxycarbonylornithyl-glycinamide (*Xb*)

The active ester was prepared from protected octapeptide-acid *IXb* (600 mg) in a similar manner to the preparation of substance *Xa* (20 ml dimethylformamide, 30 ml pyridine, 5 g bis(*p*-nitrophenyl) sulphite) with a yield of 616·5 mg (96%). The active ester was dissolved in dimethylformamide (4 ml) and 2M-HCl in ether (0·5 ml) was added. After 5 min at room temperature the mixture was diluted with ether. The hydrochloride which separated out was filtered off and washed with ether, dried and dissolved in dimethylformamide (15 ml). The latter solution was added over 6 h to 500 ml pyridine and 54 µl N-ethylpiperidine with stirring, heating to 50°C and bubbling through with N_2. After 15 h at room temperature the mixture was evaporated, the residue was ground with ether, filtered and washed with ether. After drying the product was purified by means of counter-current distribution (100 transfers of the upper phase). A peak with distribution coefficient 11·5 (tubes 81—102) was pooled, concentrated by evaporation and freeze-dried. After precipitation from methanol and ether the yield was 367 mg (73%) of a product with m.p. 159—160°C. The sample for analysis was crystallised in the same manner; $[\alpha]_D^{25}$ −46·1° (*c* 0·2, dimethylformamide); R_F 0·13 (S1), 0·72 (S3). For $C_{58}H_{78}N_{12}O_{14}S.3\,H_2O$ (1253) calculated: 55·55% C, 6·75% H, 13·42% N; found: 55·39% C, 6·35% H, 13·48% N.

The Lactam of Tyrosyl-phenylalanyl-glutaminyl-asparaginyl-
-S-(2-carboxyethyl)homocysteinyl-prolyl-ornithyl-glycinamide (*Ie*)

To a solution of *Xb* (327 mg) in acetic acid (10 ml) we added 35% HBr in acetic acid (15 ml). The mixture was heated to 50°C and left 15 min at room temperature, then diluted with ether and the hydrobromide which separated out was filtered and washed with ether. After drying, the hydrobromide was dissolved in water (20 ml) and filtered through a column of Amberlite IR-4B (25 ml, OH^--cycle). Freeze-drying of the eluate gave a yield of 277 mg of a product which was purified by gel filtration (Bio-gel P-4, 1M acetic acid). The yield was 128 mg (43%) of a product which was purified for analysis by continuous free-flow electrophoresis and precipitated from methanolic solution with ether; $E_{2.4}^{Gly}$ 0·66, $E_{5.7}^{His}$ 0·39; R_F 0·00 (S1), 0·09 (S2), 0·43 (S3); $[\alpha]_D^{25}$ −60·2° (*c* 0·24, 1M acetic acid). Amino-acid analysis: Asp 1·06, Glu 1·02, Pro 0·99, Gly 1·03, Tyr 0·92, Phe 0·99, Orn 0.93, Hcy($C_2H_4CO_2H$) 1·05. For $C_{46}H_{64}N_{12}O_{12}S.CH_3CO_2H.4\,H_2O$ (1 141) calculated: 50·50% C, 6·71% H, 14·73% N; found: 50·48% C, 6·45% H, 15·03% N.

Benzyloxycarbonyl-S-(2-methoxycarbonylethyl)homocysteinyl-prolyl-
-N^G-tosylarginyl-glycinamide (*Va*)

To a solution of benzyloxycarbonyl-N^G-tosylarginyl-glycinamide[23] (1·85 g) in acetic acid (6 ml) we added 35% HBr in acetic acid (6 ml) and after 15 min at room temperature the mixture was diluted with ether. The hydrobromide which separated out was powdered, washed with ether and dried. After dissolving in dimethylformamide (15 ml) N-ethylpiperidine was added to a pH of about 8·5 (wet pH paper) and benzyloxycarbonyl-S-(2-methoxycarbonylethyl)homocysteine 5-chloro-8-quinolyl ester[4] (1·60) g) was added. After 2 days stirring at room temperature the mixture was evaporated and the residue dissolved in chloroform (200 ml). The latter solution was sequentially washed with 1M-HCl, 0·5M-$NaHCO_3$ and water (the emulsion was separated out by centrifugation), dried with sodium sulphate and evaporated. The residue was dissolved in 2-propanol, cooled to −30 to −40°C until the crystalline turbidity was observed (if an oil starts to separate out the solution must be heated again and more solvent added) and then ether was rapidly added. After several h at +3°C the precipitate was filtered off and washed with ether and put into a desiccator over P_2O_5 and NaOH. After drying the substance is stable in air. The yield was 1·60 g (65%) with m.p. 85−87°C. The sample for analysis was precipitated in the same way, m.p. 90−93°C; $[\alpha]_D^{25}$ −22·0° (*c* 0·5, dimethylformamide). For $C_{36}H_{50}N_8O_{10}S_2.H_2O$ (837·0) calculated: 51·70% C, 6·27% H, 13·40% N; found: 51·69% C, 6·34% H, 13·70% N.

In a similar manner the tetrapeptide amide was prepared by reaction of the 5-chloro-8-quinolyl ester of N-benzyloxycarbonyl-S-(2-methoxycarbonylethyl)homocysteine with the amide of prolyl-
-NG-tosylarginyl-glycine[23]; yield 50%, m.p. 87−89°C.

o-Nitrobenzenesulphenylasparaginyl-S-(2-methoxycarbonylethyl)-homocysteinyl-prolyl-
-N^G-tosylarginyl-glycinamide (*VIb*)

To a solution of protected tetrapeptide *Va* (1·10 g) in acetic acid (5 ml) we added 35% HBr in acetic acid (5 ml). After 10 min at room temperature the mixture was diluted with ether, the hydrobromide which separated out was washed several times with ether and dried ($E_{2.4}^{Gly}$ 0·80, $E_{5.7}^{His}$ 0·45). After dissolving in dimethylformamide (40 ml) pH was adjusted to about 8·5 (wet pH paper) with N-ethylpiperidine and the 2,4,5-trichlorophenyl ester of *o*-nitrobenzenesulphenyl-asparagine[50] (0·70 g) was added. After 24 h stirring at room temperature further active ester (0·25 g) was added and after 24 h the mixture was evaporated. The residue was sequentially

ground with light petroleum, ether, 0·5M-$NaHCO_3$, 0·2M-H_2SO_4 and water and dissolved in methanol. The latter solution was cooled to a very low temperature (the substance must not separate out as an oil but as a suspension, otherwise the volume of methanol must be increased, temperature raised again and the entire procedure repeated). Ether was added, the precipitate left several hours at +3°C, filtered, washed with ether and dried over P_2O_5 in a desiccator. The yield was 1·07 g (84%) of a product with m.p. 113—115°C. The sample for analysis was precipitated in the same manner with no change in m.p.; $[\alpha]_D^{25}$ −41·7° (*c* 0·5, dimethylformamide). For $C_{38}.H_{53}N_{11}O_{12}S_3.0{\cdot}5\,H_2O$ (961·1) calculated: 47·50% C, 5·66% H, 16·04% N; found: 47·31% C, 5·54% H, 15·74% N.

o-Nitrobenzenesulphenylglutaminyl-asparaginyl-S-(2-methoxycarbonylethyl)homocysteinyl--prolyl-N^G-tosylarginyl-glycinamide (*VIIb*)

To a solution of protected pentapeptide *VIb* (3·0 g) in dimethylformamide (15 ml) we added 2M-HCl in ether (4·0 ml). After 5 min at room temperature the mixture was diluted with ether and the hydrochloride which separated out was filtered off and washed with ether; $E_{2.4}^{Gly}$ 0·74, $E_{5.7}^{His}$ 0·44. After drying, the pentapeptide hydrochloride was dissolved in dimethylformamide (50 ml) and N-ethylpiperidine was added until pH was about 8·5 (wet pH paper) and we then added *o*-nitrobenzenesulphenylglutamine 2,4,5-trichlorophenyl ester (1·6 g). After 24 h stirring at room temperature further active ester (0·8 g) was added and after 24 h the mixture was evaporated and the residue was triturated with light petroleum and ether, which converted the substance to a solid form. The product was precipitated from methanolic solution with ether in the same manner as pentapeptide *VIb*. The yield was 3·15 g (92%) of a product with m.p. 113—116°C. The sample for analysis was precipitated again in the same manner, m.p. 120 to 123°C; $[\alpha]_D^{25}$ −34·7° (*c* 0·5, dimethylformamide). For $C_{43}H_{61}N_{13}O_{14}S_3.H_2O$ (1098) calculated: 47·01% C, 5·78% H, 16·59% N; found: 46·91% C, 5·73% H, 16·34% N.

o-Nitrobenzenesulphenylphenylalanyl-glutaminyl-asparaginyl--S-(2-methoxycarbonylethyl)homocysteinyl-prolyl-N^G-tosylarginyl-glycinamide (*VIIIb*)

From protected hexapeptide *VIIb* (1·08 g) we split off the *o*-nitrobenzenesulphenyl-protecting group in the usual manner (5 ml dimethylformamide, 1·5 ml 2M-HCl in ether, 5 min); $E_{2.4}^{Gly}$ 0·63, $E_{5.7}^{His}$ 0·36. After drying the hexapeptide hydrochloride was dissolved in dimethylformamide (30 ml) and N-ethylpiperidine was added until pH reached about 8·5 (wet pH paper) and then the *o*-nitrobenzenesulphenylphenylalanine 2,4,5-trichlorophenyl ester[51] (0·50 g) was added. After 24 h stirring at room temperature further active ester was added (0·25 g) and after 24 h the mixture was evaporated and processed further as in the case of substance *VIb*. The yield was 1·18 g (96%) of a product with m.p. 131—140°C. The sample for analysis was recrystallised from methanol and ether, m.p. 145—149°C; $[\alpha]_D^{25}$ −13·9° (*c* 0·2, dimethylformamide). For $C_{52}H_{70}N_{14}O_{15}S_3.2\,H_2O$ (1263) calculated: 49·45% C, 5·91% H, 15·53% N; found: 49·45% C, 5·74% H, 15·76% N.

Phenylalanyl-glutaminyl-asparaginyl-S-(2-carboxyethyl)homocysteinyl-prolyl--N^G-tosylarginyl-glycinamide (*VIIIe*)

To a solution of protected heptapeptide *VIIIb* (1·94 g) in dimethylformamide (10 ml) we added 2M-HCl in ether (2·7 ml). After 5 min at room temperature the mixture was diluted with ether, the hydrochloride which separated out was powdered, filtered off and washed with ether; $E_{2.4}^{Gly}$ 0·64, $E_{5.7}^{His}$ 0·31. After drying the substance was dissolved in a mixture of methanol (20 ml) and

1M-NaOH (7 ml). After 1 h at room temperature the methanol was evaporated off at 20°C and the aqueous solution was transferred to a column of Dowex 50 (H^+-cycle, 45 ml). The column was washed with water and the product eluted with 10% pyridine. The eluate was acidified with acetic acid to pH 6·0—6·5, concentrated to a small volume and freeze-dried. The lyofilisate was precipitated from dimethylformamide, methanol and ether; 1·43 g (86%) of a product with m.p. 145—148°C. The sample for analysis was reprecipitated in the same manner, m.p. 148—153°C; $[\alpha]_D^{25}$ −26·9° (*c* 0·2, dimethylformamide); $E_{2.4}^{Gly}$ 0·53, $E_{5.7}^{His}$ 0·16. Amino-acid analysis: Arg 1·03, Asp 1·00, Glu 0·96, Pro 0·98, Gly 0·97, Hcy(C_2H_4COOH) 1·18, Phe 0·93. For $C_{45}H_{65}N_{13}O_{13}S_2 . 2\,H_2O$ (1096) calculated: 49·24% C, 6·34% H, 16·62% N; found: 49·15% C, 6·13% H, 16·36% N.

o-Nitrobenzenesulphenyl-O-tert-butyltyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-(2-carboxyethyl)homocysteinyl-prolyl-N^G-tosylarginyl-glycinamide (*IXc*)

To a solution of free heptapeptide *VIIIe* (0·40 g) in dimethylformamide (10 ml) we added N-ethylpiperidine (0·15 ml) and N^{α}-*o*-nitrobenzenesulphenyl-O-tert-butyltyrosine N-hydroxysuccinimide ester[52] (0·20 g). After 24 h stirring at room temperature further active ester (0·10 g) was added and after 24 h the mixture was evaporated and the residue ground with light petroleum and ether. The crystalline portion was filtered and washed with ether, dried, washed with 0·1M-H_2SO_4, water and crystallised from dimethylformamide, methanol and ether. The yield was 0·48 g (89%) of a product with m.p. 170—173°C. The sample for analysis was recrystallised in the same manner with no change in m.p.: $[\alpha]_D^{25}$ +8·7° (*c* 0·22, dimethylformamide). For $C_{64}H_{85} . N_{15}O_{17}S_3 . 1{\cdot}5\,H_2O$ (1460) calculated: 52·62% C, 6·07% H, 14·40% N; found: 52·42% C, 5·93% H, 14·70% N.

The Lactam of O-Tert-Butyltyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-(2-carboxyethyl)homocysteinyl-prolyl-N^G-tosylarginyl-glycinamide (*IXc*)

To a solution of protected octapeptide acid *IXc* (300 mg) in a mixture of dimethylformamide (10 ml) and pyridine (10 ml) 1 g of bis(*p*-nitrophenyl) sulphite was added with continual stirring and bubbling through of N_2. After 7 h at room temperature a further 1 g of reagent was added along with 5 ml pyridine, and after 15 h a further 0·5 g reagent. After 6 h the mixture was evaporated, the residue was ground with ether, filtered and washed with ether and after drying it was precipitated from methanolic solution with ether. The yield was 271 mg (83%).

To a solution of the above product in dimethylformamide (5 ml) 2M-HCl in ether (0·2 ml) was added and after 5 min the mixture was diluted with ether and the hydrochloride which separated out was filtered, washed thoroughly on the filter with ether, dried and dissolved in dimethylformamide (10 ml). This solution was added over 4 h to 200 ml pyridine and 25 µl N-ethylpiperidine with constant stirring and heating to 50°C, with bubbling through of N_2. After 12 h at room temperature the mixture was evaporated, the residue was triturated with ether, filtered and washed with ether. After drying the product was purified by counter-current distribution (117 upper phase and 175 lower phase transfers). The peak with a distribution coefficient of 7·1, contained in tubes 60 to 103, was pooled, concentrated and freeze-dried. After precipitation from methanolic solution with ether the yield was 58·6 mg (22%); R_F 0·23 (S1), 0·37 (S2), 0·78 (S3). Amino-acid analysis: Arg 0·93, Asp 0·99, Glu 1·01, Pro 1·09, Gly 1·02, Hcy(C_2H_4COOH) 0·96, Tyr 0·99, Phe 1·01. For $C_{58}H_{80}N_{14}O_{14}S_2 . 6H_2O$ (1370) calculated: 50·90% C, 6·77% H, 14·34% N; found: 50·9—97% C, 6·02% H, 14·27% N.

The Lactam of Tyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-(2-carboxyethyl)-homocysteinyl-prolyl-arginyl-glycinamide (*If*)

Protected cyclic octapeptide *Xc*(56·0 mg) was reduced in liquid hydrogen fluoride in an apparatus for reactions in non-aqueous hydrogen fluoride (Toho Kasei Co., Ltd., Osaka) at 0°C for 30 min. The hydrogen fluoride was evaporated off *in vacuo* (water pump) and the residue was dried 2 h *in vacuo* (oil pump), dissolved in 1M acetic acid (20 ml) and extracted several times with ethyl acetate. The aqueous layer was evacuated and freeze-dried. The lyophilisate was dissolved in water (5 ml) and filtered through a column of Amberlite IR-4B (1 ml, acetate cycle, pH 2·5). The eluate after freeze-drying was then purified by gel filtration (P-4, 1M acetic acid) and continuous free-flow electrophoresis. The yield was 22·3 mg (40%), R_F 0·00 (S1), 0·10 (S2), 0·51 (S3); $E^{Gly}_{2.4}$ 0·61, $E^{His}_{5.7}$ 0·36; $[\alpha]_D^{25}$ −62·0° (*c* 0·2, 1M acetic acid). Amino-acid analysis: Arg 1·03, Asp 1·05, Glu 1·01, Pro 0·94, Gly 1·00, Hcy(C_2H_4COOH) 0·93, Phe 1·00, Tyr 1·00. For $C_{47}H_{66}N_{14}O_{12}S.CH_3COOH.6\,H_2O$ (1 219) calculated: 48·30% C, 6·78% H, 16·10% N; found: 48·48% C, 6·25% H, 15·95% N.

Benzyloxycarbonyl-S-(2-methoxycarbonylethyl)homocysteinyl-prolyl-N^G-tosyl-D-arginyl-glycinamide (*Vb*)

Tetrapeptide *Vb* was prepared in a manner similar to that for tetrapeptide *Va* from 3·7 g benzyloxycarbonylprolyl-N^G-tosyl-D-arginyl-glycinamide[24], 12 ml acetic acid, 12 ml 35% HBr in acetic acid, 30 ml dimethylformamide, N-ethylpiperidine, 3·2 g of the 5-chloro-8-quinolyl ester of benzyloxycarbonyl-S-(2-methoxycarbonylethyl)homocysteine[4]. After crystallisation from 2-propanol with ether the yield was 2·95 g (60%) of a product with m.p. 86—91°C. The sample for analysis was recrystallised in the same manner, m.p. 87—90°C; $[\alpha]_D^{25}$ −4·0° (*c* 0·5, dimethylformamide). For $C_{36}H_{50}N_8O_{10}S_2.0{\cdot}5\,H_2O$ (828·0) calculated: 52·25% C, 6·21% H, 13·56% N; found: 52·23% C, 6·44% H, 13·25% N.

o-Nitrobenzenesulphenylasparaginyl-S-(2-methoxycarbonylethyl)homocysteinyl-prolyl-N^G-tosyl-D-arginyl-glycinamide (*VIc*)

Protected pentapeptide amide *VIc* was prepared in the same manner as pentapeptide *VIb* from 2·7 g substance *Vb*, 13 ml acetic acid, 13 ml 35% HBr in acetic acid; $E^{Gly}_{2.4}$ 0·80, $E^{His}_{5.7}$ 0·45; 100 ml dimethylformamide, N-ethylpiperidine, 1·7 and 0·7 g *o*-nitrobenzenesulphenylasparagine 2,4,5-trichlorophenyl ester. The residue was ground up with light petroleum, then ether and 0·5M-$NaHCO_3$. The crystalline portion was filtered off and washed on the filter with 0·5M-$NaHCO_3$ water, 0·1M-H_2SO_4, water and then dried in air. After crystallisation from methanol and ether the yield was 2·77 g (88%) of a product with m.p. 122—125°C. The sample for analysis was recrystallised in the same manner; m.p. 125—127°C; $[\alpha]_D^{25}$ −27.10° (*c* 0·5, dimethylformamide). For $C_{38}H_{53}N_{11}O_{12}S_3.0{\cdot}5\,H_2O$ (961·1) calculated: 47·50% C, 5·66% H, 16·04% N; found: 47·68% C, 5·72% H, 15·57% N.

o-Nitrobenzenesulphenylglutaminyl-asparaginyl-S-(2-methoxycarbonylethyl)homocysteinyl-prolyl-N^G-tosyl-D-arginyl-glycinamide (*VIIc*)

Protected hexapeptide amide *VIIc* was prepared similarly to hexapeptide *VIIb* from 1·9 g *VIc*, 10 ml dimethylformamide, 3·0 ml 2M-HCl in ether; $E^{Gly}_{2.4}$ 0·74, $E^{His}_{5.7}$ 0·44; 50 ml dimethylformamide, N-ethylpiperidine, 1·0 g and 0·5 g *o*-nitrobenzenesulphenylglutamine 2,4,5-trichlorophenyl ester. The yield was 2·07 g (96%) of a product with m.p. 115—130°C. The sample for analysis was crystallised from methanol and ether, m.p. 130—137°C (softening from 110°C); $[\alpha]_D^{25}$ −32·5°

(*c* 0·23, dimethylformamide). For $C_{43}H_{61}N_{13}O_{14}S_3.0{\cdot}5\,H_2O$ (1089) calculated: 47·45% C, 5·74% H, 16·75% N; found: 47·62% C, 5·90% H, 16·30% N.

o-Nitrobenzenesulphenylphenylalanyl-glutaminyl-asparaginyl-S-(2-methoxycarbonylethyl)-homocysteinyl-prolyl-N^G-tosyl-D-arginyl-glycinamide (*VIIIc*)

Protected heptapeptide *VIIIc* was prepared similarly as heptapeptide *VIIIb* from 1·83 g *VIIc*, 15 ml dimethylformamide, 2·7 ml 2M-HCl in ether; $E^{Gly}_{2.4}$ 0·68, $E^{His}_{5.7}$ 0·32; 30 ml dimethylformamide N-ethylpiperidine, 0·85 g and 0·45 g of *o*-nitrobenzenesulphenylphenylalanine 2,4,5-trichlorophenyl ester. After crystallisation from methanol and ether the yield was 1·93 g (93%) of a product with m.p. 136—141°C. The sample for analysis was recrystallised in the same manner, m.p. 139—142°C; $[\alpha]_D^{25}$ +13·2° (*c* 0·2, dimethylformamide). For $C_{52}H_{70}N_{14}O_{15}S_3.H_2O$ (1245) calculated: 50·15% C, 5·83% H, 15·74% N; found: 50·15% C, 5·76% H, 15·73% N.

Phenylalanyl-glutaminyl-asparaginyl-S-(2-carboxyethyl)homocysteinyl-prolyl-N^G-tosyl-D-arginyl-glycinamide (*VIIIf*)

Heptapeptide *VIIIf* was prepared similarly as *VIIIe* from 1·8 g *VIIIc*, 10 ml dimethylformamide, 2·3 ml 2M-HCl in ether; $E^{Gly}_{2.4}$ 0·57, $E^{His}_{5.7}$ 0·29; 15 ml methanol, 6 ml 1M-NaOH, 30 ml Dowex 50W X 2. The yield was 1·2 g (77%). The sample for analysis was recrystallised from dimethylformamide, methanol and ether; $[\alpha]_D^{25}$ −12·5° (*c* 0·22, dimethylformamide). Amino-acid analysis: Arg 1·06, Asp 1·07, Glu 0·94, Pro 1·03, Gly 1·00, Hcy(C_2H_4COOH) 1·20, Phe 0·91. For $C_{45}H_{65}N_{13}O_{13}S_2.2\,H_2O$ (1096) calculated: 49·24% C, 6·34% H, 16·60% N; found: 49·25% C, 6·04% H, 16·51% N.

o-Nitrobenzenesulphenyl-O-tert-butyltyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-(2-carboxyethyl)homocysteinyl-prolyl-N^G-tosyl-D-arginyl-glycinamide (*IXd*)

Protected octapeptide *IXd* was prepared similarly as *IXc* from 1·08 g *VIIIf*, 20 ml dimethylformamide, 0·4 ml and 0·4 ml (after 8 h) N-ethylpiperidine, 0·50 g and 0·25 g (after 8 h) and 0·25 g (after 16 h) *o*-nitrobenzenesulphenyl-O-tert-butyltyrosine N-hydroxysuccinimide ester. The yield was 1·28 g (88%) with m.p. 172—176°C. The sample for analysis was recrystallised from dimethylformamide, methanol and ether, m.p. 174—177°C, $[\alpha]_D^{25}$ +21·0° (*c* 0·2, dimethylformamide); (after removal of the *o*-nitrobenzenesulphenyl-protecting group by HCl in ether: $E^{Gly}_{2.4}$ 0·49, $E^{His}_{5.7}$ 0·12). For $C_{64}H_{85}N_{15}O_{17}S_3.1{\cdot}5\,H_2O$ (1460) calculated: 52·62% C, 6·07% H, 14·40% N; found: 52·57% C, 5·85% H, 14·54% N.

The Lactam of O-Tert-butyltyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-(2-carboxyethyl)-homocysteinyl-prolyl-N^G-tosyl-D-arginyl-glycinamide (*Xd*)

Protected octapeptide acid *IXd* (479 mg) was transformed to the active ester in the same manner as above (yield 440 mg, 85%). After removal of the *o*-nitrobenzenesulphenyl protecting group by hydrogen chloride in ether cyclisation was carried out (50°C, N_2 bubbling). The product was purified by counter-current distribution (100 transfers of the upper phase, $K = 12{\cdot}5$). The yield was 199·3 mg (47%), R_F 0·20 (S1), 0·33 (S2), 0·67 (S3). Amino-acid analysis: Arg 1·03, Asp 1·10, Glu 1·00, Pro 0·92, Gly 1·09, Hcy(C_2H_4COOH) 0·98, Tyr 0·92, Phe 0·97. For $C_{58}H_{80}N_{14}O_{14}S_2.4H_2O$ (1334) calculated: 52.20% C, 6·65% H, 14·68% N; found: 51·89% C, 6·30% H, 14·53% N.

The Lactam of Tyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-(2-carboxyethyl)homocysteinyl-prolyl-D-arginyl-glycinamide (*Ig*)

Protected cyclic octapeptide *Xd* was reduced in liquid hydrogen fluoride as described for *If*. From 99·7 mg of protected peptide after purification (P-4 in 1M acetic acid and continuous free-flow electrophoresis) we obtained 15·4 mg (19%); R_F 0·00 (S1), 0·13 (S2), 0·56 (S3); $E^{Gly}_{2.4}$ 0·66, $E^{His}_{5.7}$ 0·38. Amino-acid analysis: Arg 1·04, Gly 1·02, Pro 0·92, Hcy(C_2H_4COOH) 0·80, Asp 1·06, Glu 1·00, Phe 1·00, Tyr 1·00. For $C_{47}H_{66}N_{14}O_{12}S.CH_3COOH.7H_2O$ (1237) calculated: 47·55% C, 6·85% H, 15·86% N; found: 47·72% C, 6·71% H, 15·69% N.

Pharmacological Methods

a) *Pressor activity*: Two pressor assays were used: despinalised Wistar rats[26] and nephrectomised, ganglion-blocked rats in urethane anesthesia[27]. In both cases, activities were calculated in units from threshold values of four-point curves, using synthetic lysine-vasopressin as standard.

b) *Uterotonic activity* was assayed on rat uterus horn strips 24 h after oestradiol administration (10 μg/kg, *s.c.*). Van Dyke-Hastings solution[53] was used in a 5 ml organ bath at pH 7·4 at 30°C. Isometric contractions were measured with a permanent magnet feedback transducer[45] and recorded on a strip-chart recorder.

c) *Antidiuretic activity*: Two antidiuretic assays were used: ethanol anaesthetised rats with an oral tap water load maintained at 8—10% body weight with urine flow rate measured as drop counts recorded automatically, and continuous measurement of urine conductivity[31]. The second method was a modification of the Burn assay[32], in which trained, unanaesthetised rats, body weight 150—180 g, received an oral tap water load of 2% body weight, and T-$^1/_2$ of excretion of the load, in comparison with untreated controls run at the same time, was measured in min. Peptides were given *s.c.* at the time of oral loading. Special metabolic cages were used to ensure a quantitative collection of spontaneously voided urine with no faecal contamination, the surface of the plexiglass funnels being treated beforehand with Bryj 35 to make the surface more non-wettable. In both cases activity was taken as threshold from extrapolation of 3—4 point curves, using synthetic lysine-vasopressin and oxytocin as standards. The total pharmacological activity was measured as intensity times duration. In the latter assay, the rats were trained to the assay situation at least one week before starting to measure, and all rats were rotated through all doses of all peptides and non-treated (*s.c.* injection of saline alone) controls.

Our thanks are due to Mrs J. Cortová, Mrs H. Kovářová, Mrs J. Kellerová, Mrs M. Roubíčková for skillful technical assistance, to Mrs H. Farkašová for the amino-acid analyses and to Mrs Z. Ledvinová for the optical rotation measurements. The elemental analyses were carried out in the Analytical Laboratory of this Institute.

REFERENCES

1. Jošt K., Rudinger J.: This Journal *32*, 1229 (1967).
2. Jošt K., Rudinger J., Klostermeyer H., Zahn H.: Z. Naturforsch. *23b*, 1059 (1968).
3. Jošt K.: This Journal *36*, 218 (1971).
4. Jošt K., Šorm F.: This Journal *36*, 234 (1971).
5. Kobayashi A., Hase S., Kiyoi R., Sakakibara S.: Bull. Chem. Soc. Japan *42*, 3491 (1969).
6. Yamanaka T., Hase S., Sakakibara S., Schwartz I. L., Dubois B. M., Walter R.: Mol. Pharmacol. *6*, 474 (1970).

7. Sakakibara S., Hase S.: Bull. Chem. Soc. Japan *41*, 2816 (1968).
8. Hase S., Morikawa T., Sakakibara S.: Experientia *25*, 1239 (1969).
9. Hase S., Sakakibara S., Wahrenburg M., Kirchberger M., Schwartz I. L., Walter R.: J. Am. Chem. Soc. *94*, 3590 (1972).
10. Cort J. H.: Unpublished results.
11. *Tentative Rules on Biochemical Nomenclature.* Biochemistry *5*, 2485 (1966); *6*, 362 (1967).
12. Bodanszky M., du Vigneaud V.: J. Am. Chem. Soc. *81*, 5688 (1959).
13. Zervas L., Borovas D., Gazis E.: J. Am. Chem. Soc. *85*, 3660 (1963).
14. McKay F. C., Albertson N. F.: J. Am. Chem. Soc. *79*, 4686 (1957).
15. Anderson G. W., McGregor A. C.: J. Am. Chem. Soc. *79*, 6180 (1957).
16. Schwyzer R., Sieber P.: Helv. Chim. Acta *40*, 624 (1957).
17. Bodanszky M., Ondetti M. A., Birkhimer C. A., Thomas P. L.: J. Am. Chem. Soc *86*, 4452 (1964).
18. Zaoral M., Flegel M.: This Journal *37*, 3350 (1972).
19. Habeeb A. F. S. A.: Can. J. Biochem. Physiol. *38*, 493 (1960).
20. Mazur R. H., Plume G.: Experientia *24*, 661 (1968).
21. Schnabel E., Kostermeyer H., Berndt H.: Ann. *749*, 90 (1971).
22. Zervas L. Photaki I.: J. Am. Chem. Soc. *84*, 3887 (1962).
23. Huguenin R. L., Boissonnas R. A.: Helv. Chim. Acta *45*, 1629 (1962).
24. Zaoral M., Kolc J., Šorm F.: This Journal *32*, 1242 (1967).
25. Iselin B., Schwyzer R.: Helv. Chim. Acta *43*, 1760 (1960).
26. Krejčí I., Kupková B., Vávra I.: Brit. J. Pharmac. Chemotherap. *30*, 497 (1967).
27. Schaechtelin G., Walter R., Salomon H., Jelínek J., Karen P., Cort J. H.: Mol. Pharmacol. *10*, 57 (1974).
28. Zaoral M., Kolc J., Šorm F.: This Journal *35*, 1716 (1970).
29. Sapirstein L. A.: Circulation Res. *4*, 689 (1956).
30. Cort J. H., Albrecht I., Nováková J., Mulder J. L., Jošt K.: Europ. J. Clin. Invest., in press.
31. Pliška V., Rychlík I.: Acta Endocrinol. *54*, 129 (1967).
32. Burn H. J., Finney D. J., Goodwin L. G.: *Biological Standardization*, p. 187. Oxford University Press, Oxford 1950.
33. Zaoral M., Kolc J., Šorm F.: This Journal *32*, 1250 (1967).
34. Manning M., Balaspiri L., Acosta M., Sawyer W. H.: J. Med. Chem. *16*, 975 (1973).
35. Thorn N. A. in the book: *Handbook of Experimental Pharmacology* (B. Berde, Ed.), Vol. 23, p. 372. Springer, Berlin 1968.
36. Jošt K., Barth T., Krejčí I., Fruhaufová L., Procházka Z., Šorm F.: This Journal *38*, 1073 (1973).
37. Procházka Z., Jošt K., Šorm F.: This Journal *37*, 289 (1972).
38. Suska-Brzezińska E., Fruhaufová L., Barth T., Rychlík I., Jošt K., Šorm F.: This Journal *37*, 2289 (1972).
39. Hubbard J. I.: Physiol. Rev. *53*, 674 (1973).
40. Barth T., Krejčí I., Vaněčková J., Jošt K., Rychlík I.: Europ. J. Pharmacol. *25*, 67 (1974).
41. Frič I., Kodíček M., Jošt K., Bláha K.: This Journal *39*, 1271 (1974).
42. Hanning K.: Z. Anal. Chem. *181*, 244 (1961).
43. Prusík Z., Sedláková E., Barth T.: Z. Physiol. Chem. *353*, 1837 (1972).
44. König W., Geiger R.: Chem. Ber. *103*, 788 (1970).
45. Arold H., Barth O.: J. Prakt. Chem. *38*, 50 (1968).
46. Meienhofer J.: Nature *205*, 73 (1965).
47. Weygand F., Hunger K.: Chem. Ber. *95*, 1 (1962).
48. Sofoku S., Mizumura M., Hagitani A.: Bull. Chem. Soc. Japan *43*, 177 (1970).

49. Hope D. B., Humphries J. F.: J. Chem. Soc. *1964*, 869.
50. Poduška K.: Unpublished results.
51. Frič I., Kodíček M., Procházka Z., Jošt K., Bláha K.: This Journal *39*, 1290 (1974).
52. Gut V.: Unpublished results.
53. Munsick R. A. in the book: *Experimental Pharmacology*, (B. Berde, Ed.) Vol. 3, p. 443. Springer, Berlin 1968.
54. Ženíšek K., Barth T.: Československ. fysiol. *23*, 67 (1974).
55. Meienhofer J., Trzeciak A., Havran R. T., Walter R.: J. Am. Chem. Soc. *92*, 7199 (1970).
56. Huguenin R. L., Boissonnas R. A.: Helv. Chim. Acta *49*, 695 (1966).
57. Huguenin R. L.: Helv. Chim. Acta *49*, 711 (1966).

Translated by the author (J. H. C.).