Amino-acids and Peptides. Part 42.[1] Synthesis of a Protected Docosapeptide having the Sequence of Mast-cell Degranulating Peptide

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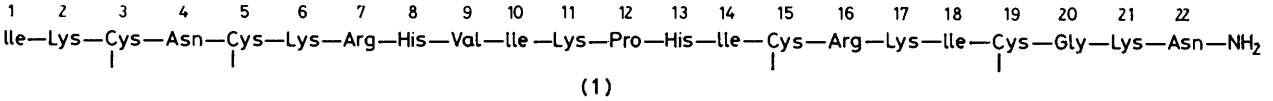
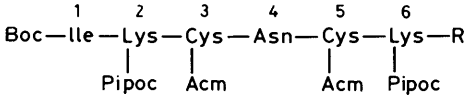
Fully protected mast-cell degranulating peptide (1) has been synthesised by the picolyl ester ' handle ' method.

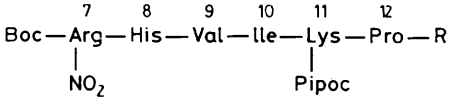
MAST-CELL degranulating peptide (MCD-peptide) (1) was isolated from bee venom by Breithaupt and Habermann [2] and proved to be identical with the anti-inflammatory ' Peptide 401 ' isolated from the same source by Vernon *et al.*[3] The amino-acid sequence was determined by Haux [4] and by Billingham *et al.*;[5] the latter authors showed also that disulphide bridges link positions 3 to 15 and 5 to 19. We embarked on a synthesis of the peptide by a scheme devised to test further the use of piperidino-oxycarbonyl [6] (more stable to acid than is benzyloxycarbonyl) for the protection of the ε-amino-group of the five lysine residues and (in the final stage) the use of electrolytic reduction [7,8] for the removal of protection at the end of the synthesis, thus allowing the use of nitroarginine until the penultimate step, despite the presence of cysteine residues, which were protected by acetamidomethyl.[9]



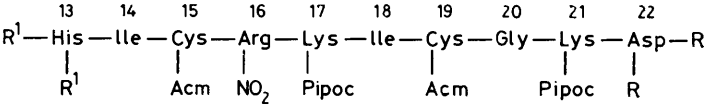
(1)



(2a) R = OPic; (2b) R = OH



(3a) R = OPic; (3b) R = OH



(4a) R = OPic, R^1 = Boc; (4b) R = NH_2, R^1 = H

Abbreviations follow the I.U.P.A.C.–I.U.B. rules, reprinted in the Chemical Society Specialist Periodical Report 'Amino-acids, Peptides, and Proteins,' ed. G. T. Young, The Chemical Society, London, 1972, vol. 4, p. 441; Pic = 4-picolyl; Pipoc = piperidino-oxycarbonyl. Amino-acids are of the L-configuration.

RESULTS AND DISCUSSION

Protected fragments with the sequences 1—6 (2a), 7—12 (3a), and 13—22 (4a) (chosen in part to facilitate the synthesis of variations of the structure) were built up using the picolyl ester ' handle ' procedure;[10] coupling was normally by means of dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole.[11] The coupling product was isolated either by extraction into aqueous citric acid or by absorption on buffered Amberlyst-15 cation exchanger in the usual way. It has been reported earlier [12] that we have in certain cases found that fragments having both piperidino-oxycarbonyl and nitro-substituents may suffer some decomposition on the Amberlyst-15 resin and therefore at the three stages after the introduction of the nitroarginine residue in the synthesis of fragment (4a) the citric acid isolation procedure was used, modified by the addition of n-butanol to the ethyl acetate to increase the solubility of the product in the organic phase. Fragment (4a) itself was isolated by gel filtration on Sephadex LH-20 with dimethylformamide as solvent, because citric acid can remove the *N-Im*-t-butoxycarbonyl protection of histidine. The overall yields, calculated on the carboxy-terminal protected amino-acids, for fragments (2a), (3a), and (4a) were 36, 40, and 32%, respectively. It may be mentioned here that further supplies of protected hexapeptides (2a) and (18) (Table 3) were obtained in a period of five days in a repetitive procedure using only t.l.c. to monitor the progress of the syntheses. Both the protected hexapeptide ester (2a) and especially its acid (2b) (see below)

TABLE 1

Synthesis of Boc–Ile–Lys(Pipoc)–Cys(Acm)–Asp(NH_2)–Cys(Acm)–Lys(Pipoc)–OPic: protected peptide intermediates

(6) Boc–Cys(Acm)–Lys(Pipoc)–OPic
(7) Boc–Asp(NH_2)–Cys(Acm)–Lys(Pipoc)–OPic
(8) Boc–Cys(Acm)–Asp(NH_2)–Cys(Acm)–Lys(Pipoc)–OPic
(9) Boc–Lys(Pipoc)–Cys(Acm)–Asp(NH_2)–Cys(Acm)–Lys(Pipoc)–OPic
(2a) Boc–Ile–Lys(Pipoc)–Cys(Acm)–Asp(NH_2)–Cys(Acm)–Lys(Pipoc)–OPic

Compound [a]	Amino-component (mmol)	Acylating component [b] (mmol)	Isolation [c]	Yield (%)	$[\alpha]_D^{20}$ [d] (°)	R_F (t.l.c.)
(6)	5.85	Boc–Cys(Acm) (6.50)	C [e]	83	+4°	0.37 (E4); 0.54 (G3)
(7)	3.3	Boc–Asp(NH_2)–OTcp (3.6)	C [f]	88	−18	0.23 (E4); 0.38 (G3)
(8)	2.7	Boc–Cys(Acm)–OTcp (3.3)	A	82	−30	0.11 (E4); 0.23 (G3)
(9)	2.10	Boc–Lys(Pipoc) (3.0) [g]	A [h]	67	−30	0.13 (E4); 0.23 (G3)
(2a)	0.83	Boc–Ile (1.5)	A [i]	82	−29	0.62 (E3); 0.17 (G3)

	Found (%)					Required (%)			
Compound	C	H	N	S	Formula	C	H	N	S
(6)	54.3	7.3	13.2	5.1	$C_{29}H_{46}N_6O_8S$	54.5	7.3	13.2	5.0
(7)	51.9	7.2	14.4	4.2	$C_{33}H_{52}N_8O_{10}S\cdot 0.5\ H_2O$	52.0	7.0	14.7	4.2
(8)	49.1	6.6	14.7	6.8	$C_{39}H_{62}N_{10}O_{12}S_2\cdot 1.5\ H_2O$	49.1	6.9	14.7	6.7
(9)	51.1	6.9	15.0	5.5	$C_{51}H_{83}N_{13}O_{15}S_2\cdot H_2O$	51.0	7.1	15.2	5.3
(2a)	52.0	7.0	14.8	4.5	$C_{57}H_{94}N_{14}O_{16}S_2\cdot H_2O$	52.1	7.4	14.9	4.9

[a] All the compounds are new. [b] Coupling was effected in dimethylformamide overnight at room temperature. [c] C = Citric acid procedure; A = Amberlyst procedure. [d] Optical rotations were measured in chloroform [compounds (6) and (7)] or dimethylformamide [compounds (8), (9), and (2a)], *c* 1.0. [e] The product was extracted into 0.7M-citric acid from solution in ethyl acetate–ether and was then extracted from the basified aqueous layer into ethyl acetate. It was recrystallised from ethyl acetate. [f] The product was extracted into aqueous citric acid from solution in ethyl acetate–dichloromethane and was extracted from the basified aqueous layer into dichloromethane. [g] Liberated from the dicyclohexylammonium salt, m.p. 135—136 °C, $[\alpha]_D^{20}$ +8° (*c* 1.1 in MeOH) (J. G. Warnke, D.Phil. Thesis, Oxford, 1974), by 0.7M-citric acid and extracted into ethyl acetate. [h] The protected peptide formed gels with dichloromethane and absorption onto the resin was from dimethylformamide solution. [i] The residue after evaporation of the filtered reaction mixture was washed with aqueous sodium hydrogencarbonate and then water, dried, and applied to the resin in dimethylformamide solution. Non-basic contaminants were washed off by dimethylformamide and the product was eluted as usual. Found after acid hydrolysis: Lys 2.00; Asp, 0.95; Ile, 1.05.

TABLE 2

Synthesis of Boc–Arg(NO_2)–His–Val–Ile–Lys(Pipoc)–Pro–OPic: protected peptide intermediates

(10) Boc–Lys(Pipoc)–Pro–OPic
(11) Boc–Ile–Lys(Pipoc)–Pro–OPic
(12) Boc–Val–Ile–Lys(Pipoc)–Pro–OPic
(13) Boc–His–Val–Ile–Lys(Pipoc)–Pro–OPic
(3a) Boc–Arg(NO_2)–His–Val–Ile–Lys(Pipoc)–Pro–OPic

Compound [a]	Amino-component (mmol)	Acylating component [b] (mmol)	Isolation [c]	Yield (%)	$[\alpha]_D^{20}$ [d] (°)	R_F (t.l.c.)
(10)	10.0 [e]	Boc–Lys(Pipoc) (12.0) [f,g]	C	95	−34°	0.64 (E4); 0.72 (G3)
(11)	6.5	Boc–Ile (9.0) [f]	C	88	−37.5	0.70 (E4); 0.71 (G3)
(12)	4.9	Boc–Val (7.0) [f]	C	92	−37	0.56 (E4); 0.80 (G3)
(13)	3.2	Boc–His(Boc) (9.6) [f,g]	C	(98) [h]	−39	0.26 (E4); 0.40 (G3)
(3a)	2.7	Boc–Arg(NO_2) (4.1) [i]	C	71 [k]	−28	0.43 (E3); 0.75 (G3)

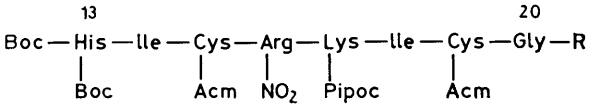
	Found (%)				Required (%)		
Compound	C	H	N	Formula [j]	C	H	N
(10)	59.3	7.7	11.9	$C_{28}H_{43}N_5O_7\cdot 0.25H_2O$	59.4	7.7	12.35
(11)	59.8	8.0	11.9	$C_{34}H_{54}N_6O_8\cdot 0.5H_2O$	59.7	8.1	12.3
(12)	59.7	7.85	12.45	$C_{39}H_{63}N_7O_9\cdot 0.5H_2O$	59.8	8.2	12.5
(13) [h]	58.8	7.6	14.9	$C_{45}H_{70}N_{10}O_{10}\cdot 0.5H_2O$	58.7	7.8	15.2
(3a)	53.55	7.15	18.45	$C_{51}H_{81}N_{15}O_{13}\cdot 2H_2O$	53.35	7.45	18.3

[a] All compounds are new. [b] Coupling was in tetrahydrofuran solution, except for compound (3a) for which the solvent was dimethylformamide. It proceeded overnight in the case of compounds (10) and (3a), but for compounds (11), (12), and (13) it was complete in 3.5, 3, and 1 h, respectively. [c] C = Citric acid procedure. The product was extracted into aqueous citric acid from ethyl acetate–ether (1 : 1). [d] *c* 1.0 in dimethylformamide. [e] The amino-component was proline 4-picolyl ester liberated from the stated quantity of dihydrobromide [R. Camble, R. Garner, and G. T. Young, *J. Chem. Soc.* (*C*), 1969, 1911] in the usual way. [f] Pre-activation was for 1 h at 0 °C and 1 h at room temperature. [g] Liberated from the dicyclohexylammonium salt by 0.7M citric acid and extracted into ethyl acetate. [h] In this case a sample was dried further before analysis. [i] Acylating agents and amino-component were mixed in dimethylformamide at 0 °C without pre-activation. [j] Found after acid hydrolysis: Compound (11), Ile, 1.00; Lys, 1.01; Pro, 0.96. Compound (12), Val, 1.00; Ile, 0.99; Lys, 1.03. Compound (13): His, 1.02; Val, 1.00; Ile, 0.98; Lys, 1.01; Pro, 0.95. Compound (3a): Arg, 0.91; His, 1.05; Val, 0.95; Ile, 0.96; Lys + Orn, 1.18; Pro, 0.95. [k] Product was extracted from the basified aqueous layer into ethyl acetate–n-butanol (1 : 1) and was finally reprecipitated from dimethylformamide by ethyl acetate.

were unstable on storage, t.l.c. showing two impurities in the former after 3—4 weeks at *ca.* 3 °C, and in the latter six contaminants after 5 d at −20 °C. It was therefore necessary to prepare batches of these intermediates as required for immediate use.

Not unexpectedly, the yield of decapeptide diamide

obtained by the action of ammonia in methanol on the decapeptide diester (4a) was low, owing to cleavage of the nitroarginine residues, and the diamide was prepared more satisfactorily by synthesis of the fragment (5a) having the sequence 13—20 and then coupling the acid



(5a) R = OPic; (5b) R = OH

derived from this ester to N^{ϵ}-piperidino-oxycarbonyl-L-lysyl-L-asparagineamide. In this, as in all the fragment couplings, the use of the trinitrobenzenesulphonate–sulphite spectrophotometric assay of amino-groups in the reaction mixture [13] was invaluable for determining the completion of the coupling reaction. Details of the syntheses of protected fragments (2a), (3a), (4a), and (5a) are given in Tables 1—4.

The hexapeptide ester (3a) was hydrolysed to the acid (3b) which was coupled to the decapeptide diamide (4b) by means of dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole. Purification of the resulting protected hexadecapeptide diamide (sequence 7—22) was made difficult by its insolubility in dimethylformamide. The *N*-t-butoxycarbonyl group was therefore removed by trifluoroacetic acid; the resulting trifluoroacetate salt dissolved in dimethylformamide on the addition of di-isopropylethylamine, and the free amino-component was readily purified on Sephadex LH-20. We have found this procedure useful in other similar cases.

The hexapeptide picolyl ester (2a) was converted into its hydrazide but surprisingly this failed to react with

TABLE 3

Synthesis of Boc–His(Boc)–Ile–Cys(Acm)–Arg(NO_2)–Lys(Pipoc)–Ile–Cys(Acm)–Gly–Lys(Pipoc)–Asp(OPic)–OPic: protected peptide intermediates

(14) Boc–Lys(Pipoc)–Asp(OPic)–OPic
(15) Boc–Gly–Lys(Pipoc)–Asp(OPic)–OPic
(16) Boc–Cys(Acm)–Gly–Lys(Pipoc)–Asp(OPic)–OPic
(17) Boc–Ile–Cys(Acm)–Gly–Lys(Pipoc)–Asp(OPic)–OPic
(18) Boc–Lys(Pipoc)–Ile–Cys(Acm)–Gly–Lys(Pipoc)–Asp(OPic)–OPic
(19) Boc–Arg(NO_2)–Lys(Pipoc)–Ile–Cys(Acm)–Gly–Lys(Pipoc)–Asp(OPic)–OPic
(20) Boc–Cys(Acm)–Arg(NO_2)–Lys(Pipoc)–Ile–Cys(Acm)–Gly–Lys(Pipoc)–Asp(OPic)–OPic
(21) Boc–Ile–Cys(Acm)–Arg(NO_2)–Lys(Pipoc)–Ile–Cys(Acm)–Gly–Lys(Pipoc)–Asp(OPic)–OPic
(4a) Boc–His(Boc)–Ile–Cys(Acm)–Arg(NO_2)–Lys(Pipoc)–Ile–Cys(Acm)–Gly–Lys(Pipoc)–Asp(OPic)–OPic

Compound [a]	Amino-component (mmol)	Acylating component [b] (mmol)	Isolation [c]	Yield (%)	$[\alpha]_D^{20}$ [d] (°)	R_F (t.l.c.)
(14)	7.5 [e]	Boc–Lys(Pipoc) (9.2) [f]	C	93	+2°	0.47 (E4); 0.56 (G3)
(15)	6.25	Boc–Gly–OTcp(8.5)	C	86	−12	0.37 (E4); 0.50 (G3)
(16)	5.9	Boc–Cys(Acm)–OTcp (7.0)	A	94	−23	0.21 (E4); 0.29 (G3)
(17)	5.25	Boc–Ile (7.0) [g]	A	71	−26	0.26 (E4); 0.40 (G3)
(18)	3.6	Boc–Lys(Pipoc) (4.8) [f]	A [h]	81	−25	0.40 (G3); 0.85 (E1)
(19)	1.0	Boc–Arg(NO_2) (1.5)	C [i,j]	85	−23	0.57 (E3); 0.62 (U)
(20)	0.6	Boc–Cys(Acm)–OTcp (0.9)	C [i]	84	−24	0.64 (E3)
(21)	0.48	Boc–Ile (0.75)	C [k]	89.5	−22	0.65 (E3); 0.59 (K)
(4a)	0.144	Boc–His(Boc) [l] (0.32) [m]	[n]	86	−22	0.50 (E3)

Compound	Found (%) C	H	N	S	Formula	Required (%) C	H	N	S
(14)	58.7	6.9	12.3		$C_{33}H_{46}N_6O_9$	59.1	6.9	12.5	
(15)	56.6	6.9	12.7		$C_{35}H_{48}N_7O_{10}\cdot H_2O$	56.4	6.9	13.15	
(16)	52.9	6.6	13.1	3.2	$C_{41}H_{59}N_9O_{12}S\cdot 2H_2O$	52.5	6.8	13.4	3.4
(17)	55.3	6.8	13.6	3.2	$C_{47}H_{70}N_{10}O_{13}S$	55.6	6.95	13.8	3.2
(18)	55.4	7.2	14.2		$C_{59}H_{91}N_{13}O_{16}S$	55.8	7.2	14.3	
(19)	52.2	7.1	16.4	2.25	$C_{65}H_{109}N_{18}O_{19}S\cdot 1.5H_2O$	52.1	7.1	16.8	2.1
(20)	50.5	6.9	16.6		$C_{71}H_{112}N_{20}O_{21}S_2\cdot 2H_2O$	50.7	6.95	16.65	
(21)	52.25	7.05	16.4		$C_{77}H_{123}N_{21}O_{22}S_2\cdot H_2O$	52.0	7.1	16.55	
(4a)	52.6	7.2	16.5	3.4	$C_{88}H_{138}N_{24}O_{25}S_2$	52.9	7.0	16.8	3.2

[a] All compounds are new. [b] Coupling was in dimethylformamide solution and proceeded overnight except in the case of compound (4a) for which it was complete in 4 h. [c] C = Citric acid procedure; A = Amberlyst procedure. [d] *c* 1.0 in dimethylformamide, except for compounds (14) and (15) for which the solvent was chloroform. [e] The amino-component was di-4-picolyl aspartate liberated from the stated amount of trihydrobromide in the usual way [R. Garner and G. T. Young, *J. Chem. Soc.* (*C*), 1971, 50]. [f] Liberated from the dicyclohexylammonium salt by 0.7M citric acid and extracted into ethyl acetate. [g] Additional dicyclohexylcarbodi-imide was required to complete the coupling. [h] Non-basic contaminants were washed off the column by ethyl acetate and dimethylformamide. [i] The product was extracted into 0.7M citric acid from dichloromethane and was extracted from the basified aqueous layer into ethyl acetate–n-butanol (1 : 1). [j] This product gelled readily and contained a contaminant (possibly amino-component) of R_F 0(E3) which was removed by chromatography on silica (Merck 60) with isopropanol–chloroform–water (60 : 40 : 2). T.l.c. solvent U is chloroform–propan-2-ol–water (60 : 90 : 6). [k] The product was extracted into 0.7M citric acid from ethyl acetate–ether (1 : 1) and was extracted from the basified aqueous layer into ethyl acetate–n-butanol (1 : 1). [l] Liberated from the dicyclohexylammonium salt by 0.1M potassium hydrogensulphate–0.1M potassium sulphate at pH 3 and extracted into ethyl acetate. [m] Acylating agents and amino-component were mixed at 0 °C without pre-activation; coupling was complete in 4 h. [n] Isolation was effected by applying the filtered, concentrated reaction mixture to a column (100 × 4 cm) of Sephadex LH-20 equilibrated and eluted with dimethylformamide; a single optically active peak was shown by the polarimetric recording and evaporation of the corresponding fractions gave pure protected decapeptide.

TABLE 4

Synthesis of Boc–His(Boc)–Ile–Cys(Acm)–Arg(NO_2)–Lys(Pipoc)–Ile–Cys(Acm)–Gly–OPic: protected peptide intermediates

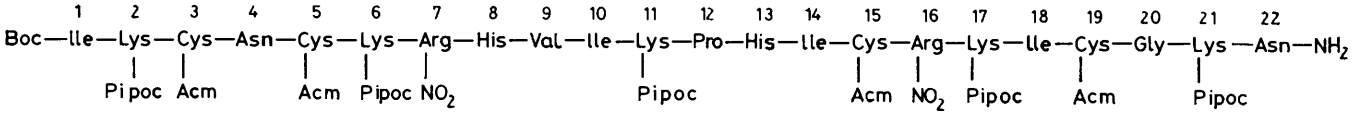
(22) Boc–Cys(Acm)–Gly–OPic
(23) Boc–Ile–Cys(Acm)–Gly–OPic
(24) Boc–Lys(Pipoc)–Ile–Cys(Acm)–Gly–OPic
(25) Boc–Arg(NO_2)–Lys(Pipoc)–Ile–Cys(Acm)–Gly–OPic
(26) Boc–Cys(Acm)–Arg(NO_2)–Lys(Pipoc)–Ile–Cys(Acm)–Gly–OPic
(27) Boc–Ile–Cys(Acm)–Arg(NO_2)–Lys(Pipoc)–Ile–Cys(Acm)–Gly–OPic
(5a) Boc–His(Boc)–Ile–Cys(Acm)–Arg(NO_2)–Lys(Pipoc)–Ile–Cys(Acm)–Gly–OPic

Compound [a]	Amino-component (mmol)	Acylating component [b] (mmol)	Isolation [c]	Yield (%)	$[\alpha]_D^{20}$ [d] (°)	R_F (t.l.c.)
(22)	40 [e]	Boc–Cys(Acm) (56.2)	C	87	−21	0.60 (E3)
(23)	34	Boc–Ile (50)	C [f]	75	−27.5	0.50 (E3)
(24)	24.4	Boc–Lys(Pipoc) (36.2)	C [f]	75	−24	0.63 (E3)
(25)	17.7	Boc–Arg(NO_2) (30) [g]	C [h,i]	62	−20	0.70 (E3)
(26)	11.4	Boc–Cys(Acm) (25)	C [h]	89	−21	0.55 (E3)
(27)	9.55	Boc–Ile (20)	C [h,i]	60 [j]	−23	0.67 (E3)
(5a)	2.2	Boc–His(Boc) (4.5) [k]	*l*	84	−19	0.58 (E3)

Compound	Found (%) C	H	N	S	Formula	Required (%) C	H	N	S
(22)	51.5	6.6	12.8		$C_{19}H_{28}N_4O_6S$	51.8	6.4	12.7	
(23)	54.1	7.2	12.5	6.0	$C_{25}H_{39}N_5O_7S$	54.2	7.1	12.65	5.8
(24)	55.0	7.6	13.5	4.2	$C_{37}H_{60}N_8O_{10}S$	54.9	7.5	13.85	4.0
(25)	50.9	6.8	17.1	3.45	$C_{43}H_{71}N_{13}O_{13}S$	51.1	7.1	18.0	3.2
(26)	48.9	7.05	17.0	5.4	$C_{49}H_{81}N_{15}O_{15}S_2 \cdot 1.25H_2O$	48.8	6.9	17.4	5.3
(27)	50.6	7.1	16.6	4.9	$C_{55}H_{92}N_{16}O_{16}S_2 \cdot H_2O$	50.2	7.2	17.0	4.9
(5a)	51.25	7.2	17.4	4.1	$C_{66}H_{107}N_{19}O_{19}S_2$	51.65	7.05	17.35	4.2

[a] All compounds are new. [b] Coupling was in dimethylformamide solution of 0.5—5 h. [c] C = Citric acid procedure. [d] *c* 1.0 in dimethylformamide. [e] The amino-component was glycine 4-picolyl ester, liberated from the dihydrobromide [R. Camble, R. Garner, and G. T. Young, *J. Chem. Soc.* (*C*), 1969, 1911] in the usual way. [f] The product was extracted into 0.7M citric acid from ethyl acetate–ether and was extracted from the basified aqueous layer into dichloromethane. [g] Acylating agents and amino-component were mixed at 0 °C without pre-activation. [h] The product was extracted into 0.7M citric acid from ethyl acetate–ether and was extracted from the basified aqueous layer into ethyl acetate–n-butanol (1 : 1). [i] A contaminant [R_F 0 (E3)] was removed by chromatography on silica using chloroform–isopropanol (1 : 1) containing 3% water. [j] Some additional product (1.16 mmol) separated as a solid during the extractions; this is not included in the quoted yield. [k] Liberated from the dicyclohexylammonium salt by aqueous potassium hydrogen sulphate–potassium sulphate and extracted into ethyl acetate. [l] Isolation was effected by applying the filtered, concentrated reaction mixture to a column of Sephadex LH-20 and eluting with dimethylformamide. Evaporation of the fractions containing the single optically active peak gave pure protected octapeptide (Found after acid hydrolysis; His, 1.01; Ile, 2.02; Arg, 0.90; Lys + Orn, 1.11; Gly, 0.99).

t-butyl nitrite under the usual conditions; the possibility that the terminal N^{ϵ}-piperidino-oxycarbonyl-lysine was intervening was eliminated by showing that *N*-t-butoxycarbonyl-*S*-acetamidomethyl-L-cysteinyl-N^{ϵ}-piperidino-oxycarbonyl-L-lysyl hydrazide reacted with t-butyl nitrite normally. The ester (2a) was therefore hydrolysed to the acid (2b), which was coupled to the hexadecapeptide diamide (sequence 7—22) by means of dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole. Analytically pure protected mast-cell degranulating peptide (28) was obtained after gel filtration of the product on Sephadex LH-20.



(28)

EXPERIMENTAL

The general instructions in Parts 36 [14] and 39 [12] apply, except that t.l.c. was on Merck silica-60, F-254 plates. Column chromatography was monitored by the u.v. absorption at 254 nm and by polarimetry (normally at 436 nm) using a micro-1-dm cell in the Perkin-Elmer 141 automatic polarimeter, coupled with a recorder. Tetrahydrofuran was purified by distillation from calcium hydride and the distillate was refluxed with equimolar amounts of naphthalene and potassium; it was distilled from this solution as required. *N*-t-Butoxycarbonyl-*S*-acetamidomethyl-L-cysteine 2,4,5-trichlorophenyl ester was originally prepared by Dr. C. F. Hayward (I.C.I. Pharmaceuticals Division, Alderley Park) by the method of Broadbent *et al.*[15] and had m.p. 139—140 °C.

N^{α}-t-Butoxycarbonyl-N^{ϵ}-piperidino-oxycarbonyl-L-lysine 4-Picolyl Ester (with R. C. ANAND).—*N^{α}*-t-Butoxycarbonyl-N^{ϵ}-piperidino-oxycarbonyl-L-lysine was liberated from its dicyclohexylammonium salt [16] as usual by means of 0.7M citric acid–ethyl acetate and was esterified with 4-picolyl alcohol in dichloromethane by the dicyclohexylcarbodi-imide method; [12] recrystallisation of the product from ether gave the *ester* (86% yield), m.p. 86—87 °C; $[\alpha]_D^{20}$ −3° (*c* 1 in $CHCl_3$); R_F 0.57 (E4) and 0.76 (G3) (Found: C, 59.3; H, 7.8; N, 12.1. $C_{23}H_{36}N_4O_6$ requires C, 59.5; H, 7.8; N, 12.1%).

N-t-Butoxycarbonylglycine 4-Picolyl Ester.—This was prepared similarly from t-butoxycarbonylglycine; recrystallisation from carbon tetrachloride–ether–light petroleum gave the *ester* (83% yield), m.p. 84—84.5 °C; R_F 0.70 (E3) (Found: C, 58.55; H, 6.7; N, 10.55. $C_{13}H_{18}N_2O_4$ requires C, 58.7; H, 6.8; N, 10.5%).

General Procedures for the Synthesis of Protected Peptides.—Exceptions to these descriptions are noted in the Tables. (1) *Removal of the* N^{α}*-t-butoxycarbonyl group and liberation of the amino-component.* This was by anhydrous trifluoroacetic acid (2—5 ml per mol of reactant) at 0 °C for 5—15 min; the solution was evaporated and the residue was triturated with ether. The residual salt was usually dissolved in dimethylformamide and the amino-component was then liberated by addition of a slight excess of dried freshly distilled di-isopropylethylamine (stored over pre-baked molecular sieve 3A); the apparent pH (moist indicator paper) was reduced to 5 by adding 1-hydroxybenzotriazole monohydrate. This procedure was used both with the active ester and dicyclohexylcarbodi-imide methods of coupling.

(2) *Coupling.* Unless otherwise stated in the Tables, coupling was by means of dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole monohydrate (both equimolar to the carboxy-component) [11] with pre-activation for 1 h at 0 °C. It should be noted that the procedure described above for the liberation of the amino-component provides 1-hydroxybenzotriazole for the catalysis of active ester couplings.[17] When detection of unchanged amino-components by t.l.c. was uncertain, the end of the reaction was determined by the trinitrobenzenesulphonate–sulphite amino-assay described in Part 38.[13] This proved to be especially important in fragment couplings.

(3) *Isolation of coupled product.* (*i*) *By the citric acid procedure.* The reaction mixture from dicyclohexylcarbodi-imide couplings was cooled, filtered, and evaporated. The residue was dissolved in ethyl acetate, the solution was washed with water, and the product was extracted into 0.7M citric acid; the aqueous layer was brought to pH 8 by the addition of solid sodium hydrogencarbonate and the product was extracted into ethyl acetate or dichloromethane; the solution was then washed with water and brine, and dried. Each extraction was repeated as needed to ensure complete transfer of the product, which was monitored by t.l.c. Evaporation (and trituration with ether or light petroleum as needed) normally gave protected peptide which, after drying at 0.1 mmHg and room temperature, was pure by t.l.c. evidence and elemental analysis. Variations in this procedure are mentioned in the Tables. (*ii*) *By use of Amberlyst-15.* The resin was saturated overnight with a 10% solution of 3-bromopyridine in the solvent to be used for the absorption (given in the Tables). After filtration and evaporation of the coupling reaction mixture, the residue was usually dissolved in dichloromethane, the solution washed with 2M potassium carbonate (to remove 1-hydroxybenzotriazole) then with brine, dried, and then the solution was shaken with Amberlyst-15 (3-bromopyridinium form; *ca.* 16 ml per mmol of protected peptide picolyl ester) until t.l.c. of a concentrated sample of the solution showed that all the product had been absorbed (1—3 h). The resin was then washed as a column with dichloromethane and then dimethylformamide. It is essential that the dimethylformamide used should be free from dimethylamine. The product was then eluted with cooled pyridine (25%) in dimethylformamide. The eluate was evaporated to dryness and bromopyridine was removed by trituration with ether (or for highly lipophilic products, with water); the product was dried for analysis at 0.1 mmHg and room temperature.

Details of the syntheses of the fragments (2a), (3a), (4a), and (5a) are given in the Tables. Assembly of each fragment was stepwise from the carboxy-end; the amino-component for each coupling step was obtained (unless otherwise noted) from the appropriate t-butoxycarbonyl derivative, the quantities taken being given in the second column of the Tables; calculated yields are based on this derivative and are for product of the stated constants and analysis. Acid hydrolysis of peptides containing the sequence Ile–Cys gave a substantial amount of *allo*-isoleucine, presumably by a mechanism analogous to that postulated for the epimerisation of the isoleucine involved with the adjacent cysteine residue in bacitracin A.[18] Peptides in which isoleucine was adjacent to lysine gave little or no *allo*-isoleucine, consistent with earlier observations with similar cases.[19]

L-*Histidyl*-L-*isoleucyl*-S-*acetamidomethyl*-L-*cysteinyl*-N^{ω}-*nitro*-L-*arginyl*-N^{ϵ}-*piperidino-oxycarbonyl*-L-*lysyl*-L-*isoleucyl*-S-*acetamidomethyl*-L-*cysteinylglycyl*-N^{ϵ}-*piperidino-oxycarbonyl*-L-*lysyl*-L-*asparagineamide* (4b).—(*a*) *By amidation of the corresponding ester.* The protected decapeptide di-4-picolyl ester (4a) (500 mg, 0.25 mmol) dissolved slowly in methanol (30 ml) saturated with ammonia at 0 °C, and overnight amide separated out and was collected. Unchanged ester was precipitated from the solution by ether and was re-treated, giving a total yield of crude diamide of 200 mg (45%). It was purified by removing the t-butoxycarbonyl group by trifluoroacetic acid as usual; the trifluoroacetate of the product was dissolved in dimethylformamide, the solution was brought to an apparent pH 10 with di-isopropylethylamine and then applied to a column of Sephadex LH-20 equilibrated with dimethylformamide. Evaporation of the fractions corresponding to the main u.v.-absorbing peak and trituration of the residue with ether gave *protected decapeptide diamide* (4b) (139 mg, 31% overall yield); $[\alpha]_D^{20}$ −27° (*c* 0.5 in Me_2NCHO); R_F 0.31 (K), 0.13 (E3) (Found: C, 47.6; H, 7.2; N, 20.2. $C_{66}H_{114}N_{24}O_{19}S_2 \cdot 3H_2O$ requires C, 47.6; H, 7.3; N, 20.2%).

(*b*) *By coupling sequence* 13—20 *to sequence* 21—22. The t-butoxycarbonyloctapeptide acid (5b) described below (1.88 g, 1.3 mmol) was activated by equimolar amounts of dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole in dimethylformamide for 0.5 h at 0 °C and 0.5 h at 21 °C and then coupled with N^{ϵ}-piperidino-oxycarbonyl-L-lysyl-L-asparagineamide (see below) (503 mg, 1.3 mmol). After 22 h the trinitrobenzenesulphonate–sulphite amino-assay [13] showed the reaction to be complete; the reaction solution was evaporated and the t-butoxycarbonyl group was removed from the residual protected peptide by trifluoroacetic acid. The trifluoroacetate was dissolved in dimethylformamide (15 ml), the apparent pH was brought to 11 by addition of di-isopropylethylamine, and the free amino-compound was purified on Sephadex LH-20 in dimethylformamide, giving protected decapeptide diamide (4b) (1.46 g, 68%) (Found after acid hydrolysis: Lys + Orn, 2.11; Arg, 0.85; His, 0.95; Asp, 1.03; Gly, 1.04; Ile + *a*Ile, 2.03).

N^{α}-*t-Butoxycarbonyl*-N^{ϵ}-*piperidino-oxycarbonyl*-L-*lysyl*-L-*asparagineamide*.— N^{α}-t-Butoxycarbonyl-N^{ϵ}-piperidino-oxycarbonyl-L-lysine was liberated from its dicyclohexylammonium salt [16] (4.59 g, 8.3 mmol) by 0.7M citric acid, extracted into ethyl acetate, and coupled with L-asparagineamide [liberated by di-isopropylethylamine from the hydrochloride (1.15 g, 6.9 mmol)] by means of dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole in dimethylformamide in the usual way. The reaction was complete in

4 h and the solution was evaporated; the residue was extracted into ethyl acetate–n-butanol (1 : 1) and washed (sodium hydrogencarbonate, citric acid, water, and brine), dried, and evaporated. The residue was recrystallised from methanol–ether, giving *protected dipeptide amide* (2.2 g, 66%), m.p. 149—151 °C; $[\alpha]_D^{20}$ −7° (*c* 1.0 in Me_2NCHO); R_F 0.48 (E3) (Found: C, 51.55; H, 7.7; N, 16.8. $C_{21}H_{38}N_6O_7$ requires C, 51.85; H, 7.85; N, 17.25%). For the coupling reaction with the acid of sequence 13—20 (described above) the t-butoxycarbonyl group was removed by trifluoroacetic acid and the free N^{ϵ}-piperidino-oxycarbonyl-L-lysyl-L-asparagineamide was obtained by passing a solution in dimethylformamide down a column of Amberlyst-21 (free base) ion-exchanger; the crude product used for the coupling reaction had m.p. 99—103 °C, $[\alpha]_D^{25}$ +4° (*c* 1.0 in Me_2NCHO), R_F 0.27 (K). Lower yields were obtained in this coupling reaction when the trifluoroacetate salt and tertiary amine were used instead of the free amino-component liberated by Amberlyst-21.

N^{α}-N(Im)-*Di-t-butoxycarbonyl-L-histidyl-L-isoleucyl-S-acetamidomethyl-L-cysteinyl-*N^{ω}*-nitro-L-arginyl-*N^{ϵ}*-piperidino-oxycarbonyl-L-lysyl-L-isoleucyl-S-acetamidomethyl-L-cysteinylglycine* (5b).—The protected octapeptide 4-picolyl ester (5a) (2.75 g, 1.8 mmol) was saponified by 1M sodium hydroxide (2.16 ml) in tetrahydrofuran–water (10 : 1) during 15 min at 21 °C. The solution was cooled to 0 °C and brought to pH 3, the solution was evaporated and the residue was triturated successively with water, acetone, and ether, and then dried giving acid (5b) (2.1 g, 79%). Careful attention to these conditions was necessary to obtain a satisfactory product. $[\alpha]_D^{20}$ −24° (*c* 0.95 in Me_2NCHO); R_F 0.34 (K) (Found: C, 48.8; H, 7.1; N, 17.2; S, 4.45. $C_{60}H_{102}N_{18}O_{19}S\cdot 2H_2O$ requires C, 48.7; H, 7.2; N, 17.05; S, 4.35%. Found after acid hydrolysis: Lys + Orn, 1.16; Arg, 0.87; His, 0.97; Gly, 0.99; Ile + *a*Ile, 2.01).

t-Butoxycarbonyl-N^{ω}*-nitro-L-arginyl-L-histidyl-L-valyl-L-isoleucyl-*N^{ϵ}*-piperidino-oxycarbonyl-L-lysyl-L-proline* (3b).—The protected hexapeptide 4-picolyl ester (3a) (2.0 g, 1.8 mmol) was saponified by 1M sodium hydroxide (10 ml) in tetrahydrofuran–water (10 : 1, 40 ml) for 20 min at room temperature. The pH was brought to 3, the solvent was evaporated off, and the product was taken up in ethyl acetate–n-butanol (1 : 1), washed (water, brine), dried, and evaporated. The residue was triturated with ether and reprecipitated from dimethylformamide by ether, giving *protected hexapeptide acid* (3b) (1.50 g, 76%); $[\alpha]_D^{20}$ −31° (*c* 1.1 in Me_2NCHO); R_F 0.23 (E3) (Found: C, 49.2; H, 7.1; N, 17.85. $C_{45}H_{76}N_{14}O_{13}\cdot 4H_2O$ requires C, 49.45; H, 7.75; N, 17.95%. Found after acid hydrolysis: Lys + Orn, 1.16; Arg, 0.87; His, 1.02; Pro, 1.00; Val, 0.97; Ile 0.95; *a*Ile, 0.04).

N^{ω}*-Nitro-L-arginyl-L-histidyl-L-valyl-L-isoleucyl-*N^{ϵ}*-piperidino-oxycarbonyl-L-lysyl-L-prolyl-L-histidyl-L-isoleucyl-S-acetamidomethyl-L-cysteinyl-*N^{ω}*-nitro-L-arginyl-*N^{ϵ}*-piperidino-oxycarbonyl-L-lysyl-L-isoleucyl-S-acetamidomethyl-L-cysteinylglycyl-*N^{ϵ}*-piperidino-oxycarbonyl-L-lysyl-L-asparagineamide* (*Sequence* 7—22).—The protected hexapeptide acid (3b) (501 mg, 0.46 mmol) described above was activated by equimolar amounts of dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole in dimethylformamide (10 ml) during 30 min at 0 °C and 30 min at 21 °C, and a solution of the decapeptide diamide (4b) (700 mg, 0.42 mmol) (described above) in dimethylformamide (10 ml) was added. The trinitrobenzenesulphonate–sulphite assay of amino-groups showed that the coupling was complete in 48 h, and the solution was then evaporated to dryness; the residue was triturated with ether and dried. The N^{α}-t-butoxycarbonyl group was removed as usual by trifluoroacetic acid, the trifluoroacetate was dissolved in dimethylformamide (7 ml) and di-isopropylethylamine added to give an apparent pH of 11. The solution was applied to a column (103 × 4.5 cm) of Sephadex LH-20 and a further gel filtration of the main fractions gave *protected hexadecapeptide diamide* (490 mg, 45%); $[\alpha]_D^{20}$ −26° (*c* 0.5 in Me_2NCHO); R_F 0.26 (K) (Found: C, 49.4; H, 7.35; N, 20.4. $C_{106}H_{180}N_{38}O_{29}S_2\cdot 4H_2O$ requires C, 49.2; H, 7.35; N, 20.55%. Found after 48 h acid hydrolysis: Lys + Orn, 3.35; Arg, 1.67; His, 1.95; Asp, 0.95; Pro, 1.05; Gly, 0.98; Ile + *a*Ile, 3.05).

t-Butoxycarbonyl-L-isoleucyl-N^{ϵ}*-piperidino-oxycarbonyl-L-lysyl-S-acetamidomethyl-L-cysteinyl-L-asparaginyl-S-acetamidomethyl-L-cysteinyl-*N^{ϵ}*-piperidino-oxycarbonyl-L-lysine* (2b).—The protected hexapeptide 4-picolyl ester (2a) was saponified as described above for the preparation of compound (5b) at 25 °C for 20 min, giving *protected hexapeptide acid* (2b) (68% yield); $[\alpha]_D^{20}$ −27.5° (*c* 1.1 in Me_2NCHO); R_F 0.32 (K), 0.60 (A2) (Found: C, 48.05; H, 7.15; N, 14.2. $C_{51}H_{89}N_{13}O_{16}S_2\cdot 4H_2O$ requires C, 48.0; H, 7.65; N, 14.25%. Found after acid hydrolysis: Lys, 1.97; Asp, 1.03; Ile, 1.00).

t-Butoxycarbonyl-L-isoleucyl-N^{ϵ}*-piperidino-oxycarbonyl-L-lysyl-S-acetamidomethyl-L-cysteinyl-L-asparaginyl-S-acetamidomethyl-L-cysteinyl-*N^{ϵ}*-piperidino-oxycarbonyl-L-lysyl-*N^{ω}*-nitro-L-arginyl-L-histidyl-L-valyl-L-isoleucyl-*N^{ϵ}*-piperidino-oxycarbonyl-L-lysyl-L-prolyl-L-histidyl-L-isoleucyl-S-acetamidomethyl-L-cysteinyl-*N^{ω}*-nitro-L-arginyl-*N^{ϵ}*-piperidino-oxycarbonyl-L-lysyl-L-isoleucyl-S-acetamidomethyl-L-cysteinylglycyl-*N^{ϵ}*-piperidino-oxycarbonyl-L-lysyl-L-asparagineamide* (28).—The protected hexapeptide acid (2b) (83 mg, 0.065 mmol), described above, was activated by equimolar amounts of dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole monohydrate in dimethylformamide (2 ml) at 0 °C for 30 min and 24 °C for 30 min and then coupled with the hexadecapeptide diamide (sequence 7—22) Arg(NO_2)–His–Val–Ile–Lys(Pipoc)–Pro–His–Ile–Cys(Acm)–Arg(NO_2)–Lys(Pipoc)–Ile–Cys(Acm)–Gly–Lys(Pipoc)–Asn–NH_2 (139 mg, 0.054 mmol) in dimethylformamide (3 ml). The trinitrobenzenesulphonate–sulphite amino-assay showed that the coupling was complete after 26 h. The solution was concentrated to 3 ml and applied to a column of Sephadex LH-20, the product being detected by u.v. absorption and polarimetry ($V_e/V_t = 0.36$). Reprecipitation from dimethylformamide by ether gave *protected docosapeptide amide* (28) (166 mg, 80%); $[\alpha]_D^{20}$ −26° (*c* 0.5 in Me_2NCHO); R_F 0.31 (K) (Found: C, 49.2; H, 7.3; N, 18.3. $C_{157}H_{267}N_{51}O_{44}S_4\cdot 8H_2O$ requires C, 49.05; H, 7.4; N, 18.6%). Found after 48 h acid hydrolysis: Lys + Orn, 5.48; Arg, 1.72; His, 2.07; Asp, 1.96; Pro, 1.04; Gly, 0.96; Val, 0.98; Ile + *a*Ile, 3.80). Material from another preparation was treated with trifluoroacetic acid as usual to remove the N^{α}-t-butoxycarbonyl group and after addition of di-isopropylethylamine to liberate the free amino-component the product was chromatographed on Sephadex LH-20 in dimethylformamide, giving N^{α}-deprotected docosapeptide amide; $[\alpha]_D^{20}$ −24° (*c* 0.6 in Me_2NCHO); R_F 0.29 (K) (Found: C, 49.6; H, 7.2; N, 18.95. $C_{152}H_{259}N_{51}S_4\cdot 5H_2O$ requires C, 49.45; H, 7.35; N, 19.35%. Found after 48 h acid hydrolysis: Lys + Orn, 5.24; Arg, 1.71; His, 2.07; Asp, 1.95; Pro, 1.08; Gly, 1.04; Val, 1.05; Ile + *a*Ile, 3.97).

N^{α}*-t-Butoxycarbonyl-S-acetamidomethyl-L-cysteinyl-*N^{ϵ}-

*piperidino-oxycarbonyl-*L*-lysyl Hydrazide.*—Hydrazine hydrate (0.1 ml, 2 mmol) reacted with the ester (6) (Table 1) (100 mg, 0.16 mmol) in methanol (2 ml) during 1 h. Evaporation of the solvent and recrystallisation from ethanol–ether gave *hydrazide* (37 mg), m.p. 141—142 °C; $[\alpha]_D^{20}$ −13.5° (*c* 1.0 in Me_2NCHO) (Found: C, 49.1; H, 7.6; N, 17.6. $C_{23}H_{43}N_7O_7S$ requires C, 49.2; H, 7.7; N, 17.45; S, 5.7%).

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